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

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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 strain was identified on the basis of 16S rRNA sequence analysis. The strain's morphological and chemotaxonomic characteristics also endorsed its identification as *Streptomyces*. As a biocontrol agent, *Streptomyces* sp. S-9 caused 70% inhibition of the pathogen and showed various attributes of plant growth promoting like production of IAA, siderophore, P-solubilization and, S-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%), catalase (CAT; 24–56%), and peroxidase (POX; 26–44%). A marked reduction in antioxidant enzymes were associated with the antagonistic effects of the different treatments. Antifungal compound was extracted from the culture broth by the results promise the use of plant growth-promoting actinomycetes (PGPA) for active induction of systemic resistance against Fusarium wilt in the plant pigeon pea. Conclusions showed that S-9 bioinocula applied as a seed coating enhanced soil availability of 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.


# Declarations

### Author contributions

AD performed the experiments. AD designed and analyzed the data of the experiments. SI as research supervisors was involved in planning, execution, and contributed reagents/ materials to carry out the experiments. All the authors have contributed towards manuscript preparation and also approved the manuscript.

### Compliance with ethical standards

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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### Data availability

Most of the data supporting the conclusions of this article are included within the article The GenBank/EMBL/DDBJ Accession Number for the 16S rRNA gene sequence of strain S-9 is MK158952. . Other datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

# Introduction

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.

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). In view of these highlights, it isn't astounding that assorted Streptomyces strains had been concentrated to control fungal and bacterial diseases of rice like Bacterial Leaf Blight caused by *Xanthomonas oryzae*, however very few *Streptomyces* are currently being developed as biocontrol products.

Plant growth-promoting actinomycetes (PGPA) are root colonizing microbes with beneficial effects including plant growth-promotion and disease control. Pigeon pea (*Cajanus cajan* (L.) Mill sp. is the most extensively used pulse (legume) crop in India grown over a 72% area of 3.9 Mha. However, pigeon pea suffers high mortality because of serious seed and soil-borne fungal pathogens like *F. udum* and *F.oxysporum*. Economically important crops like 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 *F. 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.

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* are able to 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 like 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) 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 1 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 five 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 two-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 ($CaCO_3$) was dissolved in 10 mL 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 $\mu gmL^{-1}$ each of cycloheximide and nystatin to prevent fungal contamination and incubated at 27±1°Cfor 4 days.

### Screening of actinomycetes

*In-vitro* screening for antagonistic activity was carried out on PDA plates using the dual culture technique. In the center of each plate was mounted a 5 mm agar disk of a rapidly growing culture of *F.udum*. The plates were parafilm and incubated at 27 ± 2 ° C for 5 days. 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 4 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 hours 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 spatter-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 [2] 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'(Kieser et al. 2000). Phylogenetic relatedness of the isolate was determined by constructing the dendrogram using Mega 7.

**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 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 (Dukare et al., 2011). A percentage of disease incidences were calculated based on visible wilt symptoms observed on the plant after 15

days up to 35 DAS. 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 12 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 3 replications Seven-day-old Pigeon pea seedlings (*Cajanus cajan*) var. *BDN2* was used for transplantation in earthen pots filled with field soil Plants were grown under natural greenhouse conditions, and the treatments for host plant with concerning for 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($10^8$ CFU $mL^{-1}$) for 2 h. A 100 mL spore suspension ($10^6$ CFU $mL^{-1}$) of *F. udum* was added into the pots having sterile soil, a pot with 100 mL distilled water served as control non-infested control. These pots were kept under greenhouse conditions (23-26°C and 5 h/9 h light/dark period) daily with 2% Hoagland solution ($KNO_2$ 606.60 $CaNO_2$ 656.40; $MgSO_4$ 240.76; $(NH_4)PO_4$ 115.03; $MgCl_24H_2O$ 1.81; boric acid 2.86; Mo 0.016; $ZnSO_4$ $7H_2O$, 0.22; $CuSO_4$ $5H_2O$ 0.08 $FeCr_2O_7$ 5.00) weeks of culture the root length, shoot length, dry and fresh weight was assessed for seedlings (Goudjal et al., 2016).

**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 sec. 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

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

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). The ascorbate peroxidase (APX; EC 1.11.1.3) was assayed by estimating the decrease in absorbance (290 nm; an absorbance coefficient 2.8 mM–1cm–1) for the rate of oxidation of ascorbate (Nakano and Asada 1981). Guaiacol peroxidase (EC 1.11.1.7) activity was determined by monitoring the oxidation of guaiacol (Hemeda and Klein 1990). CAT (EC 1.11.1.6) activity was estimated by the reduction in absorbance by induced decomposition of $H_2O_2$ in the presence of the enzyme (Aebi 1984). The polyphenol oxidase (EC 1.10.3.1) assay was performed and defined as the increase in absorbance (420 nm) by 0.1 min-1 with an increase in the amount of purpurogallin formed in the reaction (Patra and Mishra 1979).

