



Streptomyces sp. S-9 promotes plant growth and confers resistance in Pigeon pea (*Cajanus cajan*) against *Fusarium* wilt

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Received: 31 May 2021 / Accepted: 7 September 2021 / Published online: 9 October 2021
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Abstract

Streptomyces sp. strain S-9 was studied for its effect in inducing systemic resistance in Pigeon pea against the plant pathogen *Fusarium udum* causing wilt. The 16S rRNA gene sequencing and phylogenetic analysis indicated that S-9 is closely related to genus *Streptomyces* for which it was referred to as *Streptomyces* sp. S-9. *Streptomyces* sp. S-9 caused 85% inhibition of the pathogen and showed various attributes of plant growth-promoting such as the production of IAA, P-solubilization, and β -1, 3-Glucanase activity. Proline and malondialdehyde (MDA) content was significantly higher whereas the chlorophyll content decreased in the pathogen-infected plant when compared to S-9 treated Pigeon pea plants. The anatomical research assisted the biocontrol-mediated stress tolerance findings in the Pigeon pea plant through increased root epidermis and enhanced stress-related xylem tissues. Fungus inoculation elevated the antioxidative enzymatic activities of superoxide dismutase (SOD; 78%) and catalase (CAT; 56%). Marked reductions in antioxidant enzymes were associated with the antagonistic effects of the different treatments. Conclusions showed that S-9 bioinocula applied as a seed coating enhanced soil availability of nitrogen (N), phosphate (P), and potassium (K), indicating their suitability for direct application invigorating plant growth and persuade resistance in the plant Pigeon pea against *Fusarium* wilt.

Keywords *Fusarium* wilt · Actinomycetes · Pigeon Pea · MDA

Introduction

Soil-borne pathogenic fungi, such as *Fusarium udum*, *Fusarium oxysporum*, *Phytophthora* sp., *Alternaria* sp., etc., can cause serious plant diseases, affect crop yields, and impair product quality (Morrissey et al. 2004). Such fungal pathogens with a huge host variety ensuing into the disease of the various economically agricultural plants. Traditionally, synthetic fungicides such as Bavistin and Thiram are particularly accustomed manipulate the prevalence of *Fusarium* and different soil pathogenic fungi (Melent'ev et al. 2006). Consequently, alternate approaches are required for his or her eco-friendly and sustainable management. Consequently, alternate approaches are required for their eco-friendly and

sustainable management. Plants are quite well bestowed upon with various types of defense mechanisms to shield and protect them from many diseases. In modern times, crop farming is a complex network of interactions among plants, fertilizers, rhizobacteria, and soil. There is an earnest requirement for eco-friendly sustainable activities in the agriculture input. The ability of the rhizospheric microorganisms to give sufficient stock of fundamental supplements for the improvement of agricultural products is unquestionable (Kumar et al. 2018) As plants are known to harbor various beneficial microscopic organisms in all organs as epiphytes and endophytes, and control of these microorganisms have been demonstrated to build the profitability of harvests, we propose to call them aggregately as plant-beneficial microbes (PBR). This is the time of sustainable and evergreen agricultural food crop production; therefore, these tripartite (plant–microbe–soil) interactions in the spermosphere and rhizosphere play a humungous role.

Plant growth-promoting actinomycetes (PGPA) are root colonizing microbes with beneficial effects including plant growth promotion and disease control (Misra et al. 2017). *Streptomyces*, is the most abundant genus

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of actinobacteria representing 50% of the total population of soil actinobacteria (Parte et al. 2020). *Streptomyces* is a filamentous bacterium with a high G + C content that belongs to the Streptomycetaceae family and order Actinomycetales (Gopalakrishnan et al. 2020). It is well-known for producing a large number of bioactive metabolites. About 75% of the reported antibiotics were identified from *Streptomyces* (Olanrewaju and Babalola 2019). The filaments of *Streptomyces* promote the maximum absorption of nutrients from the soil and support colonization on substrates, particularly plant roots. As a result, *Streptomyces* populations are frequently reported in strain isolation, metagenomics, genome mining, and sequencing (Newitt et al. 2019). *Streptomyces* are known for their biocontrol potential against a variety of phytopathogens in addition to their plant growth-promoting abilities.

Numerous *Streptomyces* strains are considered biocontrol since they produce a wide scope of antimicrobials, can endure in unforgiving conditions, and proficiently colonize the rhizosphere of various plant species including rice (Qin et al. 2011; Kinkel et al. 2012). Moreover, *Streptomyces* can inspire initiated obstruction, as it has been depicted previously (Conn et al. 2008; Kurth et al. 2014). Because of these highlights, it is not astounding that assorted *Streptomyces* strains had been concentrated to control fungal and bacterial diseases of rice such as bacterial leaf blight caused by *Xanthomonas oryzae*, however, very few *Streptomyces* are currently being developed as biocontrol products.

Pigeon pea (*Cajanus cajan* (L. Mill) is one of the most significant pulse crops in the semi-arid tropics. Pigeon pea cultivation has a direct impact on the overall economic, financial and nutritional status of subsistence farmers in South and East Africa, Asia, and the South American sub-continent (Sharma et al. 2016). Pigeon pea [*Cajanus cajan* (L.) Millsp.], also known as red gram, is the second most important edible legume crop in India with an area of 5.10 hectares and a yield of 3.31 million tonnes (FAOSTAT 2019). Pea seeds are the main source of protein and vitamins, especially for the vegetarian population of the world.

In Pigeon pea cultivation, an important biological factor that causes *Fusarium* wilt is Pigeon pea wilt, which harms crop growth (30–60% of the incidence occurs in the flowering and mature period) and yield (Sharma et al. 2016). Managing *Fusarium* infection is often a difficult task, primarily due to its soil-borne nature, the deep interior of host tissues, the synthesis of persistent latent structures, and the ability to survive and sustain longer without host plants (Boukerma et al. 2017). In recent decades, the prevention and control of soil-borne diseases in crops have generally been achieved through the use of disease-resistant varieties, crop rotation, synthetic fungicides, and fumigation (Wang et al. 2018; Tang et al. 2020).

However, Pigeon pea suffers high mortality because of serious seed and soil-borne fungal pathogens such as *F. udum* and *F. oxysporum*. Economically important crops such as Pigeon pea need protection from various diseases as the demand for such crops are expected to steadily increase, particularly in the developing regions of the world. Presently, chemical fungicides such as thiram or captan are used to control wilt, but the increasing environmental awareness of pesticide-related hazards has emphasized the need for biological methods. The fungi *Fusarium udum* is a soil-borne plant pathogen and, therefore, chemical control is impractical in established cases. Moreover, extensive and inordinate use of synthetic and semi-synthetic compounds to improve Pigeon pea productivity and disease control is a growing concern. In light of the above facts, it can be observed the application of PGPA can contribute to sustainable high yield and plant protection. Therefore, the present investigation was undertaken to see the effect of PGPA strains particularly *Streptomyces* sp. S-9 on antagonistic activity against *F. udum* in vitro and further using pot, field trials in Pigeon pea.

