Chapter 5

Greenhouse study and Field trials of *Streptomyces* sp. S-9 as a Biocontrol Agent

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5.8 Introduction

It is reported and documented that soil-borne pathogens, such as *Fusarium udum*, *Phytophthora sp.*, or *Alternaria sp*., cause plant diseases, making crop yields and product quality to be negatively affected (Morrissey et al., 2004). As a result of their wide variety of host species, these fungal infections cause disease in many important crops. Traditionally, synthetic fungicides like Bavistin and Thiram have been used to control Fusarium and other soil pathogens (Melent'ev et al., 2006).

Actinobacteria are regarded as possible biocontrol agents for plant diseases, and the scientific literature has several publications on the isolation of actinobacteria of interest from the soil, the rhizosphere, and the roots of a variety of healthy plants (Goudjal et al., 2014; Patil et al., 2010; Petrolini et al., 1996; Sadeghi et al., 2012; Sardi et al., 1992). In addition, it has been claimed that plant-associated actinobacteria may be used as agents to control different soil-borne pathogenic fungi and boost plant development (El-Tarabily et al., 2009). Proposed mechanisms of action include the formation of antibiotic compounds, siderophores, hydrogen cyanide (HCN), and hydrolytic enzymes such as chitinases and glucanases (de Oliveira et al., 2010; Passari et al., 2015). In addition, actinobacteria can promote plant growth by solubilizing inorganic phosphate and improving the plant's uptake of phosphorus when its bioavailability in the soil is low (Hamdali, Hafidi, et al., 2008). Some are known to establish symbiotic interactions with crop plants, invading their internal tissues without causing disease and producing plant growth regulators including gibberellic acid (GA3) and indole-3-acetic acid (IAA) (El-Tarabily et al., 2009; Goudjal et al., 2013; Khamna et al., 2010; Ruanpanun et al., 2010).

Streptomyces is the dominating species in producing bioactive chemicals, including ivermectin, tetracycline, streptomycin, and nystatin (Ser et al., 2016). They are Gram-positive, filamentous, and sporulating actinobacteria with high G+C concentration in their DNA genomes. Secondary metabolites produced by *Streptomyces*, which are physiologically active, are considered valuable biological resources because they aid in protecting plants against diseases (Ueno et al., 2016). In preventing Fusarium wilt, *Streptomyces violaceusniger* proved to be effective (48–52%) on

potted plants, according to Getha & Vikineswary (2002). Plant height and vascular discolouration caused by Fusarium wilt can be improved by using *Pseudomonas aeruginosa* as a biological fertilizer isolated from the banana rhizosphere (Ayyadurai et al., 2006). Consequently, it is regarded as a crucial step in agricultural development, environmental safety requirements, and the prevention and management of plant disease isolation of antagonistic actinomycetes (D. Lu et al., 2016).

Arbuscular mycorrhizal fungi (AMFs) enable host plants to thrive under stressful conditions by mediating complex plant-fungal communication events, leading to higher water consumption and photosynthetic rates (Birhane et al., 2012). Due to fungal symbiosis, numerous papers demonstrate enhanced tolerance to many stressors, including drought, salinity, herbivory, temperature, metals, and pathogens (Ahanger et al., 2014; Rodriguez et al., 2008; Salam et al., 2017). Flowering plants, bryophytes, and ferns can create symbiotic relationships with AMF (Ahanger et al., 2014; Zhu et al., 2010). Vessels and arbuscules are formed in the roots by AMF, whereas spores are formed in the soil by AMF. AMF's ability to create hyphal networks with plant roots significantly enhances plant growth by providing root access to a large region of soil surface (Bowles et al., 2016). AMF improves plant nutrient availability and translocation (Rouphael et al., 2015). By improving soil structure and texture, AMF also improves plant health (Thirkell et al., 2017; Zou et al., 2016).

AMF’s relationship with plants dates back 400 million years (Selosse et al., 2015). In both natural ecosystems and agricultural biotas, such connections are formed through a series of biological processes that have a variety of beneficial impacts (M. G. A. van der Heijden et al., 2015). It is a famous example of a mutualistic interaction that regulates plant growth and development. The fungi’s mycelial network extends beneath the plant's roots, allowing nutrients that would otherwise be unavailable to be taken up. A common mycorrhizal network (CMN) is formed when the fungal mycelium colonizes the roots of numerous plants, even though the plants in question may be of different species (Begum et al., 2019).

Since it has a major impact on many plant communities, especially those occupied by invasive species (Pringle et al., 2009), this CMN serves as an essential component of the terrestrial ecosystem (Smith & Read, 2008). AMF likely improves plant tolerance to biotic and abiotic

environments since fungi and plants share nutrients (Plassard & Dell, 2010). Plant growth can be aided by modifying the soil's qualities, which they can do in both normal and stressful situations (Alqarawi et al., 2014a; Alqarawi et al., 2014b; Navarro et al., 2014). Several morpho-physiological changes generated by AMF colonisation increase plant tolerance to a variety of stresses (Alqarawi et al., 2014a; Alqarawi et al., 2014b).

Over the past two decades, various studies conducted on AMF have demonstrated their numerous benefits for soil health and crop yield. Because mycorrhizal application may successfully reduce the quantitative usage of chemical fertilizer input, particularly phosphorus, it is widely assumed that AMF could be considered as an alternative for inorganic fertilizers soon (Ortas, 2012). Continuous use of inorganic fertilizers, herbicides, and fungicides has generated a variety of difficulties for soil, plants, and human health due to their adverse effects on food quality, soil health, and air and water systems (Yang et al., 2004). It is claimed that AMF can reduce the need for chemical fertilizers by as much as 50 percent for optimal agricultural productivity, although this estimate is contingent on the plant species and the prevailing stress regimes (Begum et al., 2019).

The selection of biocontrol agents is hampered by the fact that biocontrol agents that appear to be successful *in vitro* may not be effective in greenhouse or field environments. Organic matter, pH, nitrogen levels, and soil moisture all affect the effectiveness of biocontrol agents. Biocontrol drugs that function well in vitro may fail in greenhouse or field tests due to environmental factors that change from location to location (Law et al., 2017). Consequently, it is essential to examine the local environmental parameters while deciding on the best biocontrol agents for a given situation. For successful biological control, the biocontrol agents should be isolated from and applied to regions with similar environmental variables (Suprapta & Khalimi, 2012). In addition, the formulation (e.g., powder, liquid, or granule) and application method of biocontrol agents such as soil inoculation, seed inoculation, and vegetative part inoculation should be investigated (Figure 5.1) since they have a significant impact on the outcomes of field tests (R. C. Dubey, 1993; Ou, 1980). Soil inoculation entails using drip systems to apply the biocontrol agent to sowing furrows or to mix it with the soil (Vasudevan et al., 2002). The seeds are dipped in a biocontrol agent culture or mixed with aninoculants using appropriate wetting agents before sowing (Dubey, 1993; Vasudevan et al., 2002; Yang et al., 2008). Spraying the biocontrol agent

in the air or on the leaves of a plant or treating seedlings with the biocontrol agent before transplantation are examples of vegetative part inoculation methods (Gopalakrishnan et al., 2014; Vasudevan et al., 2002) (Figure 5.1). The success of the biocontrol agents in field trials is likely to be greatly influenced by the correct application method (Suprapta & Khalimi, 2012).

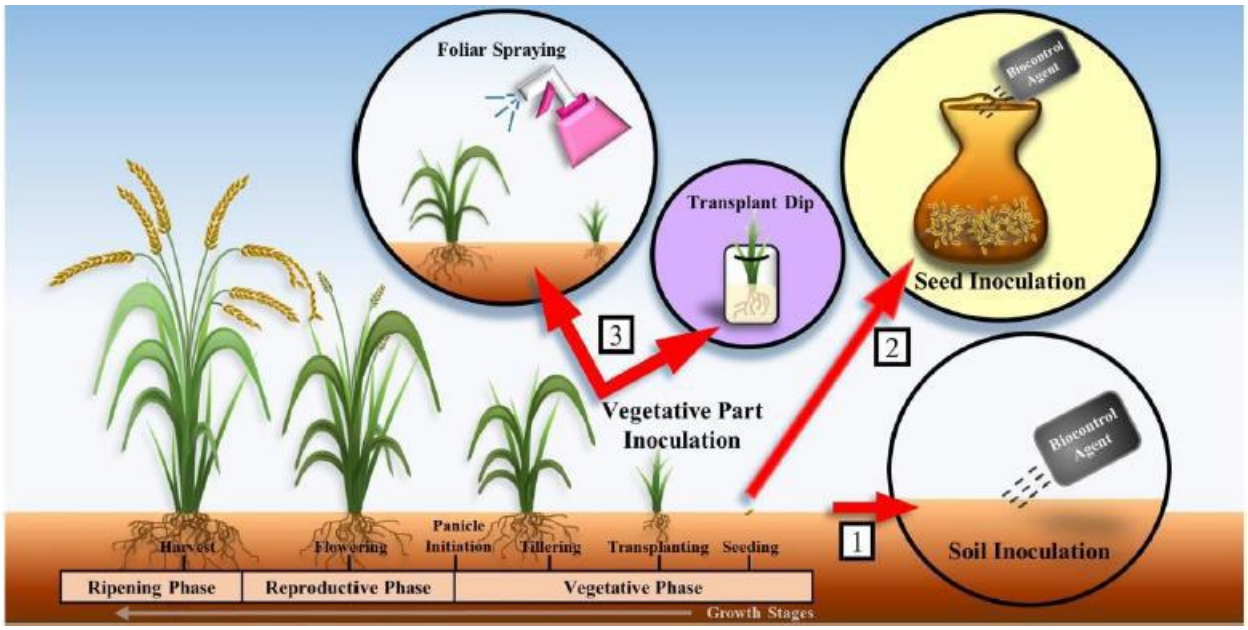

Figure 5.1: Methods of application of biocontrol agents. (1) Soil inoculation; (2) Seed inoculation; (3) Vegetative part inoculation (Law et al., 2017).

5.9 Materials and Methods

5.9.2 Development of a powder formulation of *Streptomyces* sp. S-9

Talcum powder and kaolin were used to create the powder composition. Talcum powder and kaolin were twice independently sterilized on alternating days. Using an oven set to 80°C for two days, we dried the sterilized talcum powder and kaolin. The Streptomyces sp. strain was acquired by cultivating the culture on *Streptomyces* sp. isolation agar, and the spores were mixed thoroughly with 24 g of kaolin after cooling (ISP-3). The talc-kaolin-Streptomyces sp. formulation was obtained by combining 48 g of talcum powder with the kaolin-Streptomyces sp. mixture and shaking it thoroughly. The talc-kaolin (talc) powder was kept in the dark at 4 degrees Celsius (Tamreihao et al., 2016).

5.9.3 Determining the shelf life of the powder formulation

The shelf life of the powder formulation was determined at a 1-month interval over a period of 6 months using the standard dilution plate count method.

5.9.4 *In vitro* antagonism assay of the powder formulation

The powder formulation was evaluated for its antifungal activity against pathogenic fungus *Fusarium udum* by the agar well diffusion method. Three wells of 8 mm diameter were made equidistant from the center of the plate using a sterile cork borer. *F. udum* agar disc (8mm diameter), grown on PDA at 30 °C was placed at the center of the plate. Using a sterile micropipette, 100µl of different concentrations (10, 50, 100 mg/ml) of the suspension of the powder made in sterile distilled water were poured into each well. Formulation control (FC) plate was kept separately with 3 wells (8mm diameter) each containing the suspension of the powder components without *Streptomyces* culture. A zone of fungal growth inhibition was seen after 5 days of incubation at 30 °C on the plates.