**Detection of $H_2O_2$ in Pigeon pea leaves**

Qualitative assessment of 3, 3’-diaminobenzidine (DAB) staining was performed to capture the $H_2O_2$ 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 of 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 ×40 and ×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 0-20cm depth before planting, bulked, air-dried, and sieved using 2mm sieve for analysis. The particle size analysis was done by pipette method Gee et al.33 Soil pH in water was determined using soil: water ratio of 1:2 with a glass electrode pH meter. Organic carbon was determined using Walkey 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 1M NH4 OAC (Sigma Aldrich, Banglore) 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 $HNO_3$: $H_2SO4$: $HClO_4$ acid. Total N was determined by micro–Kjeldahl method (Jackson, 1962). For P, K, samples (0.5g) were ashed, dissolved in 10% hydrogen chloride (HCl), and diluted to 50 ml. Phosphorous was determined using vandal molybdate 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 was recorded in triplicate and analyzed, using the SPPS software version 18. Analysis of variance was determined and the mean values were compared by Duncan’s multiple range test P

< 0.05. Standard error of means values is depicted in the graphs as bars.

# Results

### Isolation and characterization of actinomycetes

*Streptomyces* sp. train S-9 was isolated from rhizosphere soil of Pigeon pea and it showed (its significant superiority over the rest of the isolates); it was the most promising antagonist 85% mycelial inhibition of *F. udum* (Fig. **1**) *Streptomyces* sp. strain S-9 showed its significant superiority over the rest of the isolates; it was most promising antagonist 85% mycelial inhibition (Fig. **1**) on that basis it was selected for further investigations. 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 (Fig. **3**). The 16S rRNA gene sequence of the isolate was submitted to the GeneBank (NCBI) under the name *Streptomyces* sp. strain S-9 (MK158952).

# Phenotypic characteristics of Streptomyces sp.

The morphology of 4-week-old cultures of strain S-9 grown on ISP 3 medium indicated it was consistent with the Streptomyces sp. genus. Strain S-9 was identified as an aerobic, Gram-positive actinobacterium which produced well-developed, branched, and non-fragmented substrate mycelium but no aerial mycelium. On the substrate mycelium, non-motile and oval spores (2μ m) formed singly. (Fig. **3**).

### 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. **4** a-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. **4**. 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. **4** b and d). *Streptomyces* sp. strain S-9 had great potential as a biocontrol agent for wilt diseases of pigeon pea crop. A short summary of SEM of pathogen-bacterial interaction during the antagonism assay is presented in Fig. **4**.

### 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 glucanases and P- solubilization. The quantitative estimation of the IAA production in culture broth with tryptophan in presence of ranged from 16.8 ±0.45 $\mu gmL^{-1}$. 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 wheat plants, β-1, 3 glucanase activity (32 ± 0.20 ng glucose/min/mg protein), thereafter declined gradually. In an assessable assessment of P-solubilization of *Streptomyces* sp. strain, S-9 was 25.50±0.20 $mgL^{-1}$, indicating potential P-solubilization degradation by isolating *Streptomyces* sp. strain S-9 (Table **1**). However, there was no zone to inhibit at all around the disc of the tested isolate indicating no hydrolysis of the chitin (Table **1**).

### Root hair formation study

Results observed that the application of *Streptomyces* sp. S-9 alone and in a combination with different treatments significantly influenced germination (Fig. **5**), root and shoot growth of the test plant. Significantly highest (100%) Percentage of germination detected T2 followed by T1 ≥ T3 and T4. The same trend was also observed for the seedling length and vigor index (Table **2)**. 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 seedling, including increased root (5.9 cm) and shoot lengths (8.5 cm) and the formation of root hair.

### Effect on pre- wilt disease incidence

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%) (Fig. **6**). The significantly highest both 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 BDN2 was monitored at 30, 60, and 90 DAS, respectively, in pot conditions(Table **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 T2 followed by T3, T4, and T1, respectively (Table **4**, Fig **7)**. Pots with T3 treatment combination significantly highest fresh root (1.55 g) and shoot (1.40g) were recorded. In the case of dry weight of the root and shoot results were observed in the following order T3 > T2 > T1 and T4 (Table **4**). *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 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 absence of the 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 in relation to control in all testing. 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 T3 (16.66µ mol $g^{-1}$) > T2 (12.33µ mol $g^{-1}$) > T1 (10.66µ mol $g^{-1}$) and T4 (7.66µ mol $g^{-1}$). In case of total proline concentration it was significantly varied in following order T4 (14.83mg $g^{-1}$) > T2 (13.16mg $g^{-1}$) ≥ T3 (12.00mg $g^{-1}$) ≥ T4 (11.00mg $g^{-1}$). However, maximum chlorophyll content was found in treatment (T4) which consisted of S-9 (Fig. **8 a-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. **9 a-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. **9 a**). 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. **9 b**) compare to control. Also 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. **9 b)**. 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), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), catalase (CAT) and polyphenol oxidase (PPO). 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. **10)**. Under stress, *F.udum* challenged plants has exhibited maximum elevation in the defense enzymatic activity by 79.77%, 84.75%, 74.11%, 70.77%, and 57.75% for SOD, APX, GPX, CAT, and PPO respectively in Pigeon pea plants (Fig. **10)**. Moreover, S-9 treatment have exhibited least decrement by 54.70%, 25.24%, 30.61%, 57.78% and 51.70% for APX, CAT, SOD, GPX and PPO respectively in Pigeon pea plants (Fig. **10**).