PGPA alone or in combination can reduce the level of active free radicals through antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT). These enzymes can eliminate free radicals, thus reducing the formation of reactive oxygen species (ROS) (Manoj et al. 2016).

Many *Streptomyces* strains are considered biocontrol agents, since they produce a wide range of antimicrobials, can persist in harsh environments, and efficiently colonize the rhizosphere of different plant species including rice (Qin et al. 2011; Kinkel et al. 2012). Furthermore, *Streptomyces* can elicit induced resistance, as it has been described before (Conn et al. 2008; Kurth et al. 2014). Because of these features, it is not surprising that diverse *Streptomyces* strains had been studied to control fungal and bacterial diseases of rice such as Bacterial Leaf Blight caused by *Xanthomonas oryzae*, however, very few *Streptomyces* are currently being developed as biocontrol products.

Materials and methods

Cultivar and test pathogen used in the experiment

Pigeon pea seeds (var.BDN-2) were procured from the Pulse Research Station, Model Farm, located in Vadodara, Gujarat, India. The seeds were surface sterilized by soaking into 0.1% sodium hypochlorite solution (SDFCL, Mumbai) for 3 min followed by three washings with sterile distilled water (SDW). *Fusarium udum* (ITCC 3241) was procured from the Indian Type Culture Collection (ITCC), Indian Agriculture Research Institute (IARI), and New Delhi, India. The strain was grown and maintained on the media, viz.,

potato dextrose agar (PDA), (Hi-media, Mumbai, India) at 27 ± 1 °C for 5 days, until sporulation (200 g potato infusion, 20 g dextrose, 15 g agar) as suggested by ITCC.

Isolation and characterization of actinomycetes

Actinomycetes were isolated from soil samples procured from the rhizosphere of (about 2-month-old growing crop) Pigeon pea from Lasundra, Vadodara (N22°56'16", E73°22'20"), Gujarat (India) during July 2016. About 1 g of soil sample enriched with calcium carbonate (CaCO₃) was dissolved in 10 mL 0.85% sterile normal saline (SNS) and vortexed meticulously. These samples were serially diluted up to 10⁻³ and spread over sterilized actinomycetes isolation agar media enriched with 50 µg mL⁻¹ each of cycloheximide and nystatin to prevent fungal contamination and incubated at 27 ± 1 °C for 4 days.

Screening of actinomycetes

Fusarium udum and *Streptomyces* sp. S-9 were cultured on potato dextrose agar medium at 28 °C for 7 days. An agar block of indicator fungus was prepared using a sterile cork borer with a diameter of 8 mm and placed at the center of the plate. Inoculate an agar block containing a single colony of actinomycetes around the fungus block 10 mm from the edge of the agar block. (Bredholdt et al. 2007). The plates were incubated at 28 °C, and mycelial growth inhibition was measured after 7 days (Bredholdt et al. 2007). Inhibition of mycelial development against the bacterial isolate was indicative of antagonistic behavior. The percentage of inhibition of radial mycelial growth is determined according to Ji et al. (2013) as follows:

$$\text{Inhibition (\%)} = \frac{\text{Growth diameter in untreated control} - \text{Growth diameter in treatment}}{\text{Growth diameter in untreated control}} \times 100.$$

Phenotypic characterization of *Streptomyces* sp.

Scanning electron microscopy (JEOL JSM-6380 LV, Japan) was used to examine morphological characteristics of cultures grown on ISP 3 agar at 28 °C for 2 weeks (Jin et al. 2019). Cutting a block from an agar plate and fixing it in 2.5 percent glutaraldehyde buffer (pH 7.2) at 4 °C for around 1.5 h yielded samples for scanning electron microscopy. Samples were dehydrated through a graded sequence of ethanol, passed through tertiary-butanol, and then critically point dried after being rinsed twice with phosphate buffer. Under vacuum, the dried samples were mounted on a stub-bearing adhesive and sputter-coated with gold (Guan et al. 2015).

Amplification of 16S rDNA genes by polymerase chain reaction (PCR)

Actinomycetes (*Streptomyces* sp. strain S-9) was inoculated into ISP-1broth and kept overnight for shaking, 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 et al. 1980).

Scanning electron microscopy

For SEM, *F. udum* mycelia samples were fixed in 4% glutaraldehyde at 4 ± 1 °C overnight, then rinsed three times with 0.05 M sodium cacodylate (Sigma-Aldrich, USA) buffer (pH 7.2) for 10 min at 4 ± 1 °C. Samples were subsequently fixed with 1% osmium tetroxide (Sigma-Aldrich, USA) for 2 h at 4 ± 1 °C and washed with distilled water twice briefly. The hyphae were dehydrated in series of ethanol concentrations (50, 70, 80, and 90%) for 10 min each and then in 100% ethanol for 20 min to ensure complete dehydration. The hyphae were then placed in isoamyl acetate. After a critical point in drying, the samples were mounted on stubs and sputter-coated with gold-palladium and examined with the scanning electron microscope (Model-JEOL JSM-6380 LV, Japan) at 20 kV.

Quantification of plant growth-promoting activities

Screening for the plant growth and biocontrol activity performed following standard procedures such as IAA production (Patten and Glick 1996), P-solubilization (Pikovskaya 1948), siderophore production (Schwyn and Neilands 1987), chitinolytic activity (Vyas and Deshpande 1989). The β-1, 3-glucanase activity was assayed using laminarin (from *Laminaria digitata*) (Sigma-Aldrich) as a substrate (Liang et al. 1995).

Pre-emergence wilt incidence (%)

Pre-emergence (symptoms of the disease such as root rot/brownish lesions on root/poor/no radicle emergence) and gravity indices of post-emergence wilt disease were

determined by counting the number of germinating seeds and surviving seedlings (those seedlings that did not display any symptoms of wilt disease such as brownish lesions/premature drooping of leaves/partial or full wilting of part or wholly wilting) among those germinated. A percentage of disease incidences was calculated based on visible wilt symptoms observed on the plant after 15 days up to 35 DAS (Days after sowing). The number of plants infected with *F. udum* was counted in each plot at 90 DAS and the mortality of the plants was determined.

