Chapter 2
Isolation, Identification, and Characterization of Actinomycetes from Rhizosphere Soil of *Cajanus cajan*

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2 Isolation, Identification, and Characterizationof Actinomycetes from Rhizosphere Soil of *Cajanus cajan*

2.1 Introduction

Many soil-borne pathogenic fungi cause enormous plant infections and impair agricultural yields and product quality. These includes *Fusarium udum, Fusarium oxysporum*, *Phytophthora sp.*, and *Alterneria sp.* (Morrissey et al., 2004). Pathogenic fungi with a wide range of hosts cause disease in many economically important agricultural plants. It has been common practice to employ chemical fungicides, such as Bavistin and Thiram, to inhibit the spread of pathogenic soil fungi like *Fusarium* (Melent'ev et al., 2006).Consequently, environmentally friendly and long-term solutions are needed. A wide range of defense mechanisms is built into plants to keep them safe from various pathogens. Cultivation of crops is a complicated network of relationships among sustainable agricultural activities in current times. The rhizospheric microorganisms potential to supply essential nutrients for agricultural product enhancement is undisputed (Kumar et al., 2018).

Beneficial microbes can aid in acquiring nutrients such as nitrogen from the environment, iron from the soil, and metabolites that can enhance plant growth (Singh et al., 2011). These microorganisms encompass a diverse range of species, including nitrogen-fixing bacteria that are either symbiotic (Mendes et al., 2013) or free-living, plant growth-promoting rhizobacteria (PGPR), and fungi that participate in mycorrhizal associations (Mendes et al., 2013; Mishra & Arora, 2016), as well as biocontrol agents (Sharma et al., 2016, 2018). Numerous bacteria, such as *Azotobacter*, *Azospirillum*, *Bacillus spp*., *Pseudomonas fluorescence*, *Rhizobium*, Frankia, and other actinobacteria, are classified as PGPR due to their growth-promoting influence on plants (Nadeem et al., 2014).

Despite their near morphological resemblance to fungi, actinomycetes are a distinct category of bacteria, owing to their branched, filamentous, or hyphae-type elongated cells. These organisms have a high degree of morphological variation, reflected in their habitats and cellular output (Singh et al., 2018). Plant growth promotion has long been recognized as a critical characteristic of bacteria, particularly actinobacteria, and the subject has been extensively studied. The

agricultural sectors have rekindled interest in actinomycetes as biocontrol agents, growth promoters, and sources of agro-active chemicals, including secondary metabolites (Běhal, 2000). Plant growth-promoting actinomycetes (PGPA) are root-colonizing microorganisms that have significant results, such as boosting plant development and preventing the spread of disease (Misra et al., 2017).

Actinomycetes thrive in the rhizosphere of plants (Suzuki et al., 2000) and have a wide range of effects on plants developmental and physiological processes and a protective role against plant diseases (Ahemad & Kibret, 2014). *Streptomyces*, a genus of actinomycetes, has been extensively studied for its ability to promote plant growth against a variety of economically significant crops, including bean (Nassar et al., 2003), tomato (El-Tarabily, 2008), pea (Tokala et al., 2002), cotton (Sunita et al., 2015), wheat (Sadeghi et al., 2012), and rice (Gopalakrishnan et al., 2014). Additionally, they have been found in the root rhizosphere of medicinal plants and as novel inhibitory chemicals against phytopathogens (Sunita et al., 2015). Plant development can be stimulated in two ways: directly (by producing phyto-hormones) or indirectly (by producing cell-wall-degrading enzymes) by numerous species of *Streptomyces, Micromonospora, Corynebacterium, Frankia, Mycobacterium,* and *Rhodococcus* (Yuan & Crawford, 1995).*S.coelicolor* DE07, *S. olivaceus* DE10, and *S. geysiriensis* DE27 were isolated as drought-tolerant endophytes from arid and drought-affected areas of Rajasthan, India (Yandigeri et al., 2012). These actinobacteria produced auxin and aided in the growth and development of plants under stress situations. Additionally, it was found that chitinolytic *S. vinaceusdrappus* S5MW2 isolated from Chilika Lake, India, enhanced plant growth and biocontrol efficiency against *Rhizoctonia solani* by chitin supplementation (Yandigeri et al., 2015).

Biocontrol, plant growth promotion, and interactions with plants are all fields of focus for actinomycetes (Palaniyandi et al., 2013).Endophytic actinomycetes have attracted more interest due to their ability to produce a variety of novel antibiotics, lead compounds, and growth promoters (Shimizu, 2011).The fungal pathogen *Phytophthora cinnamomi* has also been reported to be inhibited by actinomycetes (You et al., 1996). *P. aphanidermatum* has been proven to be less harmful to cucumber plants treated with actinomycetes (El-Tarabily et al., 2009).The chitinolytic activity of five *Streptomyces* species isolated from Brazilian soil was examined against *Aspergillus* and *Colletotrichum* species (Gomes et al., 2000).It has been found that

*Streptomyces sp.*S160 can promote the growth and resilience of chickpea plants and the activity of defense-related enzymes to the charcoal rot disease caused by *Macrophomina phaseolina* in chick pea plants, resulting in the formation of defence chemicals (Yadav et al., 2014).In plants treated with biocontrol chemicals and inoculated with pathogens, higher levels of defensive enzymes and total phenol have been observed (Ashry& Mohamed, 2012; Chérif et al., 2007). Biosynthesis of phytoalexins and plant phenolic content is dependent on PAL, which works as a pathogen-inhibiting chemical (Chérif et al., 2007). Vulnerability to infection has been increased in plants with higher levels of ascorbate peroxidation (Ashry& Mohamed, 2012).