5.2.4 Pot trials of the powder formulation under natural conditions

Seeds of *Cajanus cajan* were surface sterilized with 0.1% $HgCl_2$ for 90 seconds followed by intermittent washes using sterile distilled water and germinated on a water agar plate. The plastic pot bags are filled with autoclaved garden soil artificially infected by *F. udum.* Pots containing uninfected soil were used for control treatments. 4 germinated seeds were sown in each pot containing infected and uninfected soil. Powder formulation was added to the soil and mixed well.

The following systems were prepared:

• Seeds sown in uninfected soil without powder formulation.

• Seeds sown in fungal infected soil without powder formulation.

• Seeds sown in uninfected soil containing powder formulation.

• Seeds sown in uninfected soil containing the formulation control (Talcum + kaolin).

• Seeds sown in fungal infected soil with powder formulation.

All the experiments were carried out in 3 replicate pots. The pots were kept under natural sunlight and watered daily using autoclaved distilled water. After three weeks, the plants were removed from the pots, washed with tap water, and the measurements of root lengthshoot length, fresh weight, and dry weight were taken and compared with that of the control sets (Anjaiah et al., 2003).

5.9.4.1 Mycorrhizal inoculum

The mycorrhizal product Mycozone (Agriland Biotech, Savli, Vadoadara, Gujarat) containing spore of *Rhizophagus irregularis* (syn. *Glomus intraradices*) was applied into the soil (approx. 750 spores/g trap soil)/pot as detailed else where (Hashem et al., 2016). The trap culture method was used following the technique of Stutz & Morton (1996). Pigeon pea was used as trap plant.

5.9.4.2 *In vitro* seed germination inoculation with different treatment

Strain S-9 was developed on ISP-1 Medium for 5 d, centrifuged (10,000 rpm, 10 min), and the pellet collected was washed thrice with SDW. The pellet was dissolve in SDW, and inoculum size was prepared 4.5×10^8cfu/ml. Pigeon pea seeds (Variety:BDN 2) were surface sterilized with 0.1% sodium hypochlorite solution (SDFCL, Mumbai) for 1 min, followed by three washings with sterile distilled water (SDW). Sterilized seeds were absorbed in the cell suspensions arranged before and kept under shaking conditions (150 rpm, 2 hrs). Sterilized seeds absorbed in SDW were taken as control. The seeds were then transferred to water agar plates; six seeds were kept per plate. Plates were kept at 28–30 °C, and after 7 d, the number of germinated seeds, root lengths, and shoot lengths were noted and compared with control. Three replications were done per treatment, and the analyses weredone. The vigor index was determined using the equation of Abdul-Baki & Anderson (1973) as follows;

Percent germination × Seedling length (shoot length + root length).

5.9.4.2.1 Enumeration of Treatments

T1-Control

T2- S-9 *F.udum*

T3-S-9+*F.udum*

T-4-*F.udum*

T-5-*R.irregularis*

T-6-S-9+ *R.irregularis*

T-7-S-9+ *R.irregularis + F.udum*

T-8- Trichoderma monitor

T-9- Bavistin

5.9.5 Pot trial of the powder formulation S-9 under green house conditions

The disease control and plant growth promotion effectiveness of *Streptomyces* sp. S-9 was studied in a greenhouse in an experiment with a pot in a controlled environment (32°C; relative humidity 60–70%) (Temperature-32°C; Relative Humidity-60-70 %). Pigeon pea (var.BDN-2) seeds susceptible to wilt were acquired from the Pulse Research Station, Model Farm in Vadodara, Gujarat, India. The seeds were sterilized by soaking them in 0.1% $HgCl_2$ for three minutes and rinsing them with sterile distilled water three times. The sterilised seeds soaked in a bacterial cell suspension for two hours (under aseptic conditions) were used for sowing in the pots. In certain proportions (3:1), sterilized soil and sand were employed as a growing medium for plants in pots (6 inches in diameter). Pathogen inoculums with 2×10^8 spores/ml were added to the potting medium at a rate of 160 ml/kg of soil and cultured for 4-7 days to ensure optimal germination of spore and mycelium formation (Pandey & Dubey, 1994).

In each container, four surface-sterilized seeds were planted, and germination and incidence of wilting were monitored daily. There were five replicates for each treatment. Treatment details of the pot bioassay under greenhouse conditions were as enumerated; T1-Control, T2- S-9 *F.udum,* T3-S-9+*F.udum,* T-4-*F.udum,* T-5-*R.irregularis,* T-6-S-9+*R.irregularis*, T-7- S-9+*R.irregularis*+*F.udum,* T-8- Trichoderma monitor, T-9- Bavistin.

Surface-sterilized seeds were infected with 24 hour-grown cultures (OD600 = around 1 for T -3 and T -4 treatments) 10^8 cells ml^{-1}) of *Streptomyces sp*. S-9 respectively. T-5, T-6, and T-7 surface-sterilized seeds were coated with AM fungi 5 g/pot. In the absolute control (T1), neither bacteria nor pre-fungi were added to the potting medium. Bavistin-treated seeds (4 g/kg) were employed as the indicated chemical treatment (T-2). Four weeks after seed germination, the plant defence enzyme test was conducted. Plant samples were taken 35 days after sowing (DAS), and plant samples were carefully plucked from their containers and transported to the laboratory for various biometric assessments. The pot assay experiment was done twice, each time with three replications. A completely randomized design (CRD) was used to arrange the pots in the development greenhouse.

5.9.6 Field trial of the talcum formulation S-9

Total three field trials were conducted in two locations respectively, Pulse Research Station, Model Farm Vadoadara, Gujarat, India and Dwarkapura, Samlaya, Vadoadara, Gujarat, India. Constant two years of 2018-19 and 2019-2020 filed trial was conducted in Pulse Research Station, Model Farm, Vadoadara, Gujarat, India and one field trial was conducted in 2019-2020 located at Dwarkapura, Samlaya, Vadodara, Gujarat, India.

The strain of *Streptomyces* was tested for its biocontrol efficiency against the fungal disease wilt of Pigeon pea at Pulse Research Station, Model Farm in Vadoadara, Gujarat in India. (22.19"N; 73.11"E; altitude 37.50 m) throughout the 2018–19 and 2019–2020 farming seasons. During the growing seasons, the maximum temperature range was 28.2–37.8 °C, while the lowestrangewas 11.1–17.6 °C. The soil represents the region’s soils, popularly known as “Goradu” soil. It is alluvial in origin and belongs to the order Alfisol. The texture of the soil is loamy sand. The soil is deep enough to respond well to manuring and various crops of the tropical and sub-tropical regions. The soil is low in organic carbon and nitrogen and medium in available phosphorus and

available sulphur. The potassium status was found to be high, while micronutrient status was found sufficient. Soil pH was 7.7, and organic carbon concentration was 0.36 %, making it mildly alkaline. Soil mineral composition in the top 15 cm of the rhizosphere included 0.031% nitrogen, 40.18 kg/ha of available soil phosphorus, and 450.00 kg/ha of bioavailable potassium.

Streptomyces strain was evaluated for their biocontrol efficacy against Fusarium wilt of Pigeon pea under field conditions at Dwarkapura, Samlaya, Vadodara, Gujarat, India (22.639165N; 73.6417408E; altitude 85 m), during 2019–2020 cropping seasons. During the cropping seasons, a maximum temperature range of 27.2–35.8 °C and a minimum of 12.1–16.6 °C were recorded. The soil represents the region's soils, popularly known as "Goradu" soil. It is alluvial in origin and belongs to the order Alfisol. The texture of the soil is loamy sand. The soil is deep enough to respond well to manuring and various crops of the tropical and sub-tropical regions. The soil is low in organic carbon and nitrogen and medium in available phosphorus and available sulphur. The status of potassium was high, while micronutrient status was sufficient.

Organic carbon content in the soil was 0.49 %, with a pH of 7.7. There is 0.038 % accessible nitrogen, 23.89 kg/ha phosphorous and 342.0 kg/ha potassium in the top 17 cm of rhizosphere soil mineral concentration. In the field, pigeon pea (BDN2) was sown 5 cm deep.The effectiveness of nine different treatments was examined: T1-Control, T2- S-9 *F.udum,* T3-S-9+*F.udum,* T-4-*F.udum,* T-5-*R.irregularis,* T-6-S-9+ *R.irregularis,* T-7-S-9+ *R.irregularis F.udum,* T-8- Trichoderma monitor, T-9- Bavistin. Seed bacterization (10^8 CFU/mL/h) and booster doses of Streptomyces (5 ml seedling^{-1}, 10^8cfu ml^{-1}) were used for the first five treatments; for the sixth and final treatment, soil drench was used. The second internodes of the stem were drenched with *F. udum* 10 days after 50% flowering, and the plants were infected. A randomized block design (RBD) was used for the experiment's three replications. The plot had three rows of two metres each, with 75-centimeter row spacing and a 10-centimeter plant-to-plant distance. Measurements of infection length and density were made after harvest to determine the severity of the disease.

5.9.7 Pre-emergence and post-emergence wilt incidence (%)

Wilt indices of symptom severity were estimated by numerating the sum of germinated seeds and surviving seedlings (the seedlings that did not show symptoms of wilt, such as brown

lesions/premature dropping of leaves/partial withering of part or entire seedlings) germinated under it (Dukare et al., 2011). Disease incidences were estimated based on noticeable wilt symptoms detected on the plant between 15 and 35 DAS.

5.9.8 Interaction between actinomycetes and AM fungi

The effects of the interaction between actinomycetes and AM fungus inoculation on plant growth and nutrition, AM colonization, and the dynamic population of actinomycetes in the plant rhizosphere were investigated by a pot experiment. Control, S-9+*R.irregularis*, *R.irregularis*, and S-9+*R.irregularis*+*F.udum* comprise the actinomycetes strains (10^8 cells/pot) and AM fungal therapy (5 g/pot). There were four treatments in each trial, each with three replications. On the sterilized sand-soil substrate, surface-sterilized Pigeon pea seed was germinated, and four seedlings were planted, irrigated as needed, and cultivated for 180 days. The placement of containers in a growth chamber was absolutely random. These pots were kept under greenhouse conditions in a completely randomized design (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) PO4 115.03; $MgCl_2 4H_2O$ 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 were assessed for seedlings (Goudjal et al., 2016).

5.9.9 Quantification of mycorrhizal colonization

We used 0.05% trypan blue in lactoglycerol (Phillips and Hayman, 1970) to visualise mycorrhizal colonization on segments of 1 cm-long Pigeon pea roots, which were then examined using a light microscope (BM-180, Boeco, Germany) at 10-40X magnification. Line-intersection approach was used to calculate total mycorrhizal colonization by *R. irregularis* (Giovannetti and Mosse, 1980). All roots had their number of vesicles and arbuscules measured using ImageJ (version 1.8.0_112) image editing software (Javot et al., 2011). *R. Irregularis-infested* root segments were observed on each plant.