Effect of bacterial inoculation on biocontrol and mitigation of stress under greenhouse condition

Following the characterization of bacterial strains based on their biocontrol attributes under normal and stress conditions in vitro, the bacterial strains were also tested for their competence of biocontrol under plant test using Pigeon pea (*Cajanus cajan*) plastic pot conditions (15 cm in diameter). Experiments were conducted in a completely randomized block design with three replicates in pots containing 2.0 mm sieved unsterilized field soils (2.0 kg soil per pot) of Pulse Research Station, Model Farm, located in Vadodara, Gujarat, India (latitude/longitude 73°1771' N/22°3125' E). Surface-sterilized seeds were sown in each pot (4 seeds pot⁻¹) and daily observations were taken for germination and wilt incidence. Each treatment had three replications. Seven-day-old Pigeon pea seedlings (*Cajanus cajan*) var. BDN-2 was used for transplantation in earthen pots filled with field soil. Plants were grown under natural greenhouse conditions, and the treatments for host plants with concern bacterial strains were as follows: Uninoculated, S-9, S-9 + *Fusarium udum*, and *Fusarium udum*. The surface-sterilized seeds were soaked into the culture with CMC coating broth of *Streptomyces* sp. strain S-9 (10⁸ CFU mL⁻¹) for 2 h. A 100 mL spore suspension (10⁶ CFU mL⁻¹) of *F. udum* was added into the pots having sterile soil, a pot with 20 mL distilled water served as control non-infested control. Pots were watered daily with 20 ml sterile distilled water. The plants were harvested after 30 d and selected parameters such as root length, shoot length, fresh root weight, dry root weight, fresh shoot weight, and dry shoot weight was measured.

T-1—Control (Uninoculated),

T-2—Treated with isolate S-9.

T-3—Treated with isolate S-9 + *F. udum*.

T-4—*F. udum* only.

Plant vegetative parameters, biochemical, and antioxidative assays

Lipid peroxidation assay was carried out by thiobarbituric acid (TBA) (Sigma-Aldrich, USA) method, wherein

thiobarbituric acid reacting substances (TBARS) act as an indicator of membrane lipid peroxidation that was measured in terms of malondialdehyde (MDA) (Sigma Aldrich, USA) concentration (Fazeli et al. 2007). For this purpose, about 0.2 g leaf samples were homogenized in 4 mL of 0.1% trichloroacetic acid (TCA) solution and centrifuged at 10,000 rpm for 10 min, and the supernatant was collected and 1 ml of 20% TCA containing 0.5% TBA was added to 0.5 ml of supernatant. Samples were shaken thoroughly and placed in a boiling water bath for 30 min. They were stored in a cooled ice bath. These samples were again centrifuged at 10,000 rpm for 15 min and supernatants were collected. Their absorbance was measured at 532 and 600. TBARS content was expressed in nmol per g FM.

Leaf chlorophyll was determined using a chlorophyll meter (SPAD-502, Minolta, Japan). Three measurements at random locations in the middle of the leaf were made for each plant and the average used for the analysis. Twenty leaves with incremental chlorophyll levels (determined by SPAD-502 readings) were then harvested to construct a standard curve for quantification of chlorophyll content using the method for chlorophyll analysis described by Arnon (1949).

About 0.5 g of fully expanded 'sun' leaves from field-grown Pigeon pea plants were sampled was homogenized in 10 ml of 3% aqueous sulfosalicylic acid. The homogenate was filtered through Whatman # 2 filter paper. To the 2 ml of filtrate, 2 ml acidic ninhydrin, and 2 ml of glacial acetic acid (Merck, Mumbai) were and incubated at 100 °C for 1 h the reaction was terminated in an ice bath. The reaction mixture was extracted with 4 ml toluene, mixed vigorously for 15–20 s. The chromophore containing toluene was aspirated from the aqueous phase, warmed to 28 °C ± 10 °C and the absorbance was read at 520 nm using toluene for a blank. The proline concentration was determined from a standard curve prepared with pure proline (100 µg/ml) (Bates 1973) and calculated on a fresh weight basis as follows:

$$\text{Proline (mg g}^{-1}\text{)} = \frac{\text{K Value} \times \text{Dilution factor} \times \text{Absorbance}}{\text{Weight of the sample (g)}}$$

Estimation of defense enzymes of leaf samples was done using established standard protocols. The NBT reduction was measured for evaluating the SOD (EC 1.15.1.1) activity as per the protocol of Beauchamp and Fridovich (1971). CAT (EC 1.11.1.6) activity was estimated by the reduction in absorbance by induced decomposition of H₂O₂ in the presence of the enzyme (Aebi 1984).

Detection of H₂O₂ in Pigeon pea leaves

Qualitative assessment of 3, 3'-diaminobenzidine (DAB) staining was performed to capture the H₂O₂ in Pigeon pea

leaf tissues. In short, the leaves harvested from all treatments (Control) were vacuum infiltrated for 5 min into DAB solution (PALL Life Sciences, India) with a concentration of 1 mg ml^{-1} (Rangani et al. 2016). Following this, infiltrated leaves in DAB solution were incubated for 24 h to get stained. After incubation, leaves were washed in distilled water and then, boiled in 95% ethanol for 10 min for the purpose to remove the excess stain (Thordal-Christensen et al. 1997). H_2O_2 in DAB-stained leaves were visualized as reddish-brown coloration.

Histology of Pigeon pea roots under greenhouse conditions

Microscopic examination of changes in root tissue anatomical features was observed following the protocol of O'Brien et al. (1964). Here, hand-cut sections (ca. 10–50 μ) of fresh root materials corresponding to different treatments (Control, S-9, S-9 + *F. udum*, and *F. udum*) allowed soaking for at least 2–3 min. These sections were immersed in a staining solution for 1 min. The staining solution was referred to 0.05% toluidine blue in 0.1 M phosphate buffer of pH 6.8. Following staining, sections were washed with tap water and examined at both $\times 40$ and $\times 100$ under a microscope (Olympus CX1, Leica Microsystems, GmbH, Germany). The root samples were analyzed for the thickness of endodermis; size and number of xylem cells.

Amount of estimation of the contents of N and P in soil

Soil samples were randomly taken from each pot 0–20 cm depth before planting, bulked, air-dried, and sieved using a 2 mm sieve for analysis. The particle size analysis was done by pipette method Gee et al. Soil pH in water was determined using soil: water ratio of 1:2 with a glass electrode pH meter. Organic carbon was determined using Walkley and Black method (Nelson and Sommers 1996). Total nitrogen (N) in

the soil was determined by Kjeldahl digestion Exchangeable bases in the samples were extracted in 1 M $\text{NH}_4 \text{OAC}$ (Sigma–Aldrich, Bangalore) at pH 7.0. Potassium (K) was analyzed by flame photometry. Available phosphorus (P) was determined by Bray-1 extraction and determined colorimetrically by the molybdenum blue procedure Soil samples were air-dried and ground to powder and analyzed with wet digestion method using 5:1:1 ml of $\text{HNO}_3:\text{H}_2\text{SO}_4:\text{HClO}_4$ acid. Total N was determined by micro–Kjeldahl method. For P, K, samples (0.5 g) were ashed, dissolve in 10% hydrogen chloride (HCl), and diluted to 50 ml. Phosphorous was determined using vanadomolybdate colorimetric. The Physico-chemical properties of soil used were analyzed at the Department of Agricultural Chemistry and soil science, Anand Agriculture University, Anand, Gujarat, India.