For example, *Streptomyces* sp. and other actinobacteria influence soil fertility by interacting with various components and acting as nutrient boosters. Besides producing Siderophores and phosphate solubilization enzymes, they are known to create enzymes that transform complex nutrients into simple mineral forms. These enzymes include amylase, chitinase, cellulase, invertase, lipase, keratinase, peroxidase, pectinase, protease, phytase, and xylanase. Because of this, they are suitable candidates for use as natural fertilisers (Jog et al., 2016). Indole-3-acetic acid (IAA) is a plant hormone that belongs to the auxins class. Since it causes cell elongation and division, it plays an essential function in plant growth and development (Vurukonda et al., 2018). Manulis et al. (1994) investigated the production of IAA and the methods of its synthesis by several Streptomyces species, including *Streptomyces violaceus*, *Streptomyces griseus*, *Streptomyces exfoliates*, *Streptomyces coelicolor*, and *Streptomyces lividans*. From groundnut roots, Reddy et al. (2016) identified *Streptomyces atrovirens*, which has demonstrated excellent growth-promoting activity on groundnut and various other crops. These findings were exceptionally fascinating since they demonstrate the ability of a single streptomycete to stimulate the growth of numerous plant species. In greenhouse tests, El-Sayed et al. (1987) and El-Shanshoury (1991) observed IAA synthesis in plants induced by *Streptomyces* sp., whereas El-Tarabily (2008) was successful in comparing various *Streptomyces* sp. strains. In these trials, *Streptomyces filipinensis* synthesis of IAA triggered exceptionally effective growth promotion.

1-aminocyclopropane-1-carboxylate (ACC) is essential for the endogenous production of ethylene in plants. El-Tarabily (2008) reported, in a comparison of streptomycetes, that *Streptomyces filipinensis* encouraged increased growth than *S. atrovirens* due to the production of both IAA and ACC, whereas *S. atrovirens* generated just ACC deaminase. Therefore, it has

been demonstrated that a single streptomycetes produces more than one plant hormone. These findings have significant implications for the potential use of Streptomyces as plant growth stimulants.

Pigeon pea (*Cajanus cajan* (L.) Millspaugh), commonly known as red grams, is a rain-fed crops produced in tropical and subtropical climates area of the worldwide (Pais & Bansal, 2019). Asia, South America, and East and South-East Africa farmers who depend on this grain for their daily sustenance are adversely affected by the price and availability of Pigeon peas, one of the region's most significant pulse crops (Sharma et al., 2016).The cultivation of pigeonpea is limited to emerging nations in Asia, southeast Africa, and the Caribbean. India and Myanmar account for over 80% of the global production of pigeon pea in Asia. India alone generated over 67% of the world's Pigeon pea in 2012–14. Following India and Myanmar, Malawi (6.3%), Tanzania (5.3%), and Kenya (4.6%) all produce a significant amount of Pigeon pea (Pais & Bansal, 2019). Waterlogging, frost, drought during the grain-filling stage, and soil salinity are the most significant abiotic impediments encountered during pigeon pea farming (Ali & Kumar, 2005; Desai et al., 2000; Mula & Saxena, 2010). In excessively humid conditions, undesirable plant growth is also possible (Mula & Saxena, 2010). It is possible to alleviate water logging difficulties by utilizing proper agronomic practices, such as raised-bed planting, surface drainage systems, and crop varieties with an acceptable duration (Pais & Bansal, 2019).

The most severe disease affecting Pigeon pea is Fusarium wilt, a fungal disease transmitted through the soil. It is a substantial constraint on the production of India and East African nations like Kenya, Malawi, and Tanzania (Sharma & Ghosh, 2016). Wilt can result in total crop loss before the pods mature and up to two-thirds of crop loss throughout plant maturity (Soren et al., 2012). Fungal growth is more likely to occur in fields that have been regularly grown with Pigeon pea, making them prone to the disease. Disease-resistant crop types, crop rotation, synthetic fungicides, and fumigation have traditionally been used to prevent and manage soil-borne diseases in crops throughout the recent times (Tang et al., 2020; Xiaohui Wang et al., 2018).

However, significant soil- and seed-borne fungi like *F. udum* and *F. oxysporum* cause substantial mortality rates in the Pigeon pea crop (Gopalakrishnan & Srinivas, 2019; Sharma et al., 2016).

Protecting crops like pigeon pea against illnesses is essential since the demand for these products is expected to rise in developing countries. Fungicides like Thiram and Capsan are commonly used to control plant wilting; although environmental awareness of pesticide-related hazards has increased the importance of biocontrol techniques (Aktar et al., 2009).It is impossible to control *F. udum* using chemicals because it is a soil-borne pathogen. The widespread and excessive usage of artificial and synthetic materials chemicals to boost *Cajanus cajan* effectiveness and disease management is also becoming a source of increasing concern. Because of this, PGPA can help to ensure good long-term yields and plant health. Hence, in the current study, *Streptomyces* sp. S-9, a PGPA strain, was tested in vitro and in pot field experiments in Pigeon pea to see how it affected antagonistic activity against *F. udum.*

2.2 Materials and methods

2.2.1 Sampling sites

Various soil samples from different locations in Gujarat were gathered. For more about the sampling locations, see Table 2.1 and Figure 2.1.

Table 2.1: Sampling sites and number of actinomycetes isolates from Gujarat region

Sr.No	Sampling location (Rhizosphere soil)	Latitude	Longitude	No. of isolates
1	Sanand (*C. cajan*)	22.9913°N	72.3755°E	18
2	AAU	22.5435°N	72.9580°E	10
3	Jambusar	22.2922°N	72.8013°E	13
4	Savli	22.5616°N	73.2220E	15
5	Mumbai	19.1565° N	72.9638° E	11
6	Ahmedabad	23.0063° N	72.6026° E	9
7	Jamnagar	21.8804° N	69.6734° E	10
8	Lasundra	22.9171° N	73.1453° E	36

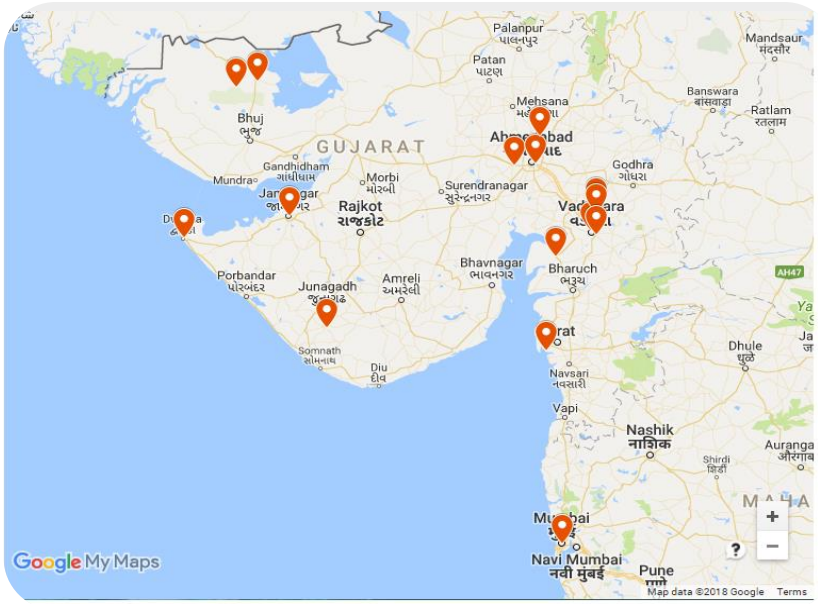

Figure 2.1: Map showing soil Sample collection sites from Gujarat region

2.2.2 Collection of soil samples

Various soil samples from different locations in Gujarat were gathered. For more about the sampling locations, see Table 2.1.A total of 15 soil samples were collected from different places in Gujarat region, India, as given in (Table 2.1). The samples were carefully taken with spatula by removing 2-3 inches top soil and kept in sterile polypropylene bags. The collected samples were taken to the laboratory for isolation of actinomycetes.