5.9.10 Evaluation of plant vegetative parameters, biochemical, and defense enzymes

Vegetative parameters comprising shoot and root length, number of leaves per plant, plant fresh and dry weight were determined using three replicates of Pigeon pea plant from each treatment. The proline content of the leaves was determined using the method of Bates et al. (1973).

5.9.10.1 Lipid peroxidation

The technique of Heath and Packer (1968) was used to investigate the changes in lipid peroxidation in terms of malonaldehyde (MDA) accumulation. Pulverized leaf tissue was placed in trichloroacetic acid 0.1 percent (w/v) and centrifuged at 10,000 rpm for five minutes. Absorbance was then measured at 532 and 600 nm using a combination that contained 15 minutes heated (95°C) 1mL of supernatant, 2mL of 20% Trichloroacetic Acid, and 0.5% (w/v) Thiobarbituric Acid. The extinction coefficient (EC) of 155 $mmol^{-1}$ cm^{-1} was used to estimate the MDA concentration.

5.9.10.2 Quantification and microscopic detection of hydrogen peroxide

Hydrogen peroxide (H_2O_2) extraction was done by crushing leaf tissue in the dark with The extraction of hydrogen peroxide (H_2O_2) was accomplished by crushing leaf tissue in the dark with cold 50 mM potassium phosphate buffer (pH 6.5) and centrifuging for 15 minutes at 6000 rpm at -4 °C. Following slight adjustments to Jana and Choudhuri's (1982) approach, we took some of the supernatant for evaluation. 24-hour incubation in diaminobenzidine (1 mg mL^{-1}, pH 3.8) solution at 30°C in the dark with leaf discs 2 cm in diameter was used to monitor in situ H_2O_2 accumulation after the leaf discs were transferred to 90 percent ethanol and boiled at 70 °C to eliminate chlorophyll. A compound light microscope with a 10x magnification was used to examine the leaf discs (Thordal-Christensen et al., 1997).

5.9.10.3 Extraction of enzyme

Enzymes such as peroxidase (POD) and polyphenol oxidase (PPO), and Phenylalanine ammonia-lyase (PAL) were extracted by homogenizing the leaf, stems, and root tissues of the Pigeon pea plants. 3 ml of 50 mM ice-cold sodium phosphate buffer (pH 6.0) was used to homogenize the

sample in a precooled mortar and pestle. The homogenate was centrifuged at 10,000 rpm for 10 min at 4 °C. Extracted crudes were used for the determination of ensuing enzyme activity.

5.9.10.4 Determination of peroxidase

In a cuvette, 0.5 ml of crude enzyme extract was put, followed by the addition of 0.5 ml of 1% guaiacol solution and 1.5 ml of 50 mM Tris buffer (pH 7.5). Then, 0.5 ml of 1% H_2O_2 was added, and the absorbance change at 470 nm was measured every 30 seconds for 3 minutes. Absorbance change min-1 per g-1 fresh weight is equivalent to one unit of peroxidase enzyme activity (Hammerschmidt et al., 1982).

5.9.10.5 Ethylene Measurements

Ethylene (Ethylene Ecocyl 2.5) was acquired from Linde. GC-FID (Hewlett Packard, Series 2, 5890 Series 2 plus, Agilent, Santa Clara, USA) linked to a headspace autosampler (Hewlett Packard, HP 7694, Agilent) was used to determine ethylene concentrations with the following parameters: Headspace sampler: Oven, sample loop, and transfer line temperature of 45°C, loop volume of 3 ml, loop fill time of 0.1 min, and injection time of 0.2 min; vial parameters: equilibration time of 0.5 minutes, pressurizing time of 0.2 minutes, and GC cycle time of 11 minutes. On a Restek Rt-QS-Bond column (30 m × 0.53 mm × 20 µm) utilising Helium as the carrier gas (3.3 ml/min constant flow), chromatographic separation was performed. Temperature programme: 30°C, hold for 1 minute, to 60°C at 30°C/min, hold for 3.5 minutes. The injector and flame ionization detector (FID) were both set to 250°C, and split injection (30:1) was used. Peak heights and external calibration (10 concentration levels between 0.17 µg/L and 10.65 µg/L) were used for quantification. The Vali Dataprogramme Version 3.02.48 was used to estimate the limit of detection (LOD = 0.67 g/L) and limit of quantification (LOQ = 2.3 g/L) based on calibration findings. Mass Hunter (Agilent Technologies, Qualitative Analysis B.06.00) was utilised for data processing. Maximum values of the quantification Ions were employed for comparative quantification, and the identity of the molecule was established by comparing its MS spectrum to those of reference standards.

5.9.10.6 Ethylene production analysis

In Pigeon Pea plants, the ethylene emissions were quantified employing a modified version of the procedure developed by Madhaiyan et al (2007). Leaves were taken from Pigeon pea plants under stress condition *F.udum* treatment and were placed in 120 ml vials and sealed with a rubber septum for 4 h. An air sample (1 ml) present in the headspace was injected into a Gas Chromatography system (Thermo Scientific, USA), having a Poropak-Q column and flame ionization detector. The quantity of ethylene emission was represented as nmol of ethylene h^{-1} g dry $weight^{-1}$ and compared to the pure ethylene reference curve.

5.9.11 Estimation of the contents of NPK in soil and plant

Using a 2mm sieve, soil samples were obtained from a depth of 0 to 20 centimetres prior to planting and air-dried. The pipette method was used to analyse particle size (Gee & Or, 2002). Using a glass electrode pH metre and a soil-to-water ratio of 1:2, the pH of water wascalculated. Walkey and Black technique wasused to quantify organic carbon (Nelson & Sommers, 1996). Total soil nitrogen (N) was assessed using Kjeldahl digestion (Bremner, 1965). The samples' exchangeable bases were extracted with $1MNH_4OAC$ at a pH of 7.0. Using flame photometry, potassium (K) was determined. Colorimetric determination of phosphorus (P) was made using the molybdenumblue technique and Bray-1 extraction (Murphy & Riley, 1962). Soil samples were air-dried, pulverised into powder, and tested with 5:1:1 ml of HNO_3:H_2SO_4:$HClO_4$ acid using the wet digestion method. For the determination of P and K, 0.5 g of samples were ashed, dissolved in 10% hydrogen chloride (HCl), and diluted to 50 ml. Using vanadium molybdate colorimetric, phosphorus was determined. Bothsoil and plant factors were examined for their physico-chemical qualities at the Department of Soil science and Chemistry, Anand Agriculture University, Anand, Gujarat, India.

5.9.12 Histology of Pigeon pea roots under greenhouse conditions

Brien et al., (1964) technique was followed for microscopic assessment of changes in root tissue anatomical characteristics. In this study, fresh root materials matching to distinct treatments (S-9, S-9 + *F. udum*, and *F. udum*) were soaked for at least 2–3 minutes before being sliced into pieces of around 10–50 microns. These sections were submerged for one minute in a staining solution.

The staining solution contained 0.5% toluidine blue in a phosphate buffer with a pH of 6.8. After staining, sections were rinsed with tap water and viewed under a microscope (Olympus CX1, Leica Microsystems, GmbH, Germany) at ×40 and ×100 magnifications. Root samples were tested for endodermis thickness, xylem cell size and number.

5.9.13 Statistical analysis

Using SPSS software (IBM SPSS Statistics Version 20), Duncan's Multiple Range Test and one-way ANOVA were used in the statistical analysis of the data. to differentiate the means. At $P \leq 0.05$, differences were deemed statistically significant. Unless otherwise specified, Data were presented as the average and standard error of the average (SEM) of three replicates. Graphpad Prism was used to produce the graphic illustrations (Version 8.0.1).

5.10 Results

5.10.2 *In vitro* seed germination test (Vigor index)

In the pre-inoculated treatments, 93.46% seed germination was observed in T-6 (S-9+ *R. irregularis*), T-4 (*Streptomyces* sp. S-9), and T-5 (*R. irregularis*) treatment .Maximal germination was seen in the absolute control (T-1) without microbial inoculation **(**Figure 5.2). The least amount of germination (29.99%) observed in *F. udum* alone inoculated pots (T-2). Seed bacterization also profoundly affected the shoot and root elongation (Figures 5.3 A & B). The highest shoot lengths of 15.58±0.02 cm and 14.24±0.04 cm were recorded in the T-6 treatment (S-9+ *R. irregularis*) and T-7 (S-9 + *R. irregularis* + *F. udum*), respectively. Only in pathogen-infected containers did we see seedlings with the shortest shoot length of 1.15±0.05cm (T-2). Overall, T-6 demonstrated the greatest improvement in shoot height compared to pathogen control (Figure 5.3A). The same tendency was also observed in terms of root length. Again, maximum root lengths of 15.26±0.03cm and 14.84±0.06cm were observed in the T-6 (S-9+ *R. irregularis*) and T-8 treatments (*Trichoderma*), respectively. The lowest root length (2.19±0.08cm) was substantially smaller in pots inoculated with *F. udum* alone than in other treatments (Figure 5.3B).

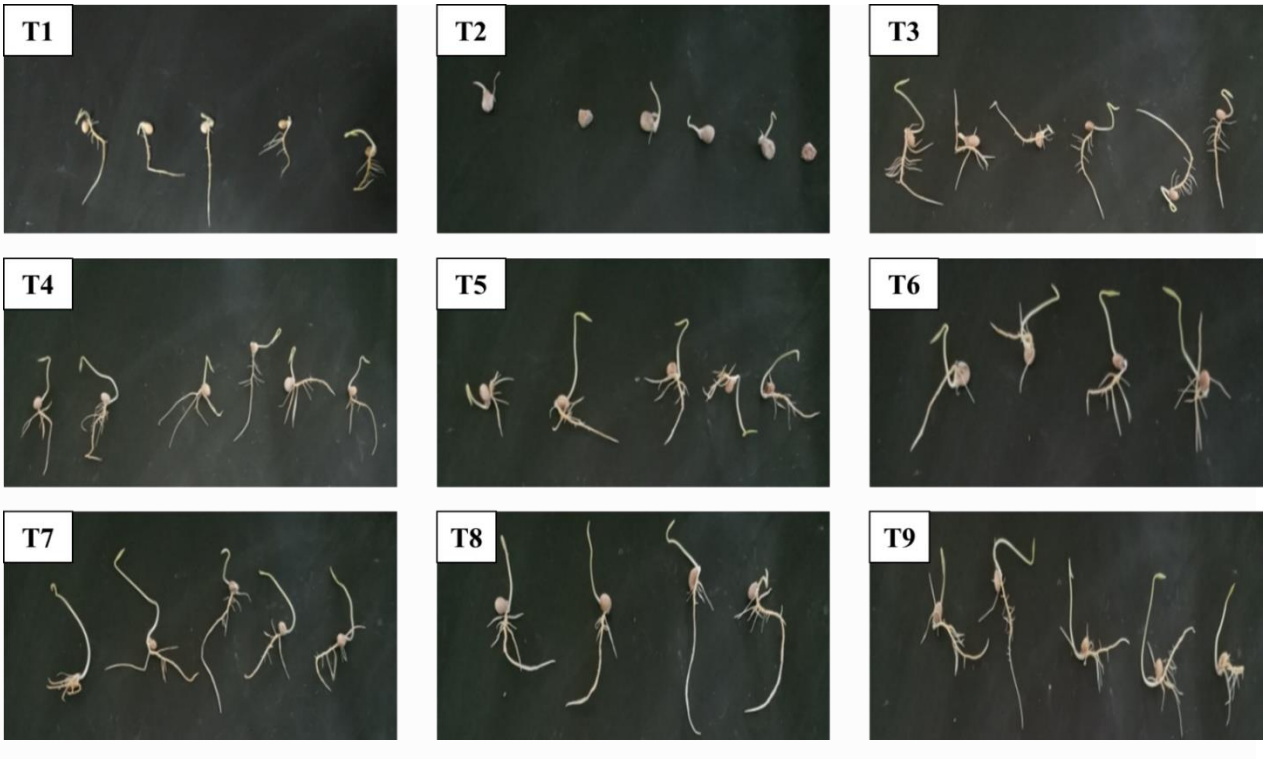

Figure 5.2: Effect of different treatments on the germination and growth of *C. cajan*(BDN-2) seeds at germination level in Petri plates.