Statistical analysis

The data obtained were statistically analyzed using one-way ANOVA and Duncan's Multiple Range Test using SPSS (Version 20). Differences were considered statistically significant at $P \leq 0.05$. Data were presented as mean \pm standard error of the mean (SEM) of three replicates except otherwise stated. The graph was prepared using Graphpad Prism (Version 8).

Results

Phenotypic characteristics of *Streptomyces* sp.

The morphology of the strain S-9 culture grown in ISP 3 medium for 4 weeks indicated that it was consistent with the *Streptomyces* genus. The S-9 strain was identified as an aerobic gram-positive actinomycete, which produced a well-developed, branched, and non-fragmented mycelial matrix, but no aerial mycelium. On the substrate mycelium,

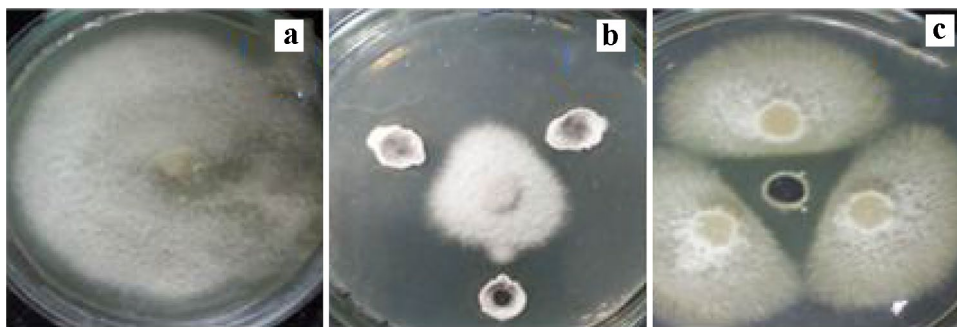


Fig. 1 Suppression of *F. udum* mycelial growth formation by selected bacterial isolates; **a** Control of *F. udum* (**b**), The mycelial growth pattern of *F. udum* in the presence of bacteria. The experiment was per-

formed with three replicates. Images were taken 15 days after inoculation (DAI) (**c**) After 7 days of bacterial treatment and incubated at 28°C , and a clear halo zone (antagonism) was observed

nonmotile and oval spores (2 μm) are formed individually (Supplementary Fig. 1).

Isolation and molecular characterization of actinomycetes

Results indicated that the inhibition zones between bacterial isolates and fungal isolates are generally confirmed by calculating the percent inhibition of radial mycelial development against bacterial isolates was observed (Fig. 1). *Streptomyces* sp. strain S-9 showed 85% mycelial inhibition of *F. udum* (Fig. 1). The 16S rRNA gene sequence of strain S-9 showed a close phylogenetic relationship with *Streptomyces spinoverrucosus* NIIST A67 and *Streptomyces pseudogriseolus* strain 99 (Supplementary Fig. 2). The sequence has been submitted to GenBank under the accession number MK158,952.

Effect of strain S-9 on *Fusarium* morphology

Results showed that the morphological alterations of *Fusarium* pathogens affected by strain *Streptomyces* sp. strain S-9, report of SEM images analysis of hyphae of *F. udum* (Fig. 2a–d). Minor developed dense hyphae revealed typically long, cylindrical cells with a smooth surface. Test strain had fewer hyphae and degenerated mycelia as shown in Fig. 2. In the degenerated hyphae, the wall intruded and formed small depressions at many sites along the hyphae. *F. udum* hyphae were also degenerated, progressively shrunken (Fig. 2b, d). *Streptomyces* sp. strain S-9 had great potential as a biocontrol agent for wilt diseases of Pigeon pea crop. A

summary of SEM of pathogen-bacterial interaction during the antagonism assay is presented in Fig. 2

Plant growth-promoting attributes of actinomycetes

Results observed that the antifungal attributes and root hair formation study according to the standard curve the strain *Streptomyces* sp. strain S-9 had plant growth-promoting traits. Results showed *Streptomyces* sp. strain S-9 able to produce IAA, β -1, 3 glucanase, and P-solubilization. The quantitative estimation of the IAA production in culture broth with tryptophan in the presence of ranged from $16.8 \pm 0.45 \mu\text{g mL}^{-1}$. In the case of P-solubilization by *Streptomyces* sp. strain S-9 observed that it was produced a varying zone of solubilization on rock phosphate around the selected strain colonies. Data showed that the P-solubilization of PGPA varied from 1.5 to 10.5 mm. Nevertheless, isolate *Streptomyces* sp. strain S-9 produced a larger (10.5 mm) zone of P-solubilization. In bacterized Pigeon pea plants, β -1, 3 glucanases activity ($32 \pm 0.20 \text{ ng glucose/min/mg protein}$), thereafter declined gradually. In an assessable assessment of P-solubilization of *Streptomyces* sp. strain, S-9 was $25.50 \pm 0.20 \text{ mg L}^{-1}$, indicating potential P-solubilization degradation by isolating *Streptomyces* sp. strain S-9 (Supplementary Table 1).

Root hair formation study and wilt incidence

S-9 treated seeds showed higher germination percentage, vigor index, and a significant increase in shoot and root

Fig. 2 Scanning electron micrographs (SEM) of pathogen-bacterial interaction during antagonism assay against *F. udum* in dual culture. **a, b** Images of *F. udum* from control plate (**c, d**) in dual culture with *Streptomyces* sp. S-9 (MK158952). Mycelial abnormality is observed along with **a** coagulation of cytoplasm, **b** mycelial shredding and shrinking, **c** leakage of cytoplasm and mycelial breakage, and **d** Perforation, breakage, and shrinking, as compared to growth in the absence of antagonistic agent *Streptomyces* sp. S-9 (MK158952)