2.2.3 Isolation and Characterization of Actinomycetes

Actinomycetes were isolated from soil samples taken from the rhizomes of pigeon peas (approximately two months old) at Lasundra, Vadodara (N22°56`16", E73°22'22"), Gujarat (India) in July 2016. 1 g of soil sample enriched with calcium carbonate ($CaCO_3$) was dissolved in 10 ml of sterile 0.85% normal saline (SNS) and vortexed. To avoid fungal infection, these samples were diluted 10^{-3} and distributed on actinomycete isolate agar enriched with 50 μgmL^{-1} of cycloheximide and nystatin and incubated at 27 ± 1°C.

2.2.4 Genomic DNA sequencing and phylogenetic analysis

Actinomycetes (*Streptomyces* sp.strain S-9) was inoculated into ISP-1 broth and kept overnight while shaking using an orbital shaker. Genomic DNA was extracted using SDS-lysozyme extraction method and PCR targeting the 16S rDNA gene amplified using universal primer gene corresponding to positions 8-27 for the forward primer and 1492-1510 for the reverse primer (Forward primer (27F): 5'- AGAGTTTGATCMTGGCTCAG-3' Reverse primer (1492R): 5'-TACGGYTACCTTGTTACGACTT -3'. The phylogeny was inferred using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980).

2.2.5 Characterization of Actinomycetes for Biocontrol and PGP traits

2.2.5.1 Phosphate solubilization by selected isolates

Tricalcium phosphate used to test the solubilization of insoluble phosphate. Spot inoculations of the isolates were performed in the centre of Pikovskaya's medium supplemented with bromophenol blue. The plates were then incubated for 48 to 72 hours at 37^{o}C. Phosphate solubilization was evaluated by seeing the formation of a distinct yellow halo around the colony, which represented the generation of organic acids as a potential mechanism for phosphate solubilization (Rao, 1982). Twenty-eight days were spent performing quantitative phosphate solubilization in Pikovskaya's media. The concentration of soluble phosphate in the supernatant was measured every seven days using the Stannous Chloride ($SnCl_2$. $2H_2O$) technique (Gaur, 1990). Using a systronics digital pH metre, a subsequent change in pH was measured in the supernatant.

2.2.5.2 Indole acetic acid (IAA) production by selected isolates

Auxin production was discovered in a medium of tryptone yeast. Bacteria was cultured for 96 hours in yeast extract broth supplemented with 1% L-Tryptophan on an orbital shaker at 200 rpm in the dark. IAA synthesis in the supernatant was determined using the Salkowsky's reagent (Sarwar & Kremer, 1995). One millilitre of culture supernatant combined with one millilitre of salkowsky's reagent, incubated in the dark for thirty minutes to acquire a pink colour, and then measured at 536 nm on a spectrophotometer. Using the usual graph of pure indole acetic acid, the amount of IAA produced was computed. The pattern of IAA production was observed and

recorded every 24 hours for up to 96 hours. TLC analysis validated the production of IAA. Compounds of Rf values near 0.56 (pure IAA) were deemed to be IAA.

2.2.5.3 Siderophore production by selected isolates

Siderophore production detected by employing the method of Schwyn & Neilands (1987) with blue agar plate contain the chrome azurol S dye (CAS). The presence of orange halos surrounding colonies on blue agar indicated Siderophore production. 2 ml of actively developing isolate with 0.6 O.D. at 600 nm were inoculated into 60 ml of succinic acid medium in 500-ml Erlenmeyer flasks for quantitative measurement. All flasks were incubated at 30^{o}C for 30 hours on an orbital shaker. After 30 hours, culture was centrifuged for 20 minutes at 5,000 rpm. The pH, fluorescence, and siderophore generation of the supernatant were measured. In addition, the development pattern of isolation was altered simultaneously. A catecholate-type siderophore was analyzed using a conventional methodology (Arnow, 1937), while hydroxymate-type siderophores were analyzed using the method of Gibson & Magrath (1969).

2.2.5.4 Ammonia production by selected isolates

The published method of Cappucino and Sherman (1992) was used to detect ammonia generation. Autoclaving at 121^{o}C was used to prepare and sterilize peptone water. All isolates were injected with 1 ml of culture solution in 30 ml of medium and cultured for ten days at 37^{o}C. After ten days, 10 ml of culture suspension was taken and centrifuged for 20 minutes at 1,000 rpm. 1 ml of supernatant was placed in a sterile test tube with 1 ml of Nessler's reagent. 9 ml of distilled water added and keep for twenty minutes. Vis/UV Systronic spectrophotometer was used to measure O.D. at 540nm. From colourless to yellow to brown precipitates, a colour change was noted during the process.

2.2.5.5 HCN production by selected isolates

On nutritional agar slants spotted with test isolates, the production of hydrocyanic acid (HCN) was identified. In the tubes, filter paper strips soaked in picric acid, and 3 percent sodium carbonate was introduced. Yellow filter paper strips whose colour changed from yellow to light brown, moderate brown or dark brown were examined to determine the presence of HCN (Askeland & Morrison, 1983).

2.2.5.6 β-1, 3 glucanase production

Using laminarin as a substrate in a minimal media, β-1, 3-glucanase formation was detected. A zone of β-1, 3 glucanase production was seen surrounding the colony when the plate was filled with 1 percent Congo red and then 1 percent NaCl. For quantitative β-1, 3 glucanase synthesis, 100 ml of minimum medium supplemented with 0.5% laminarin was added with 1 ml of an active bacterial culture and agitated at 300 degrees Celsius for 72 hours, with production monitored every 24 hours. β-1,3 glucanase was measured by incubating 0.5 ml of 0.5% (w/v) laminarin in 50 mM acetate buffer (pH 4.8) with 0.5 ml of enzyme test solution at 45 °C for 30 min, followed by measurement of reducing sugars produced using DNS (El-Katatny et al., 2000). Reducing sugar released was determined using standard curve of glucose and enzyme activity plotted in units per milliliter (U/ml).