T1-Control, T2-*F.udum*,T3-S-9+*F.udum*, T4-S-9, T-5-*R.irregularis*, T-6-S-9+ *R.irregularis*,T-7-S-9+ *R.irregularis*+*F.udum*,T-8- Trichoderma monitor and T-9- Bavistin.

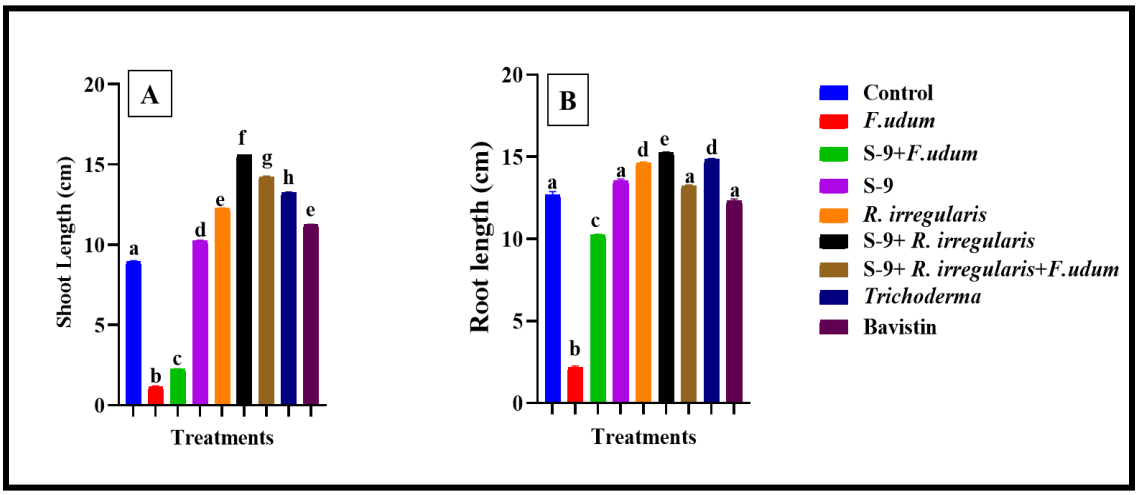

Figure 5.3: The Effect of Seed Bacterization on shoot length (A) and root length (B) of *C. cajan.* Values are mean of three replicates ± Standard Deviation (SD). Different superscripts show significant difference (P≤0.05)

5.10.3 Efficacy of the powder formulation S-9 *in vitro* antagonism assay and pot experiments

The talc-kaolin-*Streptomyces* formulation (Figure 5.4 A) at 100mg/ml showed good inhibition on the growth of *F. udum* (Figure 5.4B).

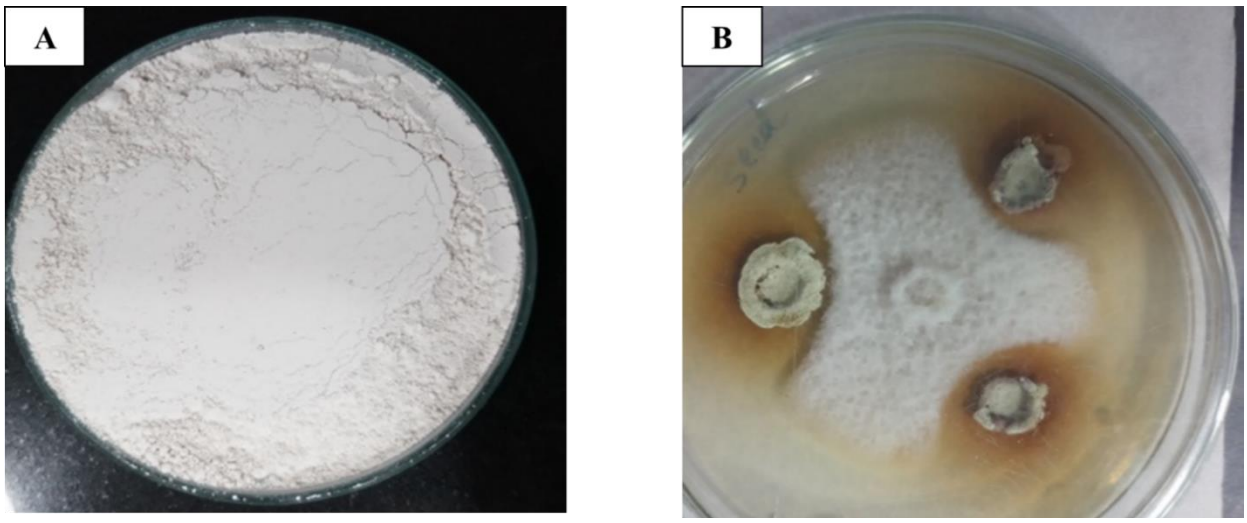

Figure 5.4: Talc-kaoline *Streptomyces* formulation (A) and its antifungal activity against *F. udum* (B).

Germinated seeds were treated with 1-month-old talc-formulation of S-9 under pot experiments. The longest root (43.27cm) and shoot (61.24cm) length were observed with T-6, which was comparing to that of the commercial biofungicide T-8 *Trichoderma* Monitor (50.47±6.25cm) (Figures 5.5 A & B). The formulation also remarkably affected the potted plants' yield (Figures 5.6A-D). The pot with T-6 treatment also had the highest fresh root weight (52.32 g) and fresh shoot weight (59.21 g). Also, the pot with T-6 treatment had the highest root and shoot dry weight of 22.26g and 36.26g, respectively. Equivalent to the synthetic fungicide Bavistin, *Streptomyces* sp.S-9 offered comparable protection. Treatment of seeds with the microbial culture boosted seedling growth, as evidenced by the increase in radicle and plumule length as well as dry weight. When seeds were treated with the Streptomyces sp. S-9 combination, the

effect was amplified. In addition to a significant (P ≤0.05) rise in root and shoot length and fresh root weight, pathogen-challenged Pigeon pea plants treated with talc powder exhibited a considerable (P ≤ 0.05) increase in root and shoot length and fresh root weight compared to pathogen-challenged plants.

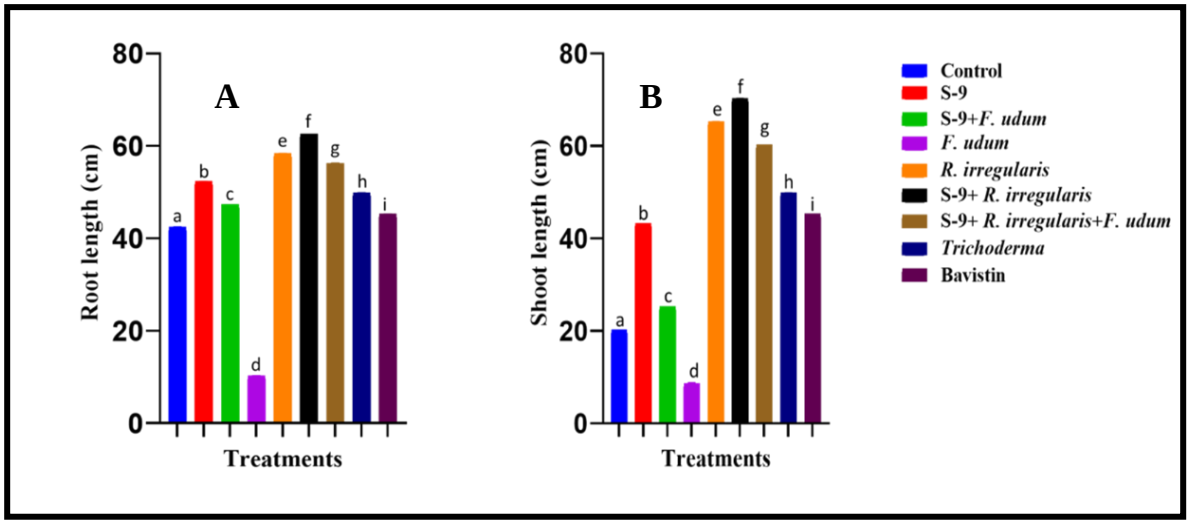

Figure 5.5: Effect of Talc-kaolin-*Streptomyces* formulation on the root length (A) and shoot length (B) of *Cajanus cajan* in the pot experiment.

Values are mean of three replicates ± Standard Deviation (SD). Different superscripts show significant difference (P≤0.05)

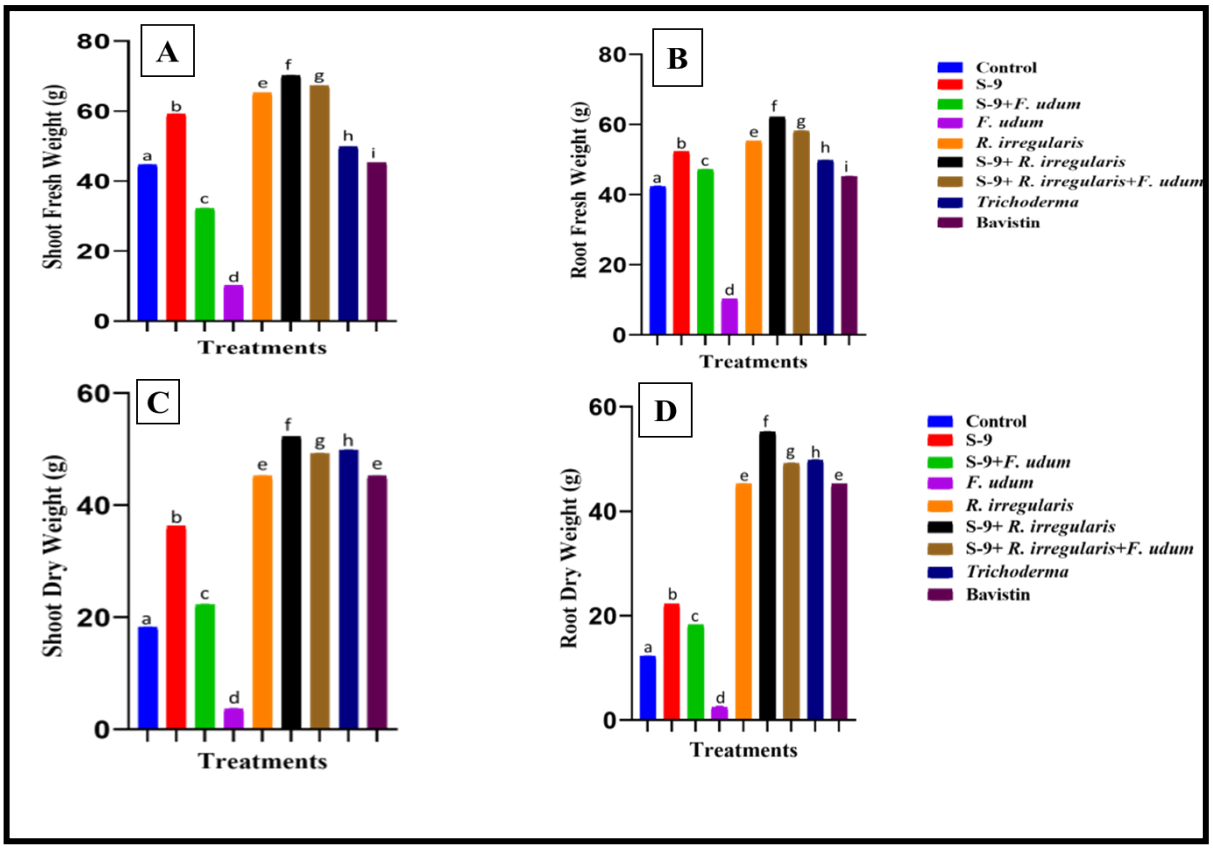

Figure 5.6: Effect of Talc-kaolin-*Streptomyces* formulation on the shoot fresh weight (A), root fresh weight (B), shoot dry weight (C), and shoot dry weight (D) of *Cajanus cajan* in the pot experiment

