Chapter 3

Materials and Methods

3.1 Sample collection

All total different soil samples were collected from cultivated lands of four different cities (Rajkot, Morbi, Veraval and Khijadiya) of Saurashtra region of Gujarat. Khijadiya (Latitude 22.348390, Longitude 70.572820),Veraval (Latitude 20.914220, Longitude 70.369130),Rajkot (Latitude 22.297680, Longitude 70.787460), Morbi (Latitude 22.824200, Longitude 70.831299).The rhizosphere region soil samples at depth of 2to3 inches below the surface of soil was collected in black polythene bags as per the standard protocol of Goswami et al.,(2014). The different plant regions like Wheat, Sorghum, Chickpea, Onion, Garlic, Maize field areas were chosen for collection. The collected soil samples were processed by sieving at preliminary stage followed by a cold storage at 4℃ in zip lock bags for further studies.

3.2 Physicochemical analysis of soil

The soil samples were undergone basic characterization as per the method of Mazeed *et al*., (2015). A series of tests pertaining to different properties like pH, Electrical conductivity, soil organic carbon and nitrogen content, available potassium, phosphorus and micronutrient estimation were carried out respectively. Soil analysis study has been carried out from Gujarat State Fertilizers and Chemical Limited.

3.2.1 Soil pH

The soil sample was dried and finely ground using a mortar and pestle. The macro-particles were separated out using a 2 mm sieve. After that, 10 grams of the soil was mixed with 40 ml of distilled water in a 15 ratio. The mixture was shaken for 2-3 minutes and left to settle for 2 minutes. Finally, the supernatant was used to measure the soil's pH using a calibrated electrode. The pH value displayed on the meter was recorded as the soil's pH (Wagh *et al*., 2013).

3.2.2 Soil electrical conductivity

10 g of air-dry soil was mixed with 50 ml of deionized water into a bottle to prepare the soil-water suspension. Then the mixer was shaken mechanically at 15 rpm for an hour to dissolve

soluble salts. At last, the supernatant was used to measure the electrical conductivity. A calibrated conductivity meter (Horiba) was used to measure the conductivity (Wagh *et al*., 2013).

3.2.3 Soil organic carbon and nitrogen

10 millilitres of 1N potassium dichromate solution were poured into a 250 millilitre conical flask, followed by a slow addition of 20 millilitres of concentrated H_2SO_4. The solution was continuously swirled and allowed to cool for 10 minutes. Subsequently, 200 millilitres of distilled water were added, along with 10 millilitres of orthophosphoric acid and 1 millilitre of indicator solution. Finally, the solution was mixed vigorously. Titration was performed using Ammonium ferrous sulphate as a titrant. Colour changed from blue to green, indicated the endpoint of titration. This recorded data served as the blank reading(Walkey and Black Method, 1979).For sample preparation, the oven-dried soil was finely ground and passed through a 0.2 mm sieve. Following this, 0.5 grams of the soil sample was added at the base of a 500 millilitreconical flask, entire titration process was repeated using soil as the sample, and the corresponding reading was noted. Soil organic carbon and nitrogen were computed utilizing the subsequent equations

$$\boldsymbol{Organic\ carbon}\ (\%) = \frac{\mathbf{0.03}\ \times (\boldsymbol{B} - \boldsymbol{T}) \times \mathbf{100}}{\boldsymbol{B}\ \times \boldsymbol{S}}$$

Organic Nitrogen (%) = 0.0862 × Organic Carbon (%)

Where,

B= Volume of ferrous ammonium sulphate required for blank titration in ml.

T= Volume of ferrous ammonium sulphate needed for soil sample in ml.

S= Weight of soil sample in grams.