2.2.5.7 Chitinase production

In minimal medium, chitinase synthesis detected utilizing colloidal chitin as a substrate. The presence of a chitinase producing zone was discovered by first adding 1% Congo red to the plate, followed by 1% NaCl. Chitinase synthesis was monitored every 24 hours for 72 hours using 100 ml of minimum medium containing 1 percent colloidal chitin and inoculated with 1 ml of active bacterial culture. The media was shaken at 30° C and the chitinase production was measured every 24 hours.0.5 ml enzyme was incubated for 45 minutes at 30°C with 0.5ml of 1% colloidal chitin in acetate buffer (50 mM, pH 5.0) as substrate. The dinitrosalicylic acid (DNS) method was used to determine the quantity of the product (Miller, 1959). The amount of enzyme that generated sugars equivalent to 1 μmol of N-acetylglucosamine per minute under the specified conditions was defined as one unit of chitinase activity.

2.2.5.8 ACC deaminase assay

Toluenized cells were resuspended in a new 1.5 ml microcentrifuge tube with 20 μl of 0.5 mol l^{-1} ACC solution, vortexed briefly, and incubated for 15 minutes at 30°C. Following the addition of 1 ml of 0.56 mol l^{-1} HCl, the mixture was vortexed and centrifuged for 5 minutes at 12,000g at room temperature. The glass tube was then filled with 300 μl of the 2, 4-dinitrophenyl hydrazine reagents, vortexed, and incubated for 30 minutes at 30°C. After adding and mixing

two millilitres of 2 N NaOH, the absorbance at 540 nm was measured using UV- visible spectrophotometer (Shimadzu UV-1800, Japan) (Penrose & Glick, 2003).

2.2.5.9 Qualitative screening for ACC deaminase activity

On the premise of the ACC deaminase (E.C. 4.1.99.4) activity, the use of 1-aminocyclopropane-1-carboxylic acid as a nitrogen source could be predicted. The ACC deaminase activity of the isolated bacterial isolates was tested as described by Glick (2005) with minor modifications. When it comes to the specifics, the overnight-grown bacterial isolates were spread out on the media, which was supplemented with 3mM of ACC (Sigma Chemical Company, USA). For four days, NFb plates were incubated at 30^{o}C after being transferred from bacterial cultures. For experimental purposes, we used a similar isolate but one that had been cultivated without the addition of a nitrogen source (Siddikee et al., 2010).

2.3 Results

2.3.1 Isolation and characterization of actinomycetes from Rhizspheric soil

The physico-chemical characterization of soil from Gujarat's four agro-climatic pigeonpea-producing zones indicated high variation in soil type, mineral and nutrient content, salinity, conductivity, and soil pH. The physico-chemical characteristics of rhizosphere and bulk soil from Central Gujarat and Saurashtra region. The soil colour varied from brown to deep black while soil consistency was clay loamy to sandy or rocky for the geographical locations.

A total of 165 actinomycetes were isolated from 8 soil samples collected from different regions of Gujarat, as given in Table 2.1. These actinomycetes were differentiated based on the colour of aerial and substrate mycelium, pigmentation, and microscopic examination (Figures 2.2 A and 2.2 B). From the different isolates, white, grey, and red mycelial pigmented actinomycetes were found dominant in Saurashtra region. The most effective culture media in the isolation of actinomycetes were Actinomycete isolation agar [Hi-media, Mumbai]. Actinomycetes colonies with various colours (aerial hyphae and spores), consistency (dry to mucoid), shape (irregular to concentric), secretions (colourless to golden), and pigmentation (reverse and diffusible melanoid) were observed in (Figures 6-12**).** When characterized based on morphology, chemo-taxonomy, and microscopic features, these isolates were broadly classified in genera *Streptomyces* sp.

Table 2.2: The Physico-chemical characterization of Rhizospheric soil

Sr.No	Sampling location (Rhizosphere soil)	Soil Colour	Soil Types	pH	No. of isolates
1	Sanand (*C. cajan*)				18
2	AAU				10
3	Jambusar				13
4	Savli				15
5	Mumbai				11
6	Ahmedabad				9
7	Jamnagar				10
8	Lasundra				36

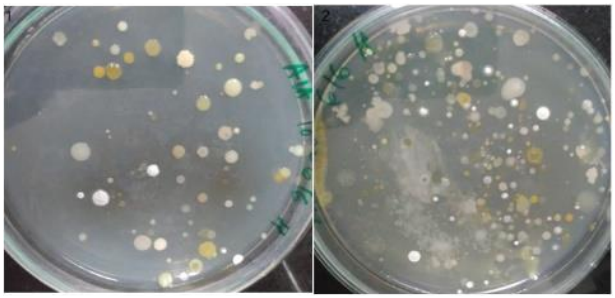

Figure 2.2A: Actinomycetes isolation from different soil sample

Figure 2.2 B: Colony morphology of different actinomycetes isolates on Actinomycetes isolation agar medium showing different pigment and colour

2.3.2 Identification of Actinomycetes by 16S rRNA Amplification

This study identified three isolates up to the genus level based on 16S rRNA gene sequences. The sequence was deposited in GenBank (NCBI) with the accession numbers MK610729.1, MK158952.1, and MK610795.1. The 16S rRNA gene was amplified and sequenced to identify actinomycete isolates up to the genus level. Genomic DNA isolated from pure cultures was amplified to obtain 1.1 kb PCR product (Figure 2.3). Purified products were sequenced, aligned, checked for chimaera, cleaned, identified (BLAST), and deposited (~700 bp) in GeneBank (NCBI) with a unique NCBI Accession number for each actinomycete sequence. The molecular characterization based on 16S rRNA partial sequencing indicated that all isolates were *Streptomyces* (Fig. 2.3 and 2.4). Thus, the correlation of classical features to molecular phylogeny was the criteria used to identify actinomycetes.