Values are mean of three replicates ± Standard Deviation (SD). Different superscripts show significant differences (P≤0.05)

5.10.4 Field trial of the talcum powder formulation of S-9

In field trials, the efficacy of *Streptomyces* sp. S-9 in reducing *Fusarium* wilt of Pigeon peawas evaluated. Treatment of Pigeon peaseeds with *Streptomyces* sp. S-9 decreased the incidence of wilt in *C.cajan* by 45.1±2.83, 51.0±3.55, and 54.9±6.58%, respectively. Individually, Streptomyces sp. S-9 was preferable to commercial biocontrol in effectiveness. Product T-2 *Trichoderma* Monitor, which reduced wilt incidence by 41.2±2.6%. *Streptomyces* sp. S-9 for seed dressing produced equivalent wilt control to the chemical fungicide Bavistin (58.8±2.0%) in the fields. Figure 5.7 displays a visual representation of the cultivated*C. cajan*. In addition to reducing the incidence of wilt, seed treatment with *Streptomyces* sp. S-9 boosted plant growth

due to their plant growth-promoting properties, as seen by the increased plant biomass (Figure 5.8) and plant height (Figure 5.9). The average number of pods per plant (Figure 5.10) and pod production increased because of increased growth (Figure 5.11). The greatest growth enhancement was observed in plants treated with *Streptomyces* sp. S-9. In all study seasons, bioinoculant-treated Pigeon pea plants displayed a substantial increase ($P \leq 0.05$) in total grain yield, the weight of 100 grains, and the number of filled grains compared to the control (Figure 5.12).

Figure 5.7: A) Effect of inoculation with different treatment growth on Pigeon pea 180 days after inoculation (DAI)

Figure 5.7 :B) Fusarium infected Pigeon pea 120 days after inoculation (DAI)

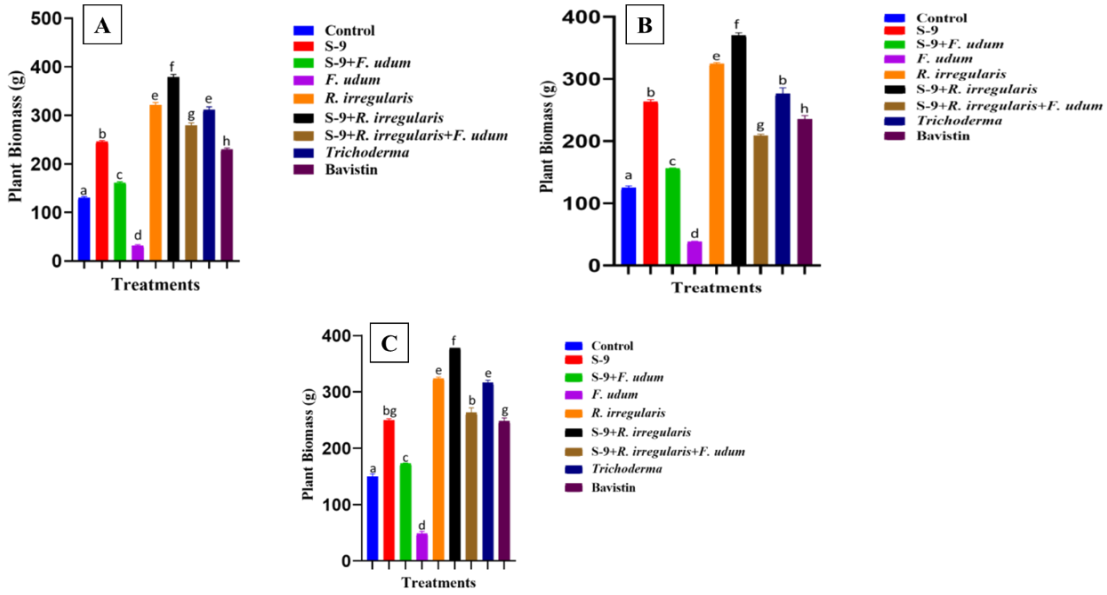

Figure 5.8: Effect of Application of BCA formulations on Plant Biomass of *Cajanus cajan* in 2019-2020 Dwarkapura (A), 2018-2019 Model farm (B), and 2019-2020 model farm (C) Values are mean of three replicates ± Standard Deviation (SD). Different superscripts show significant differences (P≤0.05)

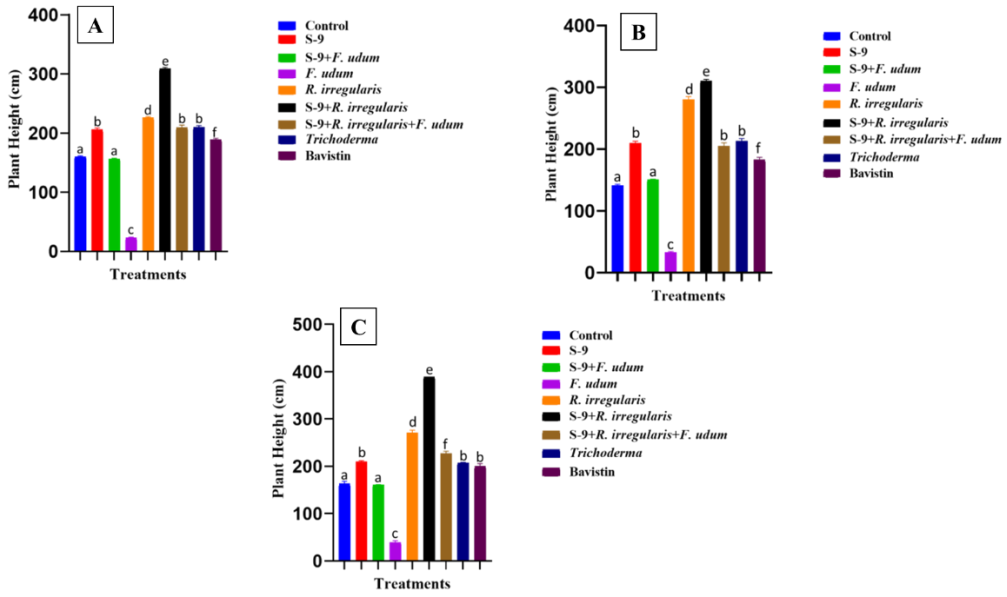

Figure 5.9: Effect of Application of BCA formulations on Plant height (cm) of *Cajanus cajan* in 2019-2020 Dwarkapura (A), 2018-2019 Model farm (B), and 2019-2020 model farm (C) Values are mean of three replicates ± Standard Deviation (SD). Different superscripts show significant differences (P≤0.05)

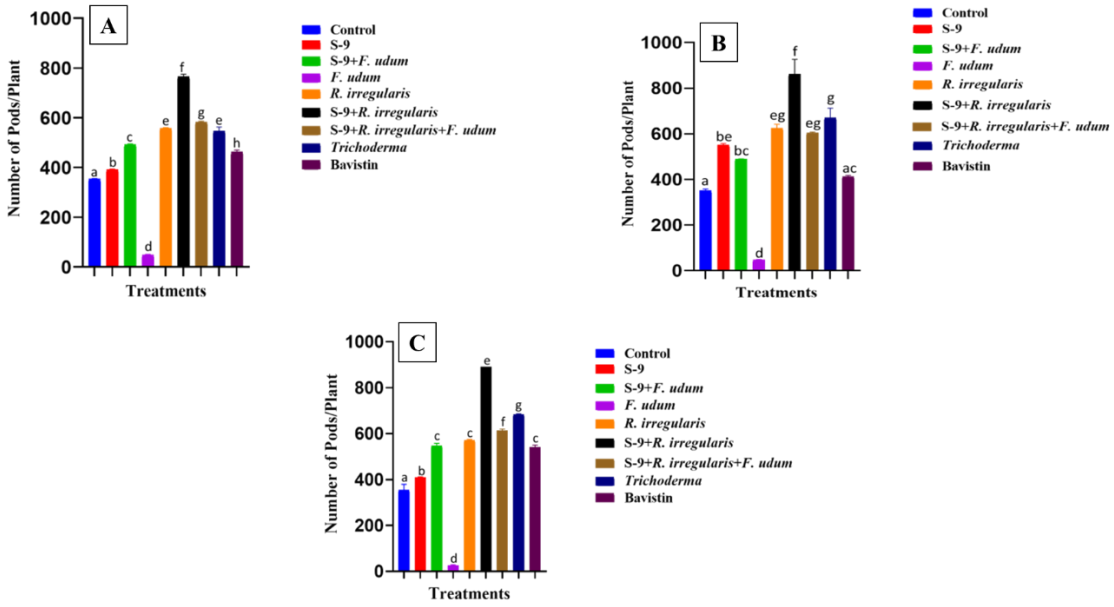

Figure 5.10: Effect of Application of BCA formulations on number of pods/plant of *Cajanus cajan* in 2019-2020 Dwarkapura (A), 2018-2019 Model farm (B), and 2019-2020 model farm (C)

Values are mean of three replicates ± Standard Deviation (SD). Different superscripts show significant differences (P≤0.05)