### Effect of fungus inoculation on histology of Pigeon pea root

In order 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 condition conditions (Fig. **11**), to protect against water loss, the root epidermis is essential and gas exchange also help 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. **11** and 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. **11** c and 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. **11** a and b). However, it showed increased xylem size under stress, only Fusarium inoculated plant root significantly higher xylem number (Fig. **11** c and d).

### Analysis of soil sample before and after treatment

The soil was slit loamy and slightly alkaline. Table **5** 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 increase 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 sp S-9 consistent. Under pot conditions, inoculation with isolates 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 conductive for phosphate solubilization, microbial phosphate solubilization would have played a role in better plant growth and nutrient uptake.

# Discussion

The plant rhizosphere is a flexible and dynamic biological condition of intense microbes plant interactions for outfitting fundamental micro-macro nutrients from a limited supplement pool (Jeffries et al., 2003). In the present investigation, one sixty- five actinomycetes isolates were tested for their antagonism nature against *Fusarium udum*, the causal agent of wilt of Pigeon pea disease. 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).In this study, the actinomycete strain S-9 was identified as *Streptomyces* sp. ; This has shown antifungal activity against several plant pathogenic fungal strains and particularly strong inhibition of the pathogen *F. udum*. 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). Microbial blends improve the growth and development of plants (Kumar et al., 2010). *Streptomyces* sp. isolate S-9 exhibited multiple PGPR traits like IAA production, phosphate solubilization besides. These isolate possessing IAA-producing trait, enhanced the growth and nutrient uptake of Pigeon pea, cultivar BDN-2, under potted condition. 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 $\mu gmL^{-1}$), which was considered superior over the rest of the isolates. Similar results were also reported by Sousa and Olivares (2016). Additionally, our results showed that the *Streptomyces* sp. strain S-9 also produces enzyme of β- 1, 3 glucanase which is responsible for indirect growth promotion of the test plant through inhibition of phytopathogenic fungi such as *F. udum* (Anupama et al., 2015). Seed bacterization of these isolates expanded the root length, plant biomass, plant height in pots essentially over control and consistently on potted conditions over the years. 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). Root apical meristems appeared to be in a better condition in seedlings inoculated with bacteria than controls treatment. Characterization of novel antagonistic bacterial strains and evaluation of their antagonistic potential is important for a better understanding of the ecological significance of the biocontrol of plant diseases. The microbial blends including *S. fredii* KCC5 and *P. fluorescens* LPK2 decreased wither illness, demonstrated the best in diminishing infection rate because of *F. udum* (Kumar et al.2010). Lipid peroxidation is commonly associated with oxidative damage (Sánchez Rodríguez et al., 2010) when the levels of ROS exceed the capacity of the antioxidant defense system (Mittler, 2002). In our study, the level of TBARS a general marker of oxidative stress–produced during peroxidation of membrane lipids was increased in the leaves of plants subjected to oxidative stress at the end of the experimental period(45dpi) ,indicating drought-induced oxidative injury. A Larger fall in the chlorophyll content may depict its susceptibility to water deficit. In the present study, proline content increased significantly in S-9-primed plants compared to CC plants. 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). Alleviation of induced oxidative damages with the use of antioxidant enzymes is an important strategy of