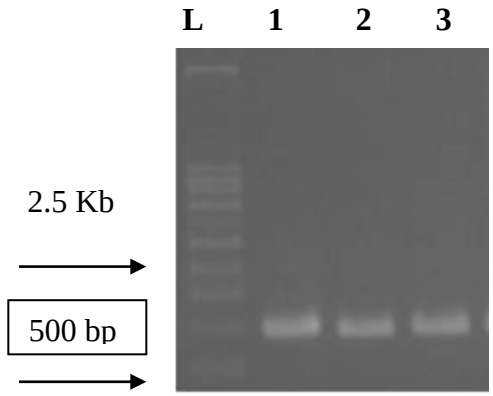

Figure 2.3: PCR amplification of 16s rDNA of actinomycetes strains

Lane Description

L-500bp DNA ladder 1) Sample S-9 2) Sample S-280 3) Sample S-107

Evolutionary analyses were conducted in MEGA 7. The phylogenetic analysis of actinomycete isolates Shown in figure 2.4 from *C. cajan* rhizosphere was done in MEGA7.0 to infer the taxonomic affiliations. The phylogenetic tree was constructed for *C. cajan* rhizosphere of South Gujarat and the Saurashtra region. The bootstrap consensus tree constructed from 1000 replicates as taken to represent the evolutionary history of taxa analyzed. Evolutionary analyses were conducted in MEGA 7.

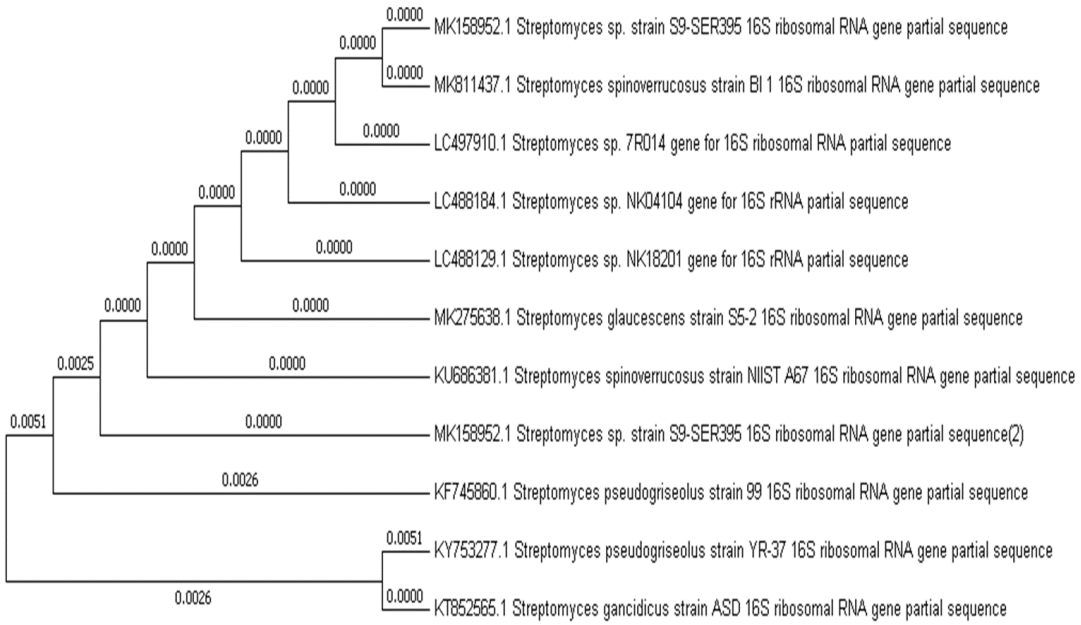

Figure 2.4: Molecular phylogenetic analysis by maximum likelihood method.

2.3.3 Characterization of Streptomyces isolates on different ISP media

S-9 spores and mycelium morphology were observed on ISP media at 28°C. The colonies were small. The colonies of different developed isolates were observed and recorded (Figure 2.5**)** as smooth with aerial and substrate mycelia of varying colours, usually with entire margins and diffusible pigment produced in ISP-7 media (Figures 2.6-12**).** The aerial mycelia were grey in colour, and the substrate mycelia were beige shown in Table 2.3.

Table 2.3: Cultural and morphological characteristics of some of the promising isolates

Strain	Colony appearance	Aerial mycelia	Aerial spore	Pigmentation	DAP Isomer	Gram staining
S-107	white, dry, powdery	White	Dark brown	None	LL-DAP	Positive, filamentous, branched
S-280	white, dry, powdery	White	Grey	None	LL-DAP	Positive, filamentous, branched
S-9	Grey, dry, Powdery	Grey	Grey	None	LL-DAP	Positive, filamentous, branched

The colour of the substrate mycelium was established by examining the plates after 7 to 10 days. It was done only after seeing the heavy spore mass surface. The present study's most important factors for classifying actinomycetes included colonial characterization, especially the ability to produce vegetative and aerial hyphae, color, and organization on defined media under standard cultivations conditions (Table 2.4; Figures 2.5-2.11). Thus, all cultures were grown on 7 ISPs (ISP-1 to ISP-7) specifically designed International Streptomyces Project media as Islam & Hernández (1966) recommended. ISP-1 (Tryptone yeast extract) and ISP-2 (Yeast malt agar) were used to classify vegetative growth; ISP-3 (Oat meal agar) and ISP-4 (Inogranic salt starch agar) promoted sporulation; ISP-5 (Glycerol aspargine agar), ISP-6 (Peptone Yeast Extract Iron Agar) and ISP-7 (Tyrosine agar) [Hi-media, Mumbai, India]. Promoted miscellaneous features like secretion of reverse and diffusible pigment and other potent antimicrobial compounds. Actinomycetes colonies with various colours (aerial hyphae and spores), consistency (dry to mucoid), shape (irregular to concentric), secretions (colourless to golden), and pigmentation were observed as shown in (Figures 2.6-2.12).