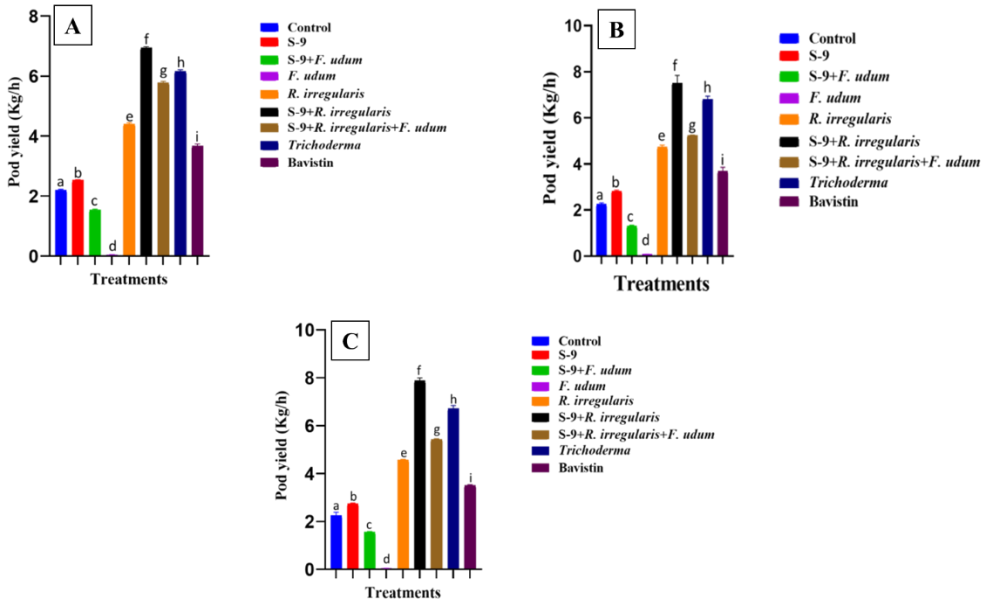

Figure 5.11: Effect of Application of BCA formulations on pod yield (kg/h) of *Cajanus cajan* in 2019-2020 Dwarkapura (A), 2018-2019 Model farm (B), and 2019-2020 model farm (C)
Values are mean of three replicates ± Standard Deviation (SD). Different superscripts show significant differences (P≤0.05)

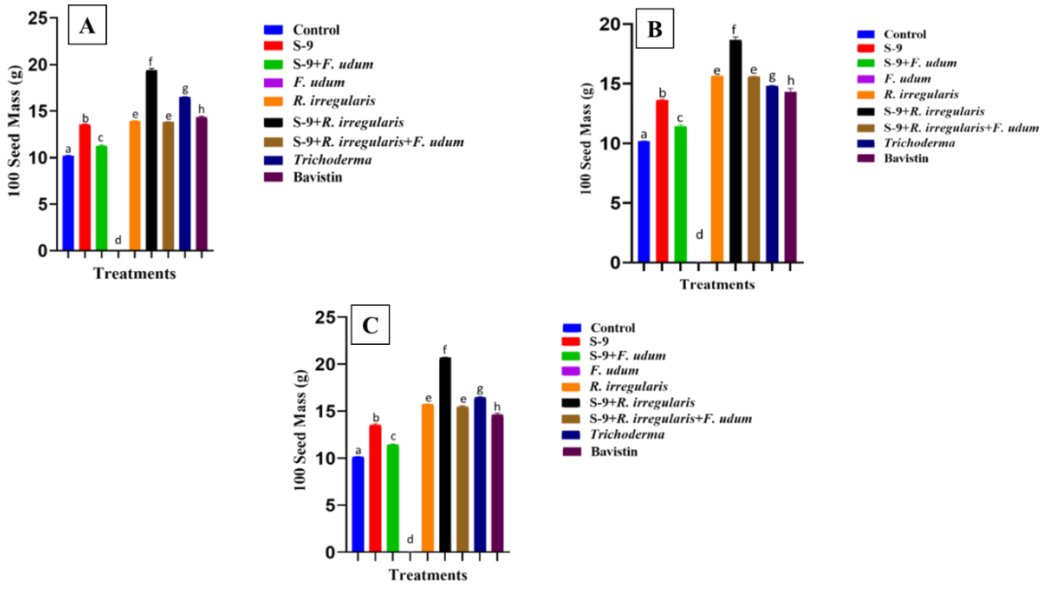

Figure 5.12: Effect of Application of BCA formulations on 100-seed mass (g) of *Cajanus cajan* in 2019-2020 Dwarkapura (A), 2018-2019 Model farm (B), and 2019-2020 model farm (C)

Values are mean of three replicates ± Standard Deviation (SD). Different superscripts show significant differences (P≤0.05)

During the field trial, the wilting symptoms were observed visibly in the stems, pods, and seeds (Figures 5.13 & 5.14). The healthy stem of *C. cajan* didn't show any symptoms when opened longitudinally (Figure 5.13A). However, infection was visible in the Fusarium-infected stem (Figure 5.13B). The fresh and dried pods from a healthy plant indicated no wilting episodes, and the seeds were well-formed (Figures 5.14 a & b). However, in the wilted plant, the dried pod and the seeds showed disease conditions (Figure 5.14c).

Figure 5.13: Symptom of Fusarium wilt in the stem of *Cajanus cajan*plant, (A) Healthy stem (B) Fusarium infected stem (arrow indicates infection by *Fusarium udum*)

Figure 5.14: Incidence of Fusarium wilt on pods of Pigeon pea plant, (a) Fresh pods from a healthy plant (b) Dried pods from healthy plants (c) Dried pods front wilted plants

5.10.5 Compatibility of *Streptomyces* sp. S-9 with *R. irregularis*

Actinomycete strain S-9 was pre-selected to investigate their PGP effects on the Pigeon pea plant. *Streptomyces* sp. S-9 was examined for compatibility with AM fungi (*Rhizophagus irregularis*). The *Streptomyces* sp. *S-9 strain* stimulated germination and mycelial development from *R. irregularis* spores *in vitro* and *in situ*. Inoculation of actinomycetes either alone or with *R. irregularis*, could be successfully established in soils for a long time of 120 days. This experiment was performed three times, and the reaction magnitudes were consistent each time. The inoculation of AM fungi accelerated the establishment of *Streptomyces* sp. S-9 in the rhizosphere of Pigeon pea. Either actinomycetes or AM fungus inoculation improved plant biomass output. Co-inoculation of both bacteria increased plant growth (especially shoot biomass at the conclusion of the experiment) by 9.7 % with individual inoculation with either organism alone. Actinomycetes inoculation enhanced the total mycorrhizal root length of *R. irregularis*-inoculated plants by 29 % at the time of harvest. The inoculation of plants with actinomycetes or AM fungi, either alone or in combination, enhanced their capability of absorbing nitrogen. Inoculation of S-9 alone and *R. irregularis*, either alone or in combination with the tested *Streptomyces* sp. S-9, also enhanced plant P absorption via a synergistic impact of co-inoculation. The root and shoot length and fresh root weight of pigeon pea plants challenged with mycorrhiza were significantly greater ($P \leq 0.05$) than those of plants tested with pathogen alone.

5.10.6 Mycorrhizal colonization of Pigeon pea

Microscopy observations enabled visualization of the colonized root segments from Pigeon pea, which primarily included intra-radicular hyphae and arbuscular formation through the cortical cells (Figure 5.15). Under these experimental conditions, the formation of vesicle structures was less evident. No symbiotic structure formation was observed in Pigeon pea roots without inoculation.

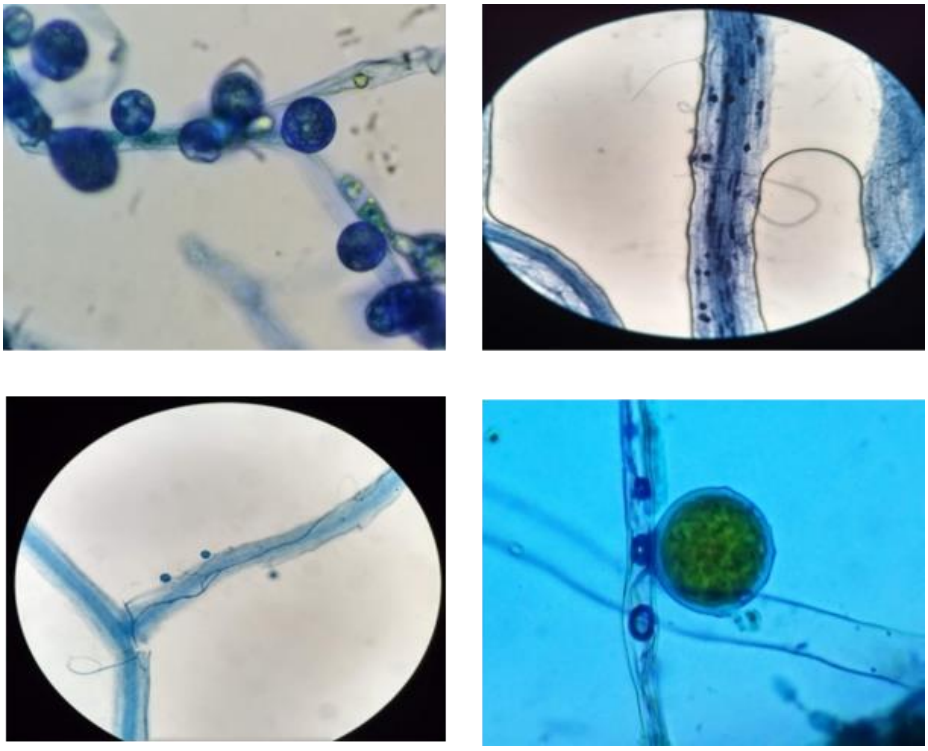

Figure 5.15: Symbiotic structures of *R. irregularis* colonization of Pigeon pea roots. Root segments of Pigeon pea-colonized plants showing Vesicle, Arbuscules, and Intraradical spore were analyzed by light microscopy after trypan blue staining**.**

5.10.7 Ethylene production

Ethylene production was increased significantly in Pigeon pea plants by 50, 37.04, and 35.18% in *F. udum* inoculated plants, respectively, compared to control under stress conditions. Ethylene production was maximum in *F. udum* inoculated plants receiving fungus treatment. However, inoculation of *F. udum* ameliorated the ethylene emission by 16.47, 29.35, and 9.40%, respectively, as compared to Uninoculated plants (Figure 5.16).

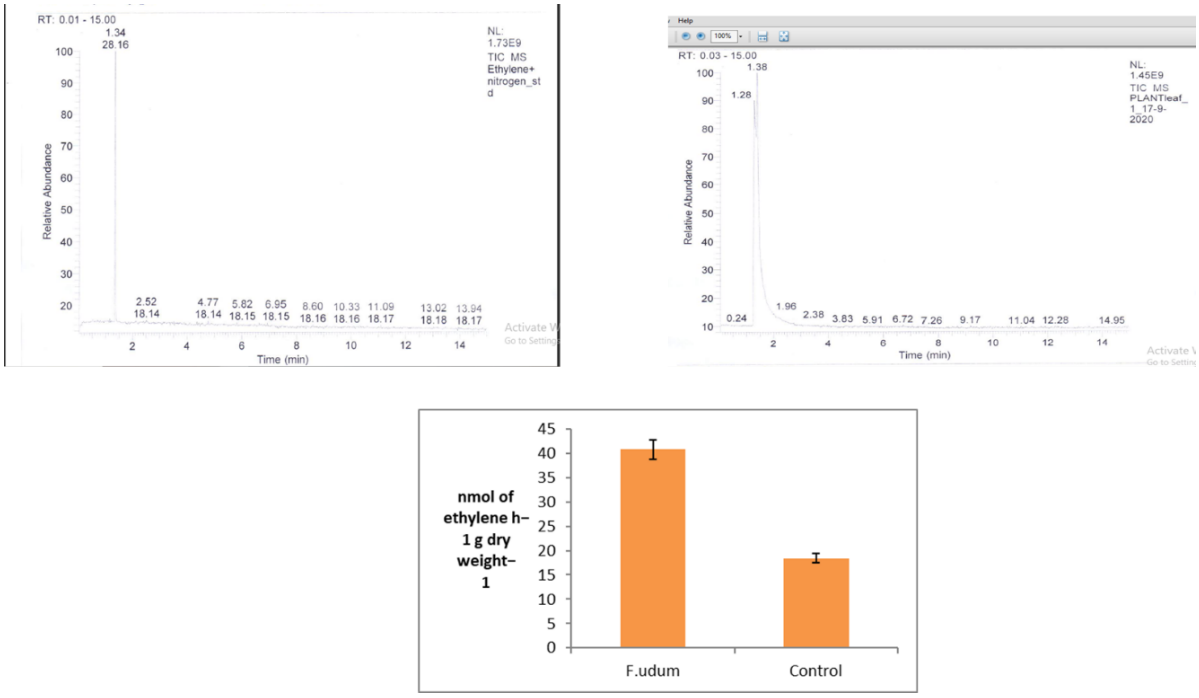

Figure 5.16: Effect of *Fusarium udum* inoculation on ethylene biosynthesis in Pigeon pea plants. The values shown here are the mean of three replicates. Errors bars represent standard errors. Significant differences according to the analysis of variance (ANOVA), followed by the Duncan test ($p \leq 0.05$) applied using the software R studio

5.10.8 Proline accumulation

According to the findings of the present investigation, *F.udum* inoculation increased the accumulation of proline. The greatest accumulation was detected on treated inoculation plants and the least on untreated Uninoculated plants (Figure 5.17).