plants for increasing their tolerance to stress conditions. In the present work, increased activities of various antioxidant enzymes following fungal inoculation illustrated that these enzymes play a crucial role in the protection of plants under stresses. *A. xylosoxidans* increase the antioxidant activity in *Catharanthus roseus* (Karthikeyan et al., 2008) and the *Solanum melongena* inoculated with *Pseudomonas* sp. DW1 (Fu et al., 2010). The bacterial SOD facilitates the removal of free radicals and plays an important role in their survival in the rhizosphere (Wang et al., 2007). The major breakdown product of SOD is $H_2O_2$, which is a toxic lipid peroxidant, but can be eliminated by activities of CAT and POX antioxidant enzymes. The POX activity plays a major role in eliminating the stress-induced $H_2O_2$ and malondialdehyde level, thus protecting the cell membrane integrity. Our data showed that activities of SOD, CAT, and POX enzymes in leaves of *Fusarium udum* inoculated plants were higher compared to uninoculated plants under stress. The increase in enzyme activities was probably because bacterial inoculation stimulated the synthesis of these enzymes (Wang et al., 2010). Moreover, in the present study, anatomical results showed that the root epidermis become thickened along with increased xylem tissues in bacteria- treated rice plants under normal and stress conditions as compared to respective controls. Bacteria-mediated increase in the thickness of rice root epidermis as well as the number of xylem tissues was demonstrated by the previous study but under normal conditions only (Rêgo et al. 2014). Increased root epidermis may result in preventing water loss, better physiological and metabolic activities of plants, and enhanced resistance to stress (Momayezi et al. 2012). An earlier report has established that rice plants with more xylem vessels allow better water conduction (Rêgo et al. 2014). Some studies showed that increasing the number and the diameter of xylem vessels promotes water stress resistance in legume crops (Choat et al. 2008; Purushothaman et al. 2013). 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. Under rain- fed, Temperature variations, pest and disease incidence, which affected the performance of Actinomycetes. As the soil was deficient in available phosphorous and soil pH was very conductive for phosphate solubilization, microbial phosphate solubilization would have played a role in better plant growth and nutrient uptake. Biological nitrogen fixation requires a bulk of the absorbed phosphate from soil to produce ATP which is required by atmospheric nitrogen fixation through Pigeon pea –Streptomyces symbiosis. Thus the continuous supply of available phosphorous was required for sustaining enhance the biological nitrogen fixation process. It would have been possible through phosphate solubilization. It has been reported earlier that inoculation of phosphate solubilizing microorganisms enhanced the growth and yield of canola but not the phosphorous uptake (De Freitas et al., 1997).The present study also indicated a similar effect of a low correlation between soil phosphorous content and P uptake in soil.

# 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 combination 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.

# Acknowledgements

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# Tables

**Table 1 Plant growth promoting traits of *Streptomyces* sp. Strain S-9**

| PGPA | IAA (µg/ml) | Phosphate solubilization ($mgL^{-1}$) | β-1,3 glucanase (ng glucose/min/mg protein) |
|---|---|---|---|
| S-9 | 16.8 ±0.45 | 25.50±0.20 | 32 ± 0.20 |

**Table 2 *In-vitro* seed germination test (7DAS)**

| Treatments | Germination (%) | Seedling length(cm) | Vigor index |
|---|---|---|---|
| **T-1** | 83.33 | 6.86 | 571.64 |
| **T-2** | 100.00 | 5.99 | 599.00 |
| **T-3** | 83.33 | 6.19 | 515.81 |
| **T-4** | 33.33 | 1.86 | 61.99 |

Treatment T-1 Control; T-2 S-9; T-3 S-9+*F.udum*; T-4 *F.udum* only.

DAS: Days after sowing

**Table 3** Effect of Streptomyces sp. Strain S-9 inoculation on seed germination under *F. udum* challenged condition

| Treatment | Disease severity(%) |
|---|---|
| T-1 | 10.23 |
| T-2 | 12.67 |
| T-3 | 16.89 |
| T-4 | 57.50 |

T-1 Control, T-2 S-9, T-3 S-9+*F.udum*, T-4 *F.udum* only

MAS: Months after sowing Data is presented as mean ± SD, n=3 according to Duncan multiple range test (DMRT) ($P < 0.05$).

**Table 4** 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±0.72 | 26.67±1.53 | 1.18±0.08 | 0.93±0.06 | 0.22±0.02 | 0.23±0.02 |
| T-2 | 13.77±0.44 | 31.33±1.00 | 1.55±0.05 | 1.40±0.12 | 0.42±0.02 | 0.29±0.01 |
| T-3 | 12.50±0.61 | 31.00±1.53 | 1.46 ±0.06 | 0.93±0.10 | 0.39±0.03 | 0.25±0.02 |
| T-4 | 10.90±0.36 | 24.67±0.58 | 0.82±0.07 | 0.80±0.10 | 0.18±0.01 | 0.15±0.01 |

Data is presented as mean ± 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

**Table 5: Nutrient status under potted conditions during rainy seasons at harvest**

| Treatment | Organic Carbon(%) | Nitrogen(%) | Phosphorous(kg/ha) | Potassium(kg/ha) | pH | Elctro conductivity |
|---|---|---|---|---|---|---|
| T-1 | 0.15 | 1.62 | 8.11 | 249 | 7.8 | 0.22 |
| T-2 | 0.17 | 2.68 | 32.46 | 259 | | 0.2 |
| T-3 | 0.12 | 0.18 | 8.49 | 240 | | 0.2 |
| T-4 | 0.11 | 0.16 | 7.23 | 228 | | 0.20 |