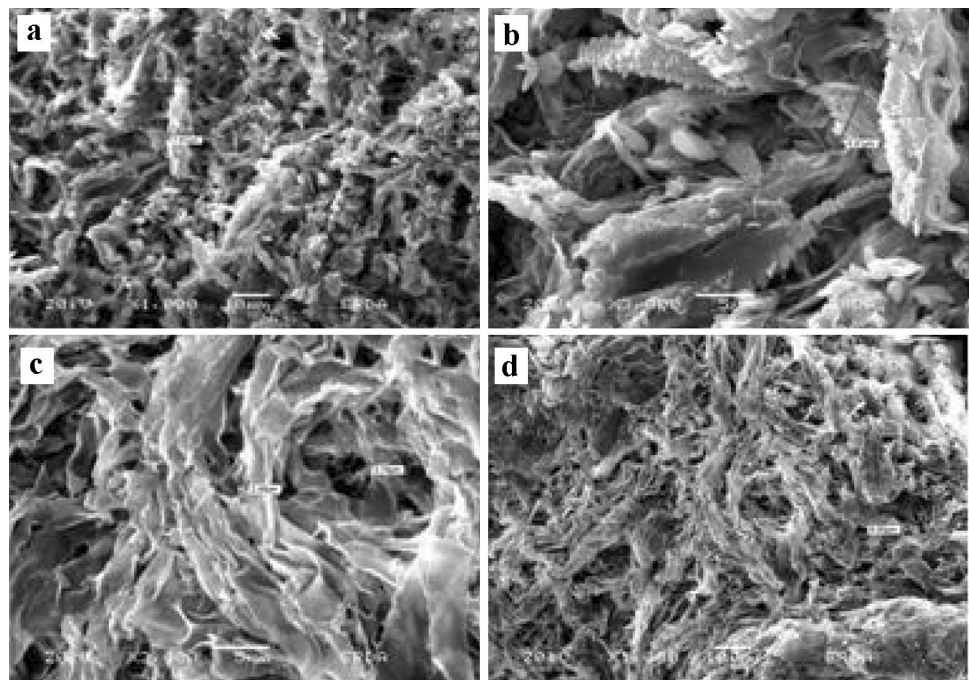


Table 1 In vitro seed germination and Effect of *Streptomyces* sp. Strain S-9 inoculation on seed germination under *F. udum* challenged condition

Treatments	Germination (%)	Seedling length (cm)	Vigor index	Disease severity (%)
T-1	83.33	6.86	571.64	10.23
T-2	100.00	5.99	599.00	12.67
T-3	83.33	6.19	515.81	16.89
T-4	33.33	1.86	61.99	57.50

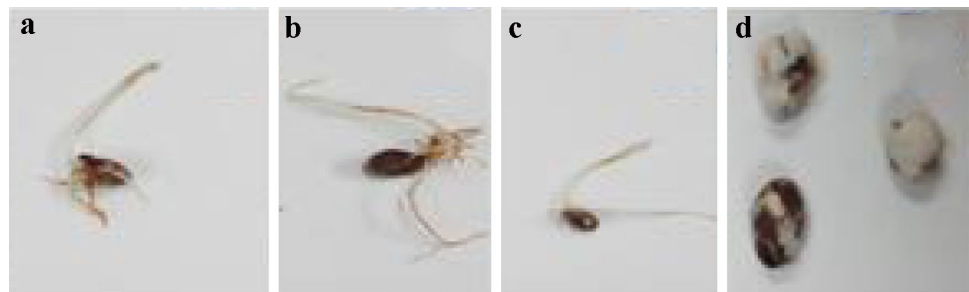
T-1—Control; T-2—S-9; T-3—S-9 + *F. udum*; T-4—*F. udum* only
 DAS Days after sowing, MAS Months after sowing data are presented as mean \pm SD, $n=3$ according to Duncan multiple range test (DMRT) ($P < 0.05$)

lengths. Results observed that the application of *Streptomyces* sp. S-9 alone and in a combination with different treatments significantly influenced germination. Significantly highest (100%) percentage of germination detected T-2 followed by T-1 \geq T-3 and T-4. The same trend was also observed for the seedling length and vigor index (Supplementary Fig. 3; Table 1). Inoculation with bacteria onto seeds promoted a positive effect. Strain S-9 significantly promoted root hair formation in treated seedling roots of Pigeon pea vis-à-vis controls (without bacteria). Seeds treated with *Streptomyces* sp. S-9 showed abundant production of long root hairs. This isolate also encouraged the development of seedlings, including increased root (5.9 cm) and shoot lengths (8.5 cm) and the formation of root hair.

Under pathogen-challenged conditions, the lowest (12.67%) pre-emergence disease incidence was observed in T-2 treatment (*Streptomyces* sp. S-9) followed by T-3 treatment (*Streptomyces* sp. S-9 + *F. udum*) (16.89%), and T-1 (Control) (10.23%). The significantly highest pre-emergence disease incidence (57.50%, respectively) was observed in T-4 treatment having only *F. udum* pre-inoculation. The incidence of wilt in Pigeon pea cultivar BDN-2 was monitored at 30, 60, and 90 DAS, respectively, in pot conditions (Table 2). S-9 treated seedlings challenged with pathogens also exhibited higher germination percentage, vigor index, and a significant increase in root and shoot length over untreated seedlings challenged with pathogens (Table 1; Fig. 3).

Effect of bacterial inoculation on biocontrol and stress mitigation under greenhouse condition

Data showed that the highest root (13.77, 12.50, 10.90, and 12.10 cm) and shoot (31.33, 31.00, 26.67, and 24.67 cm) length was observed with T-2 followed by T-3, T-4, and T-1, respectively (Table 2; Fig. 4). Pots with T-3 treatment combination significantly highest fresh root (1.55 g) and shoot (1.40 g) were recorded. In the case of the dry weight of the root and shoot results were observed in the following order T-3 > T-2 > T-1 and T-4 (Table 2). *Streptomyces* sp. strain S-9 treated Pigeon pea plants revealed a significant increase in the root (13.70 cm) and shoot lengths (31.00 cm), fresh root (1.46 g) and shoot (0.93 g), and dry shoot weights (0.25 g) over the control, it was also observed

Fig. 3 Disease severity of *F. udum* on Pigeon pea with different treatments. **a** Control, **b** T-2 S-9, **c** T-3 S-9 + *F. udum*, **d** T-4 *Fusarium udum***Table 2** Different growth parameters of Pigeon pea plant (BDN2) inoculation (3 MAS)

Treatment	Length (cm)		Fresh weight (g)		Dry weight (g)	
	Root	Shoot	Root	Shoot	Root	Shoot
T-1	12.10 \pm 0.72	26.67 \pm 1.53	1.18 \pm 0.08	0.93 \pm 0.06	0.22 \pm 0.02	0.23 \pm 0.02
T-2	13.77 \pm 0.44	31.33 \pm 1.00	1.55 \pm 0.05	1.40 \pm 0.12	0.42 \pm 0.02	0.29 \pm 0.01
T-3	12.50 \pm 0.61	31.00 \pm 1.53	1.46 \pm 0.06	0.93 \pm 0.10	0.39 \pm 0.03	0.25 \pm 0.02
T-4	10.90 \pm 0.36	24.67 \pm 0.58	0.82 \pm 0.07	0.80 \pm 0.10	0.18 \pm 0.01	0.15 \pm 0.01