5.10.9 Malonaldehyde and H_2O_2 Content

Accumulation of malonaldehyde and generation of reactive oxygen species indicate membrane damage due to stress within plant tissue. Accumulation of H_2O_2 (Figure 5.19) and malonaldehyde (Figure 5.18) increased significantly 72 h following *Fusarium* inoculation. However, in PGPA-treated infected plants, H2O2 and malonaldehyde accumulation was relatively lesser than in untreated infected plants, indicating a reduction in spot blotch-induced oxidative damage.

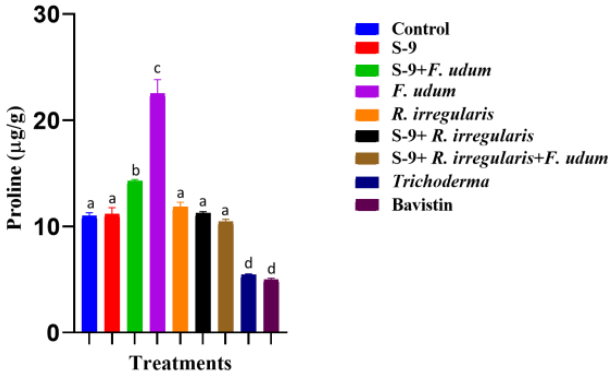

Figure 5.17: Proline accumulation in Pigeon pea plant; Values are mean of three replicates ± Standard Error of Mean (SEM). Different superscripts show significant differences (P≤0.05)

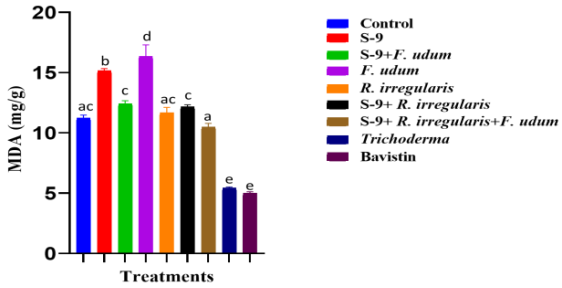

Figure 5.18: MDA content in wilted Pigeon pea leaves;;Values are mean of three replicates ± Standard Error of Mean (SEM). Different superscripts show significant differences (P≤0.05)

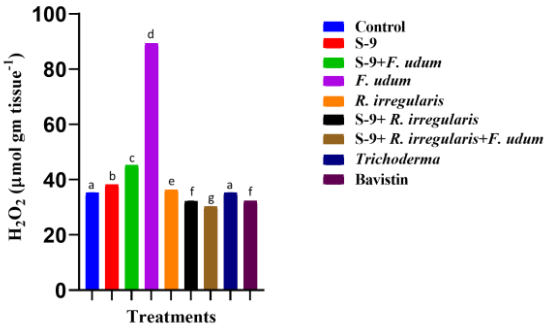

Figure 5.19: H_2O_2 accumulation in wilted Pigeon pea leaves Values are mean of three replicates ± Standard Error of Mean (SEM). Different superscripts show significant differences (P≤0.05)

5.10.10 Histochemical detection of H_2O_2 accumulation

The histochemical detection of H_2O_2 in leaf tissue was accomplished by observing dark brown patches of DAB-H_2O_2 complex in leaf tissue. Microscopical studies revealed that 72 hours after F. udum inoculation, there was an H_2O_2 eruption around the necrotic site, as evidenced by dark brown spots. However, PGPR-treated plants exhibited fewer dark brown spots, indicating that the decline in H_2O_2 accumulation within infected plant tissue was significantly less than in untreated plants, demonstrating the beneficial role of PGPA in reducing spot blotch-induced membrane damage (Figure 5.20).

Figure 5.20: Representative photograph of *in vivo* DAB staining for visualization of H_2O_2formed in Pigeon pea leaf at the end of the experiment**.** The reddish-brown colored spots in the leaves attested to the take-up and polymerization of DAB to capture H_2O_2

5.10.11 Determination of defense-related enzymes

The estimation of defense-related enzymes revealed that inoculated seedlings had significantly higher enzyme activity ($P \leq 0.05$) than control. However, the enzyme concentrations in both treatments were significantly different in the root, shoot, and leaf. The inoculation of *F.udum* significantly increased all defense enzymes, and antioxidant properties in fungal pathogen challenged plants compared to control plants. It was observed that pathogen-challenged treatment increased maximum POD (79 %) contents in the root (Figure 5.21). In the case of *Streptomyces* sp. S-9, the induction of defense enzymes and antioxidant activity was poor in

plants challenged with *F.udum.* However, elevated levels were observed with *the F. udum*-challenged plants similar to those observed for plant growth.

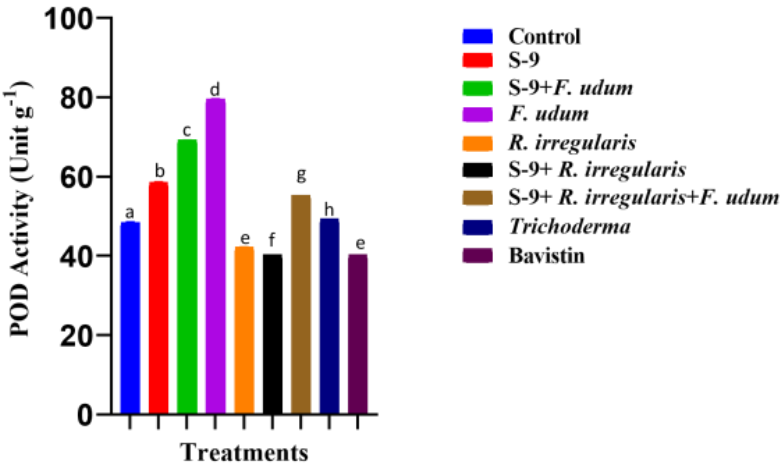

Figure 5.21: Defense enzyme activities in Pigeon pea leaves. Values are mean of three replicates ± Standard Error of Mean (SEM). Different superscripts show significant differences (P≤0.05)

5.10.12 Physicochemical Investigations of Cultivated Plants

Soil analysis showed increased TOC in the soil inoculated with isolate S-9. Compared to uninoculated controls, the phosphorus content also increased in the soil treated with S-9+ *Rhizophagus irregularis*. Soil treated with isolate S-9+ *R. irregularis*showed an increase in potassium and phosphorus content which was beneficial to plant growth (Figures 5.22-5.25). The increase in phosphorus and potassium content in the soil treated with isolates *R. irregularis* is due to their potential to solubilize insoluble P and release K from silicate in soil and thus enhance mineral uptake by plants

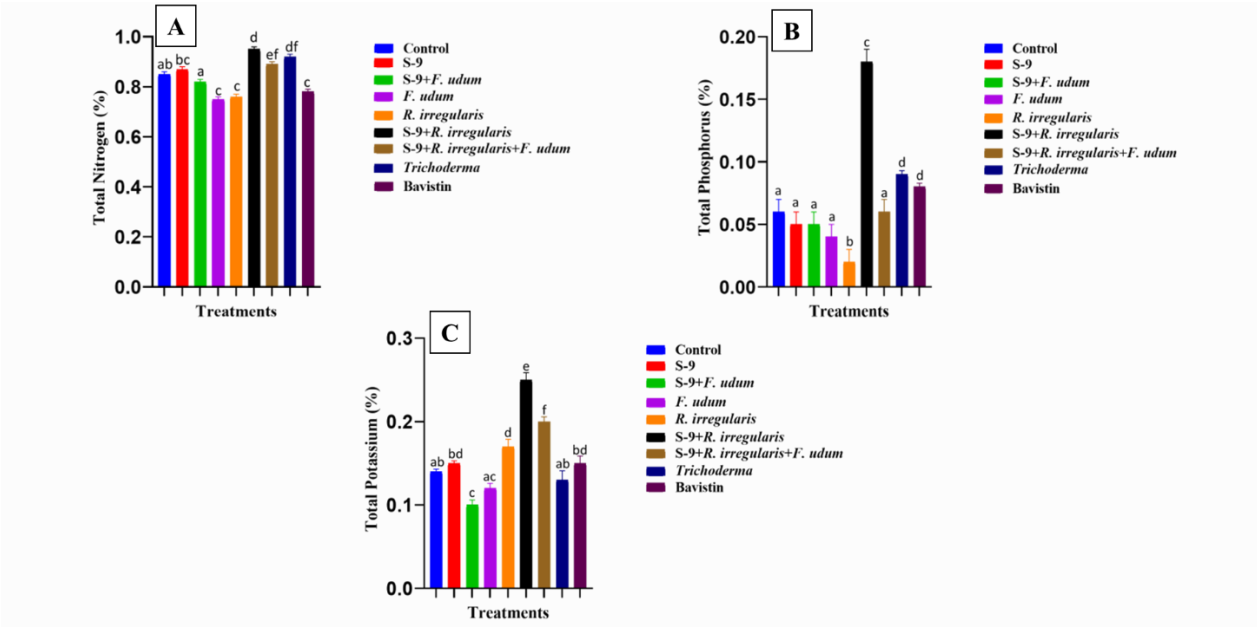

Figure 5.22: Physicochemical properties 2018-2019 Model Farm-cultivated *Cajanus cajan*Plant roots; A- Total Nitrogen; B- Total Phosphorus; C- Total Potassium.