T-1 Control; T-2 S-9; T-3 S-9+*F.udum*; T-4 *F.udum* only

# Figures

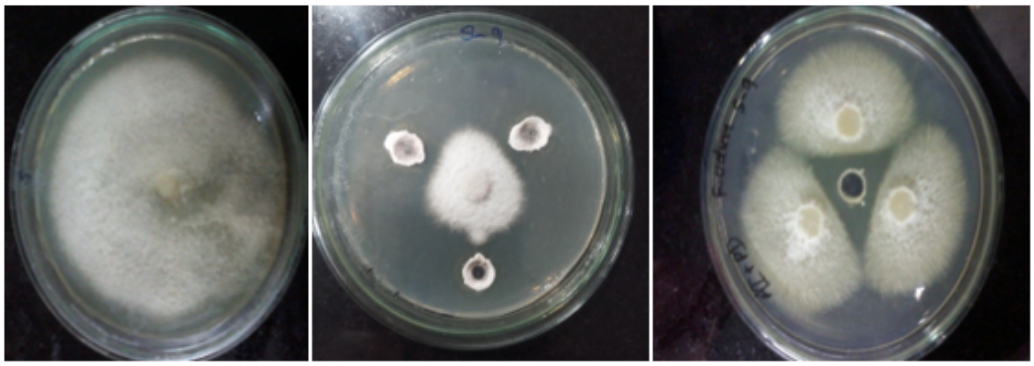



**Figure 1**

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 performed with 3 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.

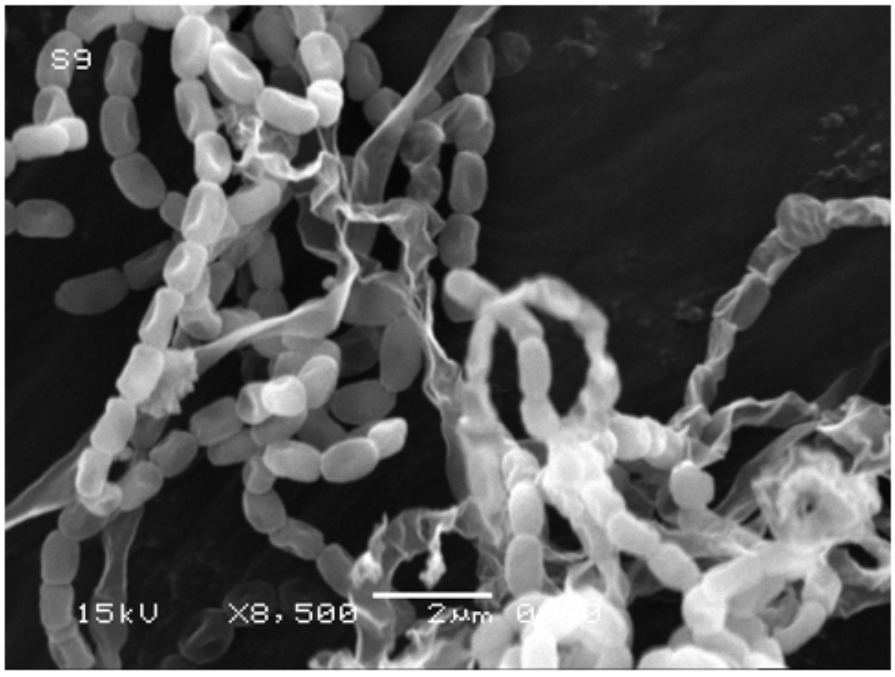
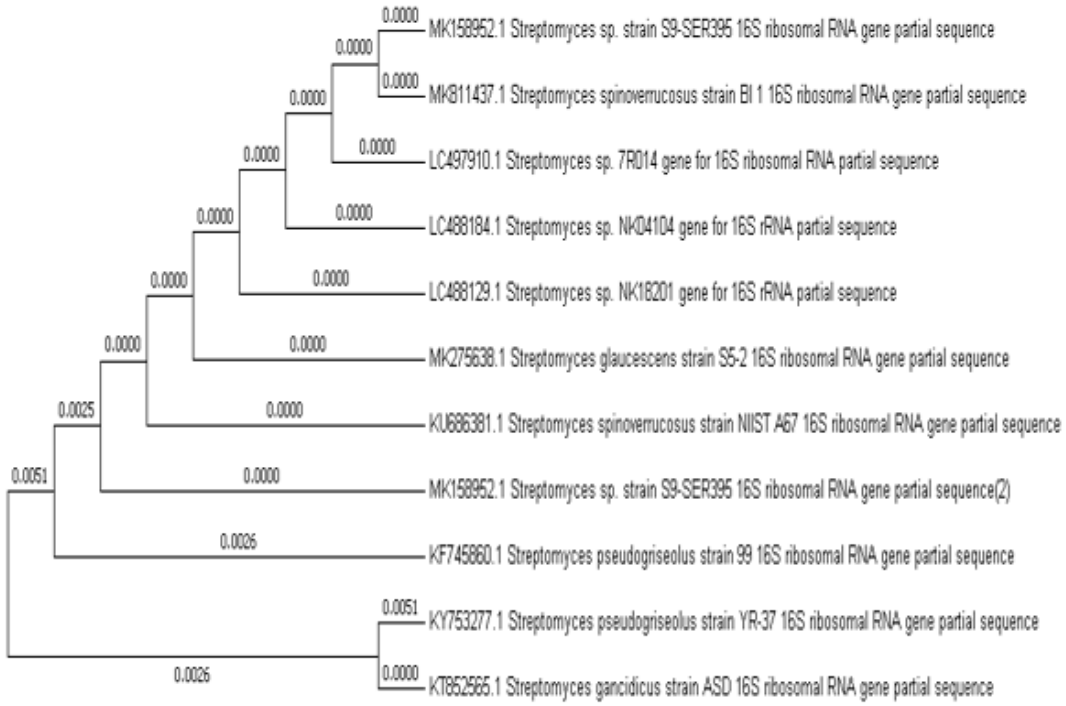



**Figure 2**

Scanning electron microscopy of strain S-9 grown on ISP 3 for 4 weeks at 28 °C. Bar, 2.0 µm




## Figure 3

Molecular phylogenetic analysis by maximum likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura 1980). Evolutionary analyses were conducted in MEGA 7.