Data are presented as mean \pm SD, $n=3$ according to Duncan multiple range test (DMRT) ($P < 0.05$). T-1—Control; T-2—S-9; T-3—S-9 + *F. udum*; T-4—*F. udum*

MAS Months after sowing

Fig. 4 Effect of inoculation with different treatment on the disease incidence and severity of Fusarium wilt and growth on Pigeon pea (90 DAI (days after inoculation) sown in autoclaved soil. Treatments: **a** T-1—Control, **b** T-2—S-9, **c** T-3—S-9 + *F. udum*, **d** T-4—*Fusarium udum*

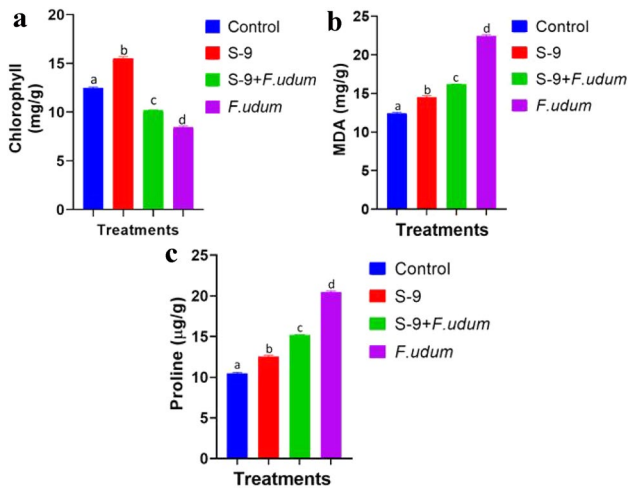


Fig. 5 a–c Chlorophyll, proline, and MDA content in Pigeon pea leaves. The data obtained were statistically analysed using one-way ANOVA and Duncan's Multiple Range Test using SPSS (Version 20). Differences were considered statistically significant at $P \leq 0.05$. Data were presented as mean \pm standard error of the mean (SEM) of five replicates except otherwise stated. The graph were prepared using Graphpad Prism (Version 8)

that the *Streptomyces* sp. strain S-9 treated plants under-challenged inoculation condition resulted in better plant growth and vigor vis-à-vis plants challenge with pathogens in the absence of seed bacterization.

Physiological and biochemical evaluation of plant

The degree of MDA, the last disintegration result of lipid peroxidation inside the leaves test plant treated with *F. udum* was altogether unique to control in all treatments. MDA aggregation in leaves was critical after 8 h of treatment and expanded bit by bit and topped at 48 h, at that point declined subsequently. It was observed in the following order T-3 ($16.66 \mu\text{mol g}^{-1}$) > T-2 ($12.33 \mu\text{mol g}^{-1}$) > T-1 ($10.66 \mu\text{mol g}^{-1}$) and T-4 ($7.66 \mu\text{mol g}^{-1}$). In case of total Proline concentration it was significantly varied in following order T-4 (14.83mg g^{-1}) > T-2 (13.16mg g^{-1}) \geq T-3 (12.00mg g^{-1}) \geq T-4 (11.00mg g^{-1}). However, maximum



Fig. 6 Representative photograph of in vivo DAB staining for visualization of H_2O_2 formed in Pigeon pea leaf at the end of the experiment. The reddish-brown colored spots in the leaves attested the take-up and polymerization of DAB to capture H_2O_2

chlorophyll content was found in treatment (T-4) which consisted of S-9 (Fig. 5a-c).

Effect of bacterial and fungal inoculation on H_2O_2 accumulation of Pigeon pea plant

Biocontrol mediated response of Pigeon pea plant towards H_2O_2 accumulation and modulation of defense enzymes under normal and stress conditions (Fig. 6a–c). In the present study, we observed more accumulation of H_2O_2 in leaves of fungus-treated Pigeon pea plant than in other treatments as indicated through high retention of DAB stain (Fig. 6a). Moreover, bacteria S-9 inoculation had reduced the formation of H_2O_2 in Pigeon pea leaves with fewer reddish-brown spots.

Effect of fungal inoculation on H_2O_2 accumulation and defense enzymes of Pigeon pea

In the present study, we found that in leaves of fungus-treated Pigeon pea plants, DAB stain captured more H_2O_2 than in other treatments (Fig. 6b) compare to control. Besides, Pigeon pea leaves inoculated by bacteria

substantially decreased the formation of H_2O_2 as conferred by the close non-availability of reddish-brown spots (Fig. 6b). At the same time, to analyze the quenching of accumulated H_2O_2 and other oxidative stress in Pigeon pea plants, we conducted defense enzyme assays such as superoxide dismutase (SOD) and catalase (CAT). Fungus-treated plants had observed significantly maximum activity for all antioxidant enzymes considered. However, all four biocontrol treatment has significantly lowered the activity for all the antioxidant enzymes under stress (S-9, S-9 + *F. udum*) when compared with only fungus treatment (Fig. 7). Under stress, *F. udum* challenged plants have exhibited maximum elevation in the defense enzymatic activity by 79.77% and

70.77% for SOD and CAT, respectively, in Pigeon pea plants (Fig. 7). Moreover, S-9 treatment has exhibited the least decrement by 25.24% and 30.61% for CAT and SOD, respectively, in Pigeon pea plants (Fig. 7).

Effect of fungus inoculation on histology of Pigeon pea root

To research the adaptation acquired by the plant in stress environments, the anatomical changes in the root are unique and of utmost importance. We observed some anatomical changes in Pigeon pea root in the current microscopic analysis through various treatments under normal as well as stress