Table 2.4: Colony characteristics of actinomycete isolates on different ISP Medium

Strain no	ISP-1	ISP-2	ISP-3	ISP-4	ISP-5	ISP-6	ISP-7
S-107	Dark brown	Light brown	light yellow	white to light brown,	chalky, round	light yellow	Dark brown
S-280	Chocolate brown (chalky)	White chalk	Dark brown	White chalk	Dark brown	Chocolate brown (chalky)	Light Yellow
S-9	White chalk	Dark white	Dark grey	Light grey	Chalky, brown, round	Dark white	Black

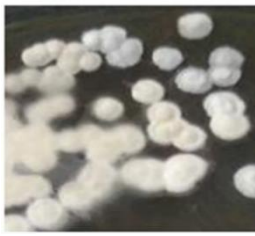

Figure 2.5: Colony characterization of actinomycete isolates: Variation in *Streptomyces* isolates aerial hyphae and spores colour and texture on ISP-1. A: S-107 Strain; B: S-280 Strain; C: S-9 Strain

Figure 2.6: Colony characterization of actinomycete isolates: Variation in *Streptomyces* isolates aerial hyphae and spores colour and texture on ISP-2. A: S-107 Strain; B: S-280 Strain; C: S-9 Strain

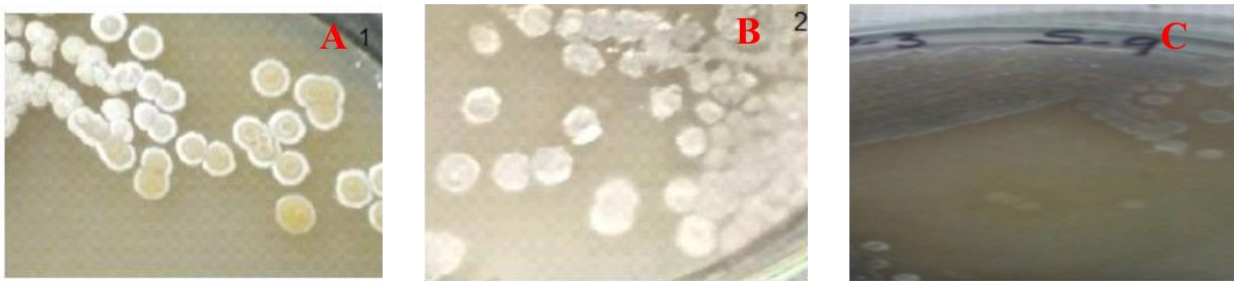

Figure 2.7: Colony characterization of actinomycete isolates: Variation in *Streptomyces* isolates aerial hyphae and spores colour and texture on ISP-3. A: S-107 Strain; B: S-280 Strain; C: S-9 Strain

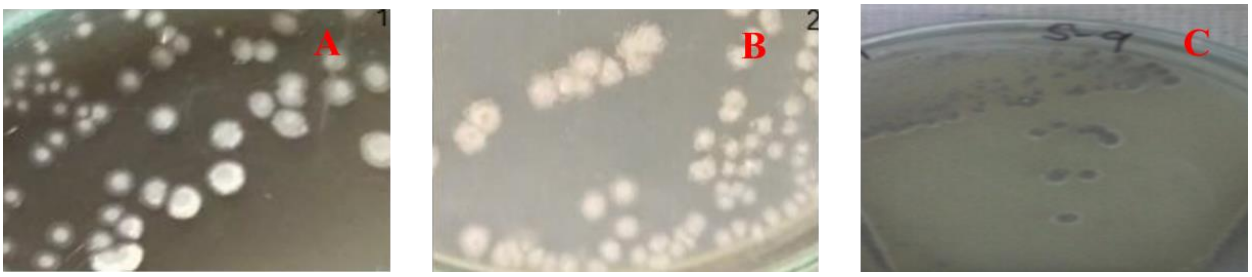

Figure 2.8: Colony characterization of actinomycete isolates: Variation in *Streptomyces* isolates aerial hyphae and spores colour and texture on ISP-4. A: S-107 Strain; B: S-280 Strain; C: S-9 Strain

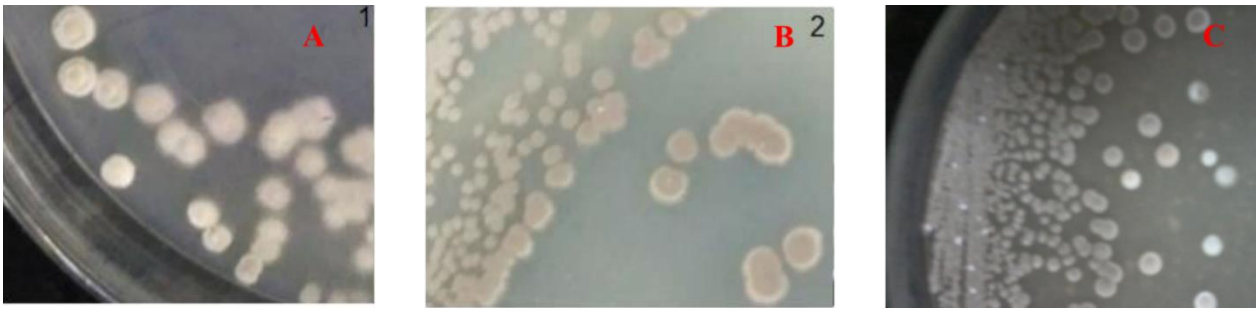

Figure 2.9: Colony characterization of actinomycete isolates: Variation in *Streptomyces* isolates aerial hyphae and spores colour and texture on ISP-5. A: S-107 Strain; B: S-280 Strain; C: S-9 Strain

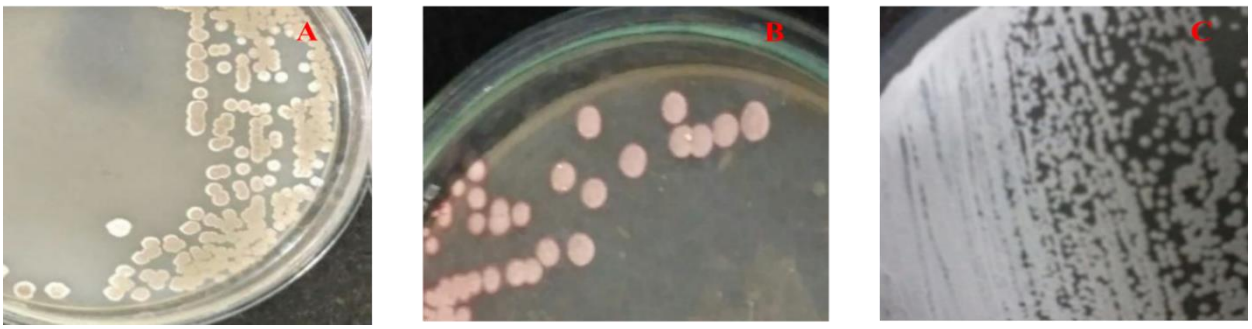

Figure 2.10: Colony characterization of actinomycete isolates: Variation in *Streptomyces* isolates aerial hyphae and spores colour and texture on ISP-6. A: S-107 Strain; B: S-280 Strain; C: S-9 Strain

Figure 2.11: Colony characterization of actinomycete isolates: Variation in *Streptomyces* isolates aerial hyphae and spores colour and texture on ISP-7. A: S-107 Strain; B: S-280 Strain; C: S-9 Strain

2.3.4 Phenotypic characterization of *Streptomyces sp.*

Morphology is a crucial attribute for the description of taxa, although it is not sufficient to distinguish between many genera on its own. In fact, in many early descriptions, it was the only character that was ever used. Staining revealed the varied morphologies of the isolates (Figure 2.12).