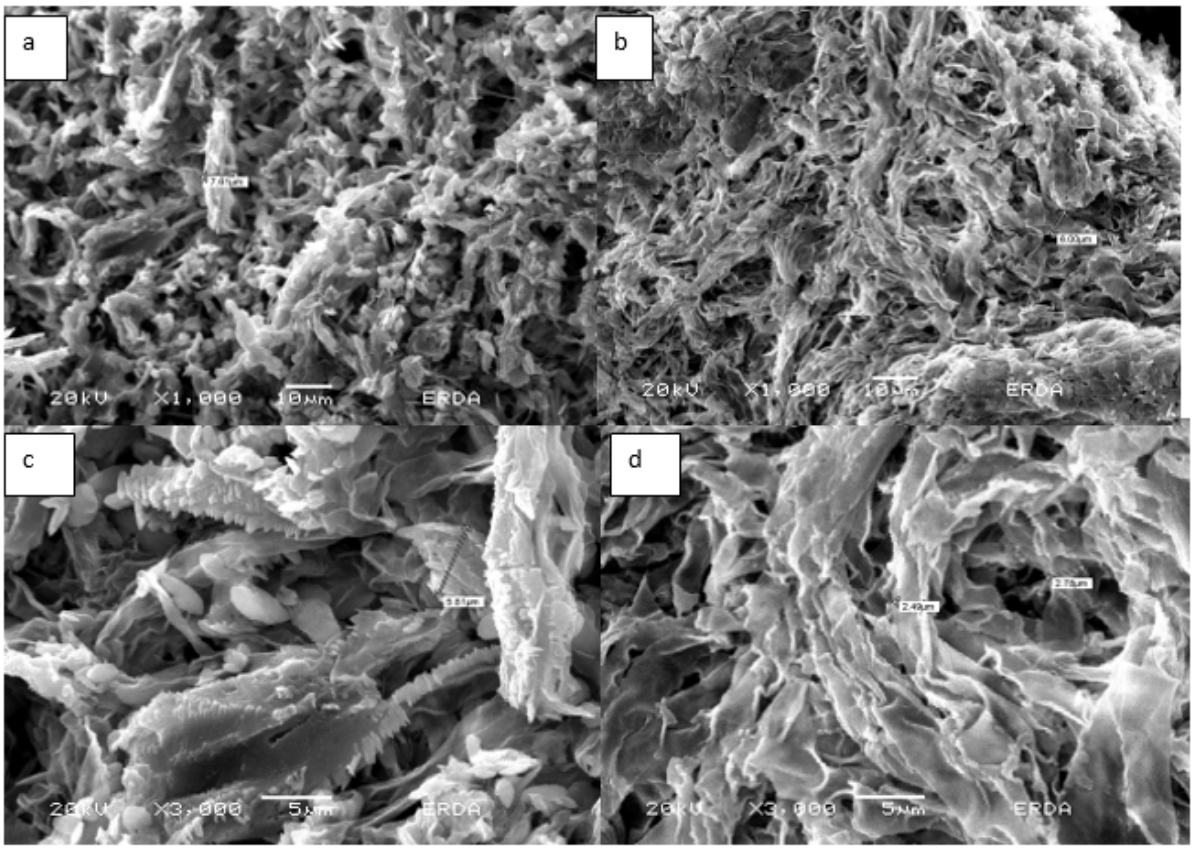



**Figure 4**

Scanning electron micrographs (SEM) of pathogen-bacterial interaction during antagonism assay against F. udum in dual culture. (a and b) Images of F. udum from control plate (c and 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, (d) Perforation, breakage and shrinking, as compared to growth in absence of antagonistic agent Streptomyces sp. S-9 (MK158952)