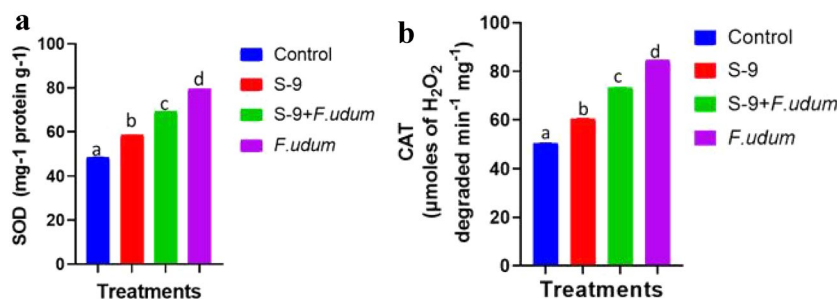
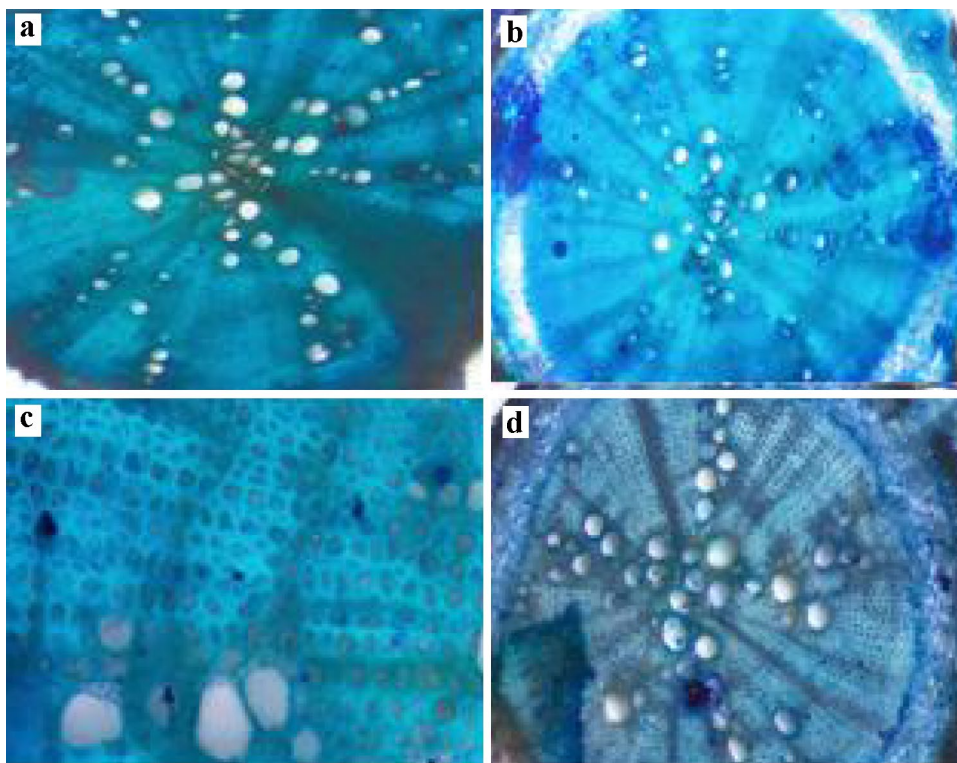


Fig. 7 Defense enzyme activities in Pigeon pea leaves. The data obtained were statistically analysed using one-way ANOVA and Duncan's Multiple Range Test using SPSS (Version 20). Differences were considered statistically significant at $P \leq 0.05$. Data were presented

as mean \pm standard error of the mean (SEM) of five replicates except otherwise stated. The graph were prepared using Graphpad Prism (Version 8)

Fig. 8 Effect of Fungus inoculation on Pigeon pea root anatomy (vascular bundle), root anatomy (epidermis) under normal and stress condition. **a** Root anatomy of Pigeon pea plant under control conditions. **b** Pigeon pea root anatomy (vascular bundle) under control condition. **c** Root anatomy of Pigeon pea plant under stress conditions. **d** Pigeon pea root anatomy (vascular bundle) under stress condition



condition conditions (Fig. 8), to protect against water loss, the root epidermis is essential and gas exchange also helps in the absorption of water and nutrients. In the current analysis, plants treated with bacterial strains showed an improvement in root epidermis thickness under usual and stressful conditions relative to the respective controls. (Fig. 8a, b). In normal conditions, S-9 treated plant roots showed the maximum thickness of endodermis while *Fusarium* inoculated plants showed maximum thickness in stress conditions (Fig. 8c, d). Xylem is an essential vascular tissue from root to stem and leaves to provide water and nutrients. It is fascinating that bacterial treatment has not only increased the size of xylem cells but also increased the number of xylem cells as opposed to regulation (Fig. 8a, b). However, it showed increased xylem size under stress, only *Fusarium* inoculated plant root significantly higher xylem number.

Analysis of soil sample before and after treatment

The soil was slit loamy and slightly alkaline. Supplementary Table 1 shows the values of the soil physicochemical properties of the pot sample soil. There was a decrease in NPK and organic carbon content in the soil treated with *F. udum*. NPK content was higher in S-9 treated soil. Since many of the actinomycete isolates had a phosphate solubilizing activity, the available phosphorous content in the soil increases significantly due to the inoculation of *Streptomyces* sp. S-9. N and P content in the soil as compared to control by the inoculation of *Streptomyces* sp S-9 consistent. Under pot conditions, inoculation with isolate produced significant improvement in N and P content in the soil. Consistently there were seasonal variations among treatments over the year. This might be attributed to variations in bacterial population due to rapid wetting and drying of the soil. As the soil was deficient in available phosphorous and soil pH was very conducive for phosphate solubilization, microbial phosphate solubilization would have played a role in better plant growth and nutrient uptake.

Discussion

In this study, the actinomycete strain S-9 was identified as *Streptomyces* sp. This has shown antifungal activity against plant pathogenic fungal strain and particularly strong inhibition of the pathogen *F. udum*.

Streptomyces sp. isolates S-9 exhibited multiple PGPR traits such as IAA production, phosphate solubilization. This isolate possessing an IAA-producing trait enhanced the growth and nutrient uptake of Pigeon pea, cultivar BDN-2, under potted conditions. The plant rhizosphere is a flexible and dynamic biological condition of intense microbe plant interactions for outfitting fundamental micro-macro

nutrients from a limited supplement pool (Jeffries et al. 2003). The plant advantageous qualities of these microorganisms are associated with various lytic enzymes and metabolites. Either these molecules are liable for the concealment of pathogens using of Production of lytic enzymes and antimicrobial compounds or through ISR mediated plant boosting resistance just as the advancement of plant development through controllers creation (Lutgenberg and Kamilova 2009; Toumatia et al. 2015; Barka et al. 2016; Bubici, 2018). As soil conditions were conducive for IAA production and P-solubilization, this trait could have been involved in inhibiting pathogens in the rhizosphere and thus, suppressing the incidence of diseases (Zhao et al. 2013). If it is believed that IAA affects plant height due to hormonal impact and if increased plant height has a strong positive association with biomass, IAA may be presumed to be involved in boosting growth. The IAA yields of *Streptomyces* sp. strain S-9 were maximum ($60.5 \mu\text{g mL}^{-1}$). Similar results were also reported by Sousa and Olivares (2016). Additionally, our outcomes confirmed that the *Streptomyces* sp. S-9 additionally produces an enzyme of β -1, 3 glucanase that is responsible for indirect growth promotion of the test plant via inhibition of phytopathogenic fungi consisting of *F. udum* (Anupama et al. 2015).