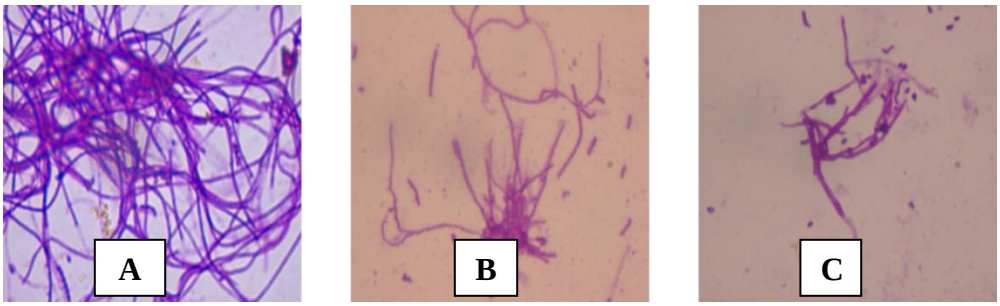

Figure 2.12: Gram staining of rhizospheric actinomycetes isolates. A: S-280; B: S-9; C: S-107

2.3.5 Plant Growth Promoting (PGP) Potential of Studied Actinomycetes Isolates

In our study, the PGP potential of the isolates studied revealed that S-9 isolate had the highest zone of inhibition, while S-280 had the lowest inhibition zone (24) (Table 2.5). In the present study, the isolates had no activity on chitinase, while they exhibited β-1,3-glucanase, P-solubilization, and IAA production. Siderophore production was only recorded in S-280 and S-107 (Table 2.5).

Table 2.5: PGP potential of Actinomycetes isolates

Strain no	Zone of inhibition	Chitinase	β-1,3-glucanase	P-Solubilization	Siderophore production	IAA Production
S-280	24	-	+	+	+	+
S-107	32	-	+	+	+	+
S-9	34	-	+	+	-	+

- = No Production; + = Production

2.3.5.1 Phosphate solubilization

On Pikovskyaya's medium, all three isolates exhibited a zone of phosphate solubilization after three days of incubation. In the instance of P-solubilization by *Streptomyces sp.* strain S-9, a variable solubilization zone formed on rock phosphate surrounding the colonies of the selected strain. According to the data, the P-solubilization of PGPA varied between 1.5 and 10.5 mm. However, isolate *Streptomyces* sp. strain S-9 exhibited a wider (10.5 mm) P-solubilization zone.

2.3.5.2 IAA production

In the presence of 1 % tryptophan, a qualitative study of the culture supernatant of selected actinomycete isolates demonstrated the synthesis of varied quantities of IAA. In our investigation, IAA production ranges from 16.8 to 18.0 μgmL^{-1}.

2.3.5.3 Ammonia and HCN production

After 24 hours of incubation at 37°C using the picrate assay method, there was no evidence of HCN generation. In the current investigation, no ammonia production was found.

2.3.5.4 β-1, 3 glucanase production

There was a zone of synthesis of β-1, 3-glucanase. Surrounding the colony when the plate was filled with 1 % Congo red and then 1 % NaCl. The DNSA method was used to investigate the quantitative synthesis of β- 1, 3 glucanase in laminarin-embedded media. The activity of β -1, 3 glucanase (32 ± 0.20 ng glucose/min/mg protein) decreased steadily.

2.3.5.5 Chitinase production

No chitinase-producing regions were assessed in this study by adding 1% Congo red and 1% NaCl to a plate of colloidal chitin, and then evaluating the area around the colony.This may be due to the methodology used.

2.3.5.6 ACC Deaminase Activity

The presence of ACC deaminase was verified by assaying for α-ketobutyric acid. Isolate S-9 produced 2.8 ±0.03nmol α-Ketobutyrate mg $protein^{-1}h^{-1}$. This confirms that *Streptomyces* sp. S-

9 strain produced ACC deaminase. Furthermore, the strain was subjected to evaluating ACC deaminase activity. S-9 exhibited maximum ACC deaminase (Figure 2.13).

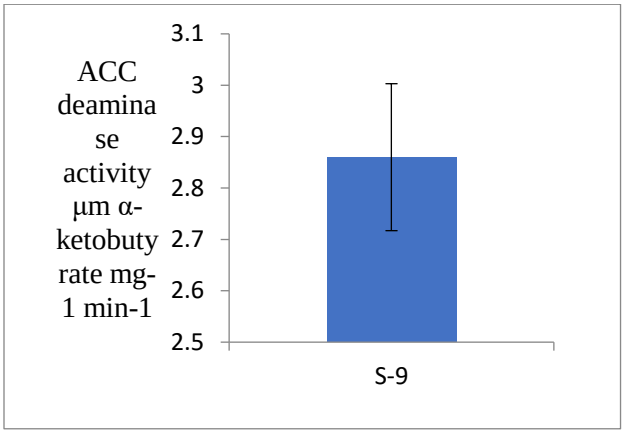

Figure 2.13: ACC deaminase production by *Streptomyces* sp. S-9

2.4 DISCUSSION

The variations in the soil can also affect the bacteria present in the rhizosphere of soil, as opined by Kuramae et al., (2012), who reported that several soil bacterial taxa were strongly correlated to physicochemical soil characteristics. Also, according to Khandan & Janardhana (2013), actinomycetes in soils are directly linked to soil type, pH, organic carbon, and mineral elements.