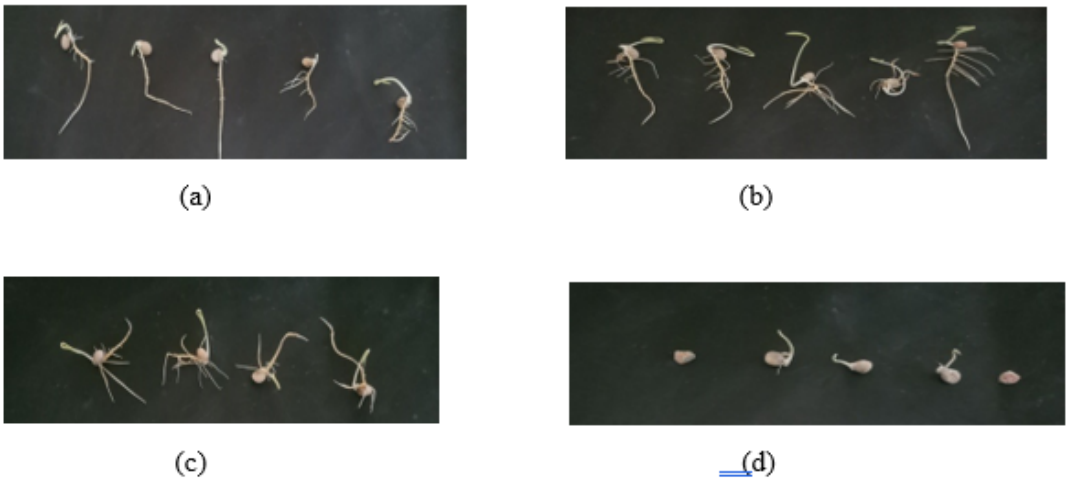



**Figure 5**

Seed germination of Pigeon pea (7DAS) with different treatment a) T-1 Control; (b) T-2 S-9; (c) T-3 S-9+F.udum; (d) T-4 Fusarium udum