Values are mean of three replicates ± Standard Error of Mean (SEM). Different superscripts show significant differences (P≤0.05)

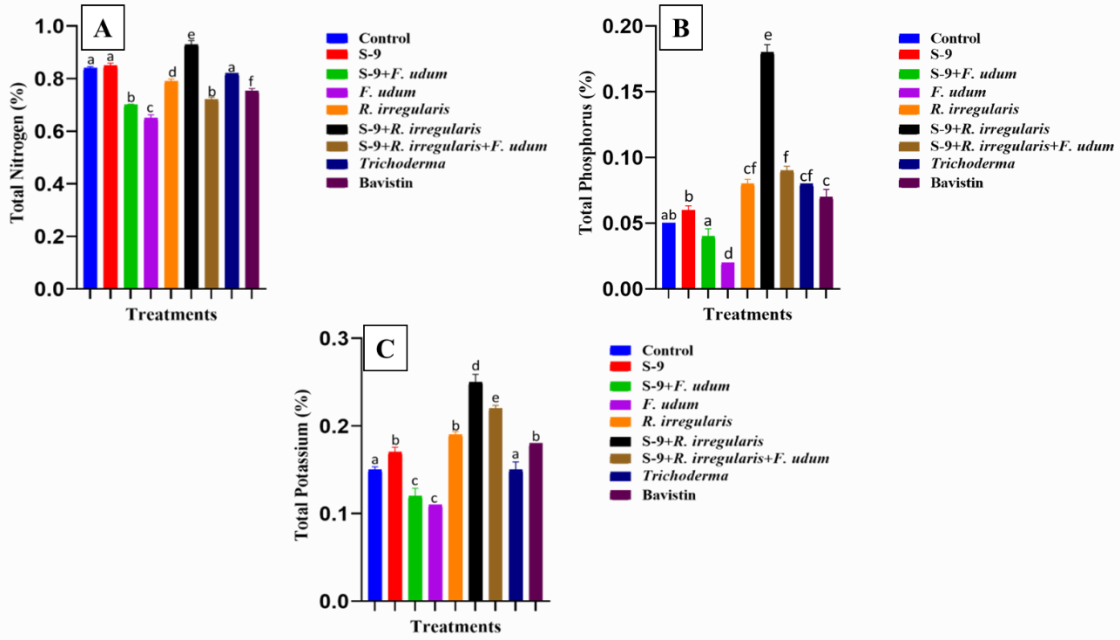

Figure 5.23: Physicochemical properties 2019-2020 Model Farm-cultivated *Cajanus cajan* Plant roots; A- Total Nitrogen; B- Total Phosphorus; C- Total Potassium.

Values are mean of three replicates ± Standard Error of Mean (SEM). Different superscripts show significant differences (P≤0.05)

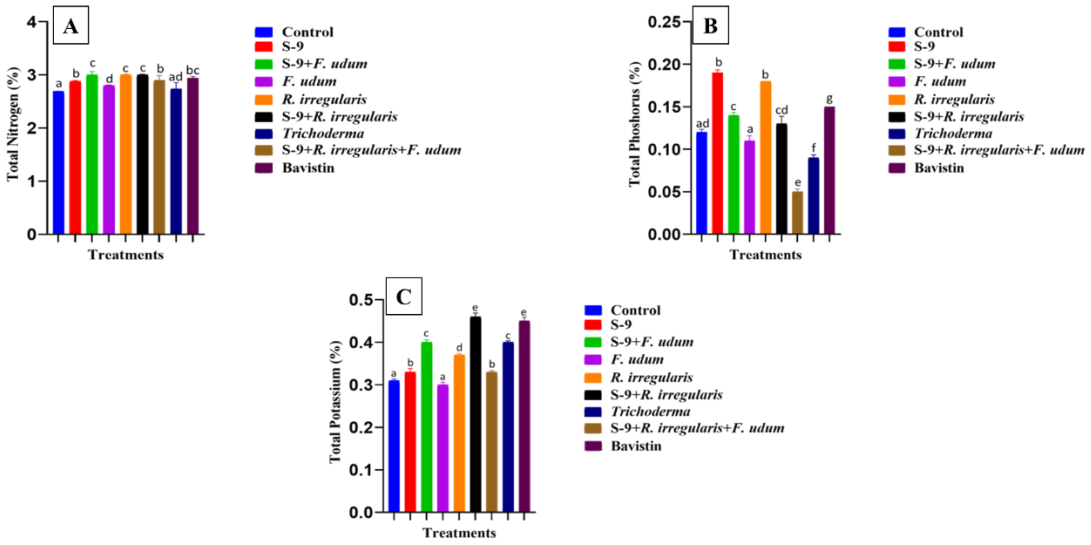

Figure 5.24: Physicochemical properties 2019-2020 Dwarkapura-cultivated *Cajanus cajan* Plant leaves; A- Total Nitrogen; B- Total Phosphorus; C- Total Potassium.

Values are mean of three replicates ± Standard Error of Mean (SEM). Different superscripts show significant differences (P≤0.05)

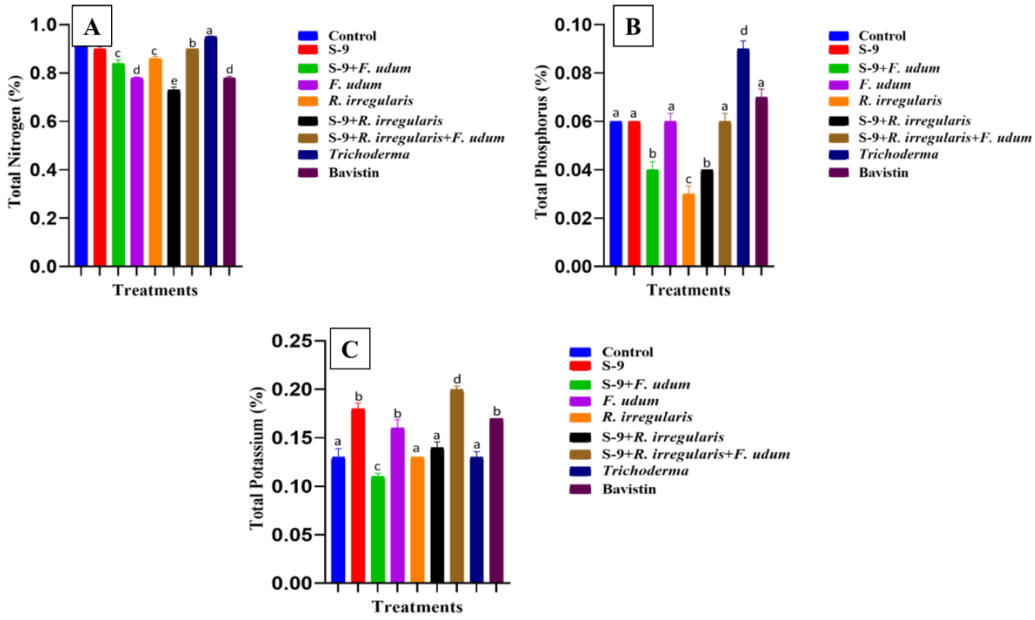

Figure 5.25: Physicochemical properties 2019-2020 Dwarkapura-cultivated *Cajanus cajan* Plant Roots; A- Total Nitrogen; B- Total Phosphorus; C- Total Potassium.
Values are mean of three replicates ± Standard Error of Mean (SEM). Different superscripts show significant differences (P≤0.05)

5.11 Discussion

Various biocontrol agents (BCAs), including bioherbicides, have been formulated in liquid, solid, and powdered forms (Green et al., 1998; Lewis, 1991; Lumsden et al., 1995). Biofertilizers and biocontrol agents can be developed using actinomycetes, notably *Streptomyces* sp., because of their ability to produce spores that aid in dispersal and give resistance to various threats (Chater 1993; Goodfellow & Williams, 1983). There has been insufficient research into the potential of *Streptomyces* species as bacteria that promote plant growth despite the fact that Streptomyces species have a long history in biocontrol and the ability to stimulate plant growth (Aldesuquy et al., 1998). Our research effectively investigated the impact of microbial inoculation on the germination of various *C. cajan* treatments. The microbial inoculations had a considerable impact on seedling emergence and vigour. Seed vigour and viability are essential elements affecting seedling germination, plant growth, and crop output (Miller & Copeland, 1997). Seeds can be treated with fungicides to prevent crop failure caused by seed- or soil-borne plant diseases. Because it introduces inoculum into the rhizosphere, where pathogens such as *Pythium* and *Rhizoctonia* are active and cause seed rots and seedling damping down, seed-applied plant pathogen antagonists are an ideal form of administration. Antimicrobial drugs have been evaluated and commercialized for this purpose, although their usage as seed treatments is uncommon (Butt & Copping, 2000; McQuilken et al., 1998; Nelson, 2004).

In the present study, the seeds treated with *F. udum* and S-9+*F. udum* had stunted growth which resulted in their shoot length being short. However, the root length of S-9+*F. udum*-treated seeds had remarkable germination with long root length but not as long as other treatments. The inoculation with other treatments resulted in long shoot and root lengths. A typical reaction to gibberellins was an increase in seed germination percentage and the length of the seedling shoots. These products can mimic exogenous GAs application. A more significant initial emergence of inoculated plants may be attributable to increased phytohormone synthesis, affecting seed germination (Mia et al., 2012). For example, the aleurone layer's α-amylase plays

a crucial role in hydrolyzing endosperm starch into metabolizable sugars during cereal seed germination, providing energy for root and shoot growth (Akazawa & Hara-Nishimura, 1985; Beck & Ziegler, 1989). Meanwhile, the physiological roles of GAs in seeds of dicotyledonous plants have revealed that developing seeds of leguminosae contain a substantial amount of GAs(Nakayama et al., 2002).

The use of live microbial cultures in agriculture is not encouraged. As a result, several solid, liquid, and powder formulations based on bacterial agent spores have been created (Tamreihao et al., 2016). According to Martinez-lvarez et al. (2016), the formulation process has a crucial impact in the efficacy of goods by increasing the durability and potency of the antibacterial component. Dry formulations, such as powders and granules, are preferred over liquid formulations due to their extended shelf life and portability. Additionally, most dry formulations can be turned into liquid or water-based solutions for spray, drench, or root-soak applications (Lumsden et al., 1995). It's been proposed that bacteria-based formulations for disease management and plant growth promotion could replace chemical chemicals used in agriculture (Martnez-lvarez et al., 2016; Soe & De Costa, 2012). Multiple studies have revealed the screening of microorganisms with biocontrol activity. Due to privacy constraints enforced by commercial companies, relatively few of these publications contain information regarding the formulation of microorganisms (Herrmann & Lesueur, 2013). The talcum powder formulation of S-9 significantly improved the root length of seedlings, as we and these findings are consistent with several studies highlighting the enhancement of plant development by spore-forming bacteria-based biopesticides (Martínez-Álvarez et al., 2016).

In the present study, S-9+*R. irregularis* formulation exhibited the best biocontrol activity when compared to other treatments. The native microorganisms, *R. irregularis,* in the experimental set-up soil had a synergy with S-9 in the biocontrol of *F. udum*. These results concur with Goudjal et al. (2014, 2016) about the biocontrol of soil-borne phytopathogenic fungi, who hypothesized the presence of indigenous antagonistic microorganisms. Using *R. irregularis* can reduce the negative impacts of drought by regulating signalling pathways and protein production in plants' responses to drought (T. Li et al., 2014; Porcel et al., 2006). Due to increased competition for nutrients and the formation of secondary metabolites that can impair either PGPR or AMF populations, competitive interactions may emerge among inoculants (Trivedi et al., 2012; Xiao et

al., 2008). Varied species (Jäderlund et al., 2008) and diverse abiotic environments lead to different interactions. A combination of plant species, bacteria, and fungi can be used to increase plant growth under adverse circumstances (Larsen et al., 2009).