Morphological characterization showed that all isolates were Gram-positive filamentous bacteria. Diaminopimelic Acid (DAP) isomers are a critical component of the cell wall of Gram-positive bacteria and actinomycetes. The majority of bacteria have a distinctive wall envelope comprised of peptidoglycan. The 2, 6- Diaminopimelic Acid (DAP) is cosmopolitan as key amino acid and its optical isomers (Sharma et al., 2014). In our study, the LL form of DAP isoform was present within the peptidoglycan of the studied isolates (S-107, S-280, and S-9). From their DAP type, it is evident that the isolates belong to *Streptomyces* group. This finding conforms to Poomthongdee et al. (2015) report that the presence of LL-DAP is an indication of the group *Streptomyces.*

Molecular biology techniques have grown increasingly popular to overcome the significant constraints of culture-dependent methods in identifying bacterial diversity (Amroyan et al., 1999; Giovannoni et al., 1990; Torsvik et al., 1990; Ward et al., 1990). Where cultivation has failed, 16S rRNA sequences have been used to identify and define actinomycete groups (Niner et al., 1996; Relman et al., 1992; Rheims et al., 1996). The use of molecular techniques to isolate previously uncultured bacteria with known rRNA sequences can aid in the future study or use of these microorganisms (Kwok et al., 1990; Teske et al., 1996). The evolutionary history could be deduced from the maximum likelihood method based on Kimura's two-parameter model (Kimura, 1980).

Actinomycetes in the rhizosphere of plants can enhance plant development by releasing hormones such as auxins or gibberellin. Auxin is a class of indole chemicals that stimulates shoot elongation, root initiation, seed germination, and plant biomass (Anwar et al., 2016). Indole-3-Acetic Acid (IAA) is a naturally occurring auxin produced by many microorganisms during the metabolism of L-tryptophan (Khamna et al., 2010). Rhizosphere bacteria produce auxin more efficiently than bacteria isolated from bulk soils (Mohite, 2013). The majority of *Streptomyces spp*. strains discovered in the rhizosphere of diverse plants are capable of generating IAA. *Streptomyces sp.*-generating IAA has also been reported to be used as growth promoters in tomatoes (El-Tarabily, 2008), wheat (Sadeghi et al., 2012), rice (Gopalakrishnan et al., 2013), and pomegranate (Poovarasan et al., 2013).

Additionally, actinomycetes can enhance plant growth by generating phytohormones, iron chelation, phosphate solubilization, and nitrogen fixation (Gopalakrishnan et al., 2013; Wahyudi et al., 2019). An increase in adventitious roots, which aids the plant's uptake of nutrients and water and increases root exudates, is primarily due to IAA synthesized by most PGPR actinomycetes (El-Tarabily, 2008). Diverse actinomycetes species can synthesize the auxin phytohormone IAA; these actinomycetes synthesize auxins in the presence of a suitable precursor, such as L-tryptophane (Abd-Alla et al., 2013). It is worth mentioning that all the three isolates tested had the ability of produce IAA. Actinomycetes from a wide range of crop rhizosphere soils have been demonstrated to have this ability, according to several studies (El-Tarabily & Sivasithamparam, 2006; Tsavkelova et al., 2006).

Phosphate solubilizing ability is present in over a quarter of all actinomycetes, including *Streptomyces* and *Micromonospora* (Barreto et al., 2008; El-Tarabily, 2008; Hamdali et al., 2008). Actinomycetes as P-solubilizers have garnered increased attention for their ability to produce agro-active metabolites such as phytohormones, siderophores, and antibiotics (Errakhi et al., 2009; Errakhi et al., 2007; Hoster et al., 2005), and their tolerance for a variety of stressor molecules (Fabre et al., 1988; Hamdali et al., 2008). According to the literature, microbial solubilization of mineral phosphate may occur due to the external media becoming acidified (Whitelaw, 1999) or due to the excretion of chelating chemicals (Hamdali et al., 2008; Welch et al., 2002), hence boosting phosphate solubilization.

Due to their structural complexity, rock phosphates are often less receptive to microbial solubilization, whereas orthophosphates (HAP and TCP to a lesser extent) are more solubilizable (Dastager & Damare, 2013; Kumari et al., 2008). P-solubilizing bacteria (PSB) convert the insoluble inorganic phosphate forms into soluble forms through various processes. The best-understood process involves the excretion of strong chelating organic acids (Farhat et al., 2009) or siderophore-like compounds (Hamdali et al., 2008; Rungin et al., 2012). Numerous PSB excrete an assortment of low-molecular-weight organic acids (Vassilev et al., 2006), which acidify the growth media. Several Gram-negative pathogens, such as *Burkholderia cepacia* and *Serratia marcescens* CTM 50650, were previously discovered to excrete gluconic acid (GA) (Farhat et al., 2009; Song et al., 2008). In these various species, the excretion of GA was positively linked with the release of soluble P (Park et al., 2010), as was demonstrated for the first time in the Gram-positive *Streptomyces* sp.CTM396 and CTM397 strains (Farhat et al., 2015). We have also demonstrated that the *Streptomyces* strains studied are all phosphate solubilizers.

Bacteria can produce ammonia and provide nitrogen to the host plant, according to (Marques et al., 2010). The increased virulence of opportunistic plant diseases is also a result of ammonia overproduction. In the fight against plant disease, HCN generation is critical, as is the creation of ammonia, a precursor in the production of HCN (Anwar et al., 2016). However, no Ammonia production was studied as per the method of Dye (1968). β -1,3-glucanase production was observed in the studied strains. The same was reported for *P.membranefaciens* and *C.guilliermondii* in Lilly-Barnett minimal salt media added with glucose as the carbon source (Q.

Fan et al., 2002). Due to their ability to create diverse antibiotics, actinomycetes are well-known for their antagonistic actions against numerous diseases. Actinomycetes are prolific producers of chitinases in addition to antibiotics. Both mesophilic and thermophilic taxa, including *Streptomyces*, *Saccharothrix*, *Microbispora*, *Thermobifida* and *Streptosporangium*, have been reported to generate chitinases (Dhole et al., 2021; Mathew et al., 2021). *Saccharothrix yanglingensis* Hhs.015 was isolated from cucumber roots as a chitinase-producing actinomycete that produced eight distinct chitinases (Y. Lu et al., 2018). However, the reason for the non-production of chitinase by the studied *Streptomyces* should be thoroughly investigated.

ACC deaminase is an enzyme found in *Bacillus*, *Enterobacter*, *Pseudomonas*, and *Streptomyces*, among other PGP-bacteria. It increases plant stress tolerance to drought, flooding, salinity, and phytopathogens (Jaemsaeng et al., 2018; Kruasuwan & Thamchaipenet, 2018; Saleem et al., 2018; Toklikishvili et al., 2010).