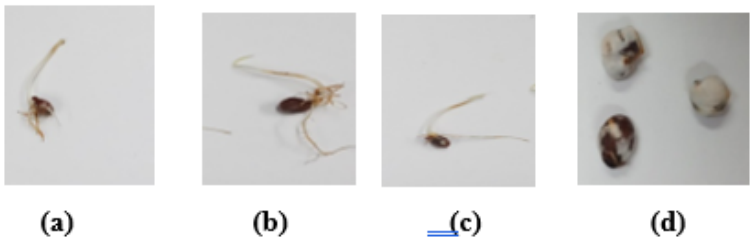



**Figure 6**

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

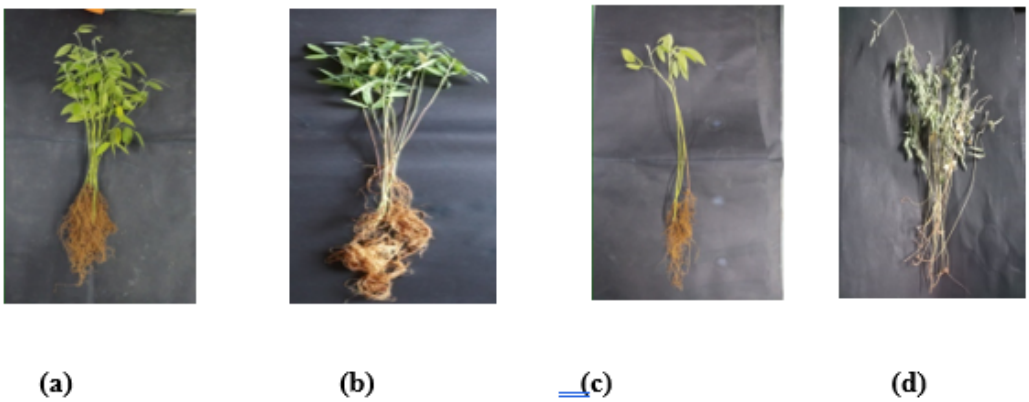



**Figure 7**

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

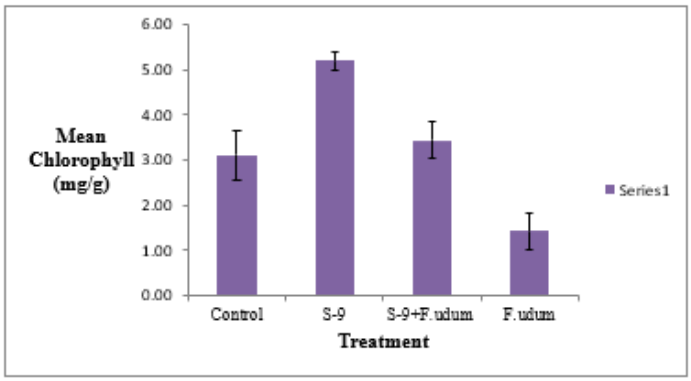



Fig. 8 (a) Total Chlorophyll content

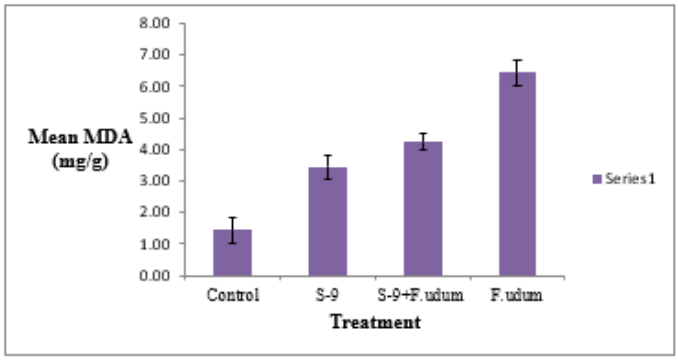



Fig. 8 (b) Total MDA content

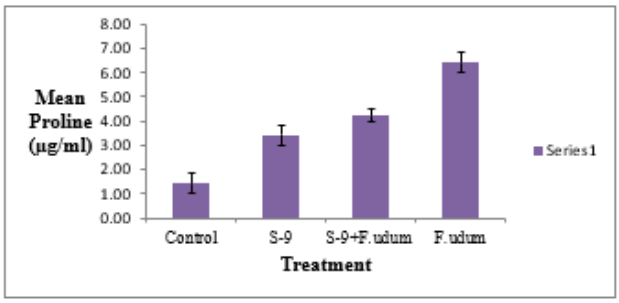



Fig. 8 (c) Total Proline content

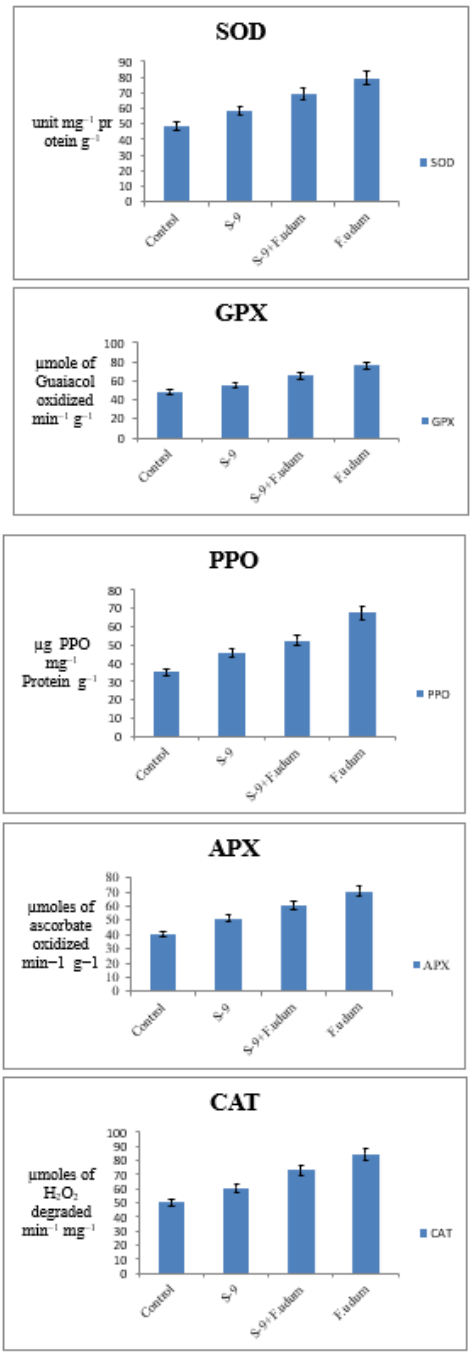
## Figure 8

(a-c) Chlorophyll, proline, and MDA content in Pigeon pea leaves. The values shown here are the mean of three replicates. Errors bars represent standard errors. Diferent letters above the bars represent signifcant diferences according to the analysis of variance (ANOVA), followed by the Duncan test ($p \leq 0.05$) applied using the software SPSS ver 20.

a. Control | b. S-9 | c. Fusarium infected

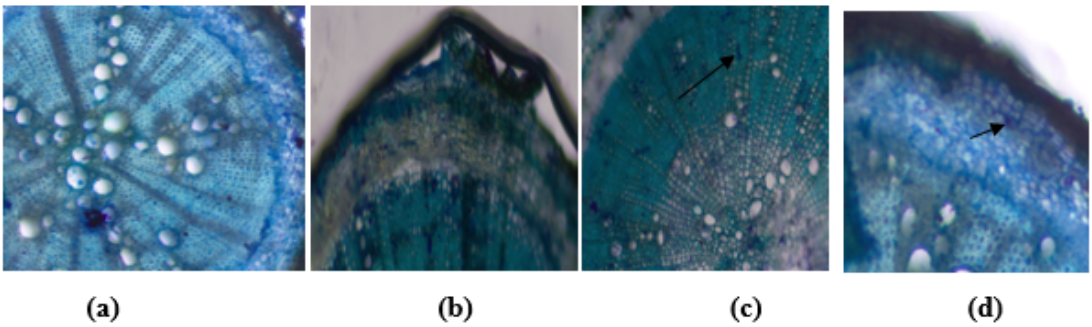
**Figure 9**

Representative photograph of in vivo DAB staining for visualization of H2O2 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 H2O2




## Figure 10

Defense enzyme activities in Pigeon pea leaves. The values shown here are the mean of three replicates. Errors bars represent standard errors. Different letters above the bars represent signifcant diferences according to the analysis of variance (ANOVA), followed by the Duncan test ($p \leq 0.05$) applied using the software SPSS ver 18




## Figure 11

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