3.2.4 Soil potassium content

The soil sample was dried and finely ground using a mortar and pestle. The macro-particles were separated out using a 2 mm sieve. After that, 10 gm of soil was mixed with 50 ml of the ammonium acetate solution into a bottle and kept in a shaker for 30 minutes. The soil particles were allowed to settle down and the supernatant was filtered through a dry Whatman filter paper No. 2. Flame Photometer was set at 100 using the 100ppm potassium solution. The calibration graph was constructed by drawing data points from aspirating standard

potassium solutions at concentrations of 20, 40, 60, and 80 ppm. Subsequently, the potassium content within the soil extract was assessed by aspirating the solution. The concentration of potassium was then determined using the calibration graph (Rawal *et al.,* 2022).

3.2.5 Soil phosphorus content

The process began with air-dried soil samples being sifted through a 2 mm sieve. Then, 2 grams of activated charcoal were combined with 5 grams of the soil sample. This mixture was then blended with 50 ml of NH_4C0_3 solution in a bottle and placed on a shaker for 30 minutes. Once the shaking concluded, the soil particles settled, and the resulting supernatant was filtered through a dry Whatman filter No. 2. To this filtered solution, 5 ml of ammonium molybdate solution was added and thoroughly mixed until the evolution of CO_2 ceased. Following this, 10 ml of distilled water and 1 ml of stannous chloride solution were introduced. The Optical density at 660nm was measured subsequently (Rawal *et al.,* 2022).

3.2.6 Estimation of Micronutrients

10 grams of soil sample were moved into 100 ml polythene shaking bottles. Approximately 20 ml of the DTPA extracting solution was introduced and vigorously shaken for a duration of two hours. The contents were subsequently filtered through Whatman No. 42 filter paper. The resulting filtrates were utilized for determining the levels of copper, zinc, ferrous, and manganese using the appropriate hollow cathode lamps within an Atomic Absorption spectrophotometer (Lindsay and Norvell, 1978).

3.3 Isolation of bacteria from soil sample

All stored soil samples were taken and settle down at room temperature (28±1°C).Spread plate technique were employed for isolation of PGPR.

3.3.1 Spread plate method

For the isolation of bacterial strain collected soil sample were serially diluted followed by 10^{-1} to 10^{-6} and placed on nutrient agar plates and subjected to incubation at 37°C for 24- 48 hours. Single colony was picked up and streaked on sterile nutrient agar plate to get pure culture. Pure isolated, purified colonies were observed for morphological and microscopic characterization and maintained for further studies. Likewise,

KW– Khijadiya Wheat

KS- Khijadiya Sorghum

KC – Khijadiya Chickpea

VG – Veraval Garlic

VO-Veraval Onion

MC-Morbi Corn

RC- Rajkot Corn

3.4 Identification of bacterial isolates

The selected isolated bacterial colonies were examined for a detailed study of colonycharacteristics employing basic colony inspection on Nutrient Agar medium. A series of colony morphology identification in terms of size, shape, margin, elevation, pigmentation, opacity, consistency etc.(Aneja, 2005) were performed. Also, the Gram's Staining carried out as per the standard procedures given in Claus (1992).

3.5 Screening of PGPR isolates based on evaluation of different traits Qualitative characterization of PGPR traits of the isolated rhizobacteria (PGPR)

All total 41 isolates of rhizobacteria were undergone the screening for potent isolates selection for further experiments to be carried out.

3.5.1 HCN Production

HCN production is the major factor to suppress pathogens from plants. The bacterial culture was inoculated on a nutrient medium enriched with 4.4 g/l glycine. Sterile filter paper saturated with picric acid solution (composed of 2.5 gm of picric acid, 12.5 gm of sodium carbonate, 1000 ml d/w)was positioned on the upper lid of petri-plate and then subjected to the incubation at 37°C for 2 days. Observing the colour change of filter paper from yellow to light brown, brown-reddish, and brown was recorded as weak, moderate, and strong respectively (Bhatt *et al.,* 2020).

3.5.2 Chitinase Assay

Colloidal chitin was prepared, Take the ten grams of chitin crab shell and was mixed with 150mL concentrated hydrochloric acid, after that the mixture was put in shaker for continuous stirring for 2 hours at 4°C. The suspension was diluted with 1 litre of D/W and filtered using

filter paper. This process was repeated four to five times and the pH of the resulting suspension