Streptomyces sp S-9 decreased the infection in plants and it demonstrated the best in diminishing infection rate because of *F. udum* (Kumar et al. 2010). It has been observed in our experiments that *Streptomyces* sp. isolate S-9 which consistently enhanced growth, and the use of nutrients Pigeon pea under potted conditions had multiple plant growth-promoting traits. For a certain point in time, all the PGPR features could not be expressed. Thus the continuous supply of available nutrients was required for sustaining plant growth and development (Verma et al. 2015).

The potential of *Streptomyces* sp. S-9 was further verified by conducting potted plant growth experiments. Improved plant biological characteristics, such as shoot length, root length, and biomass, establish the PGP potential of *Streptomyces* sp. S-9.

Besides conferring protection against *Fusarium* infection, *Streptomyces* sp S-9 increased growth of *Cajanus cajan* seedlings as evidenced by an increase in plumule and radicle lengths and overall weight. Treatment of seeds with cultures especially, *Streptomyces* sp. S-9 resulted in a considerable increase in radical length. The effect was enhanced when the seed was treated with *Streptomyces* sp. S-9. An increase in radical length is important for seedlings as the increase in the radicle length increase the root surface area that results in increased water and mineral absorption from soil hence faster and enhanced growth of the plants (Fassler et al. 2010).

Streptomyces sp. S-9 effectively reduced the wilt rate on the *Cajanus cajan* plant. In this study, *Streptomyces*

sp. S-9 has been shown to significantly reduce Fusarium head blight and populations in the rhizosphere. Numerous microorganisms colonize the rhizosphere improve the harmful impacts of biotic stress, and upgrade plant development and advancement.

Plants infected with *F. udum* in the absence of *Streptomyces* strain as biocontrol, agents reduced the defense-related antioxidant enzyme activity (CAT and SOD). In response to pathogen infection, numerous studies have revealed that PGPA can activate a variety of defense responses in host plant structure, including the activation of antioxidant defense enzymes (Senthilraja et al. 2013; Bano and Muqarab 2017).

In the present study, we found that inoculating plants with *Streptomyces* sp S-9 significantly increased nutritional and biochemical indices such as chlorophyll content (total). Furthermore, under stressful conditions, Proline accumulation was significantly reduced. Previous studies have shown that inoculating PGPA from other host plants under stress circumstances has similar effects (Ullah and Bano 2015; Vurukonda et al. 2016; Li and Jiang 2017). The bacterial strain S-9 (*Streptomyces* sp.) was more effective in improving plant vegetative qualities, which could play a role in stress reduction and growth promotion.

Proline accumulation in plants is an important process that plays an important role in maintaining the osmotic balance between the intracellular and extracellular spaces while minimizing damage from stress (Lei et al. 2016).

Antioxidant enzymes regulate ROS production and play an important role in stressed plants. The results of this study showed that the number of antioxidant enzymes was significantly reduced in the Pigeon pea plants that were inoculated with bacteria under stress. In addition, in this study, bacterially treated Pigeon pea plants under upper and stress conditions had thicker root epidermis and increased xylem tissue as an anatomical result compared to their respective control groups. Bacterial-mediated increases in the thickness of the epidermis of pigeon roots and the number of xylem tissues have been demonstrated by previous studies, but only under normal conditions (Rêgo et al. 2014). Some studies have shown that increasing the number and diameter of all vessels promotes water-stress tolerance in legumes (Choate et al. 2008; Purushothaman et al. 2013).

Soil microbes play a very important role in agricultural productivity, mainly by improving soil quality (Barea et al. 2013; Lugtenberg 2015). This study sees that PGPA increases soil fertility, which positively affects that indicator. This is consistent with several previous studies that showed that the total organic carbon content of the soil was directly proportional to PGPA (Valarini et al. 2003; Wu et al. 2005). pH has been reported as an important factor affecting phosphate availability and mineralization from soil (Wu et al. 2005). This discovery was made by

Srivastava et al. 2014. PGPA improved soil organic matter content and soil properties, such as total nitrogen, and texture (Wu et al. 2005). Soil pH and EC are commonly used as soil health indicators and have been reported to alter the biological composition of the soil (XiuMei et al. 2008). In addition, based on the results of pot experiments, the possibility of the strain *Streptomyces* sp. S-9 for improving the growth of pigeon bean plants was evaluated. The results of the greenhouse experiment showed that *Streptomyces* sp. S-9 inoculation resulted in better plant growth compared to the control group and the 100% recommended NPK.

Conclusions

Application of *Streptomyces* sp. strain S-9 on a soil–plant system under greenhouse/field conditions can be a valuable tool for increased Pigeon pea growth and development; therefore, in this study, an examination of *Streptomyces* sp. strain S-9 with different treatment combinations to plant growth-promoting traits was carried out under in vitro and pot conditions. Influence of *Streptomyces* sp. strain S-9 production of plant growth traits such as antagonistic activity against the fungal pathogen *F. udum* and in controlling the wilt disease in Pigeon pea. We also reported that plant development advancing traits IAA and P-solubilization stimulated the vegetative and reproductive growth of Pigeon pea. Strains were able to efficiently plant growth promotion, therefore, showed great potential for use as novel biofertilizers. Overall, the efficient application of PGPA can be an alternative and promising technology to Pigeon pea crop sustainability under sustainable agriculture. In short, these strains of actinomycetes may be potential biological control candidates for the ecological management of Fusarium wilt and, therefore, may limit the excessive use of synthetic fungicides and their associated harmful effects on humans and the environment.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s13205-021-02989-0>.

Acknowledgements This work was supported in part by University Grants Commission (UGC) for providing Basic Science Research (BSR) fellowship. We are also grateful to Department of Microbiology and Biotechnology Centre, The Maharaja Sayajirao University of Baroda for providing basic infrastructure to support this research.

Author contributions SI conceived and co-ordinate the research. AD conducted experiments and analyzed the data. AD and SI wrote the manuscript. Both authors read and approved the manuscript.

Funding This work was supported in part by University Grants Commission (UGC) for providing Basic Science Research (BSR) fellowship (Grant No. F. 25-1/2014-15(BSR)/7-128/2007(BSR)). We are also

grateful to Department of Microbiology and Biotechnology Centre, The Maharaja Sayajirao University of Baroda for providing basic infrastructure to support this Research.

Data availability All data generated or analysed during this study are included in this published article and additional supplementary file.

Declarations

Conflict of interest The authors declare no conflicts of interest.

Human and animal rights This article does not contain any studies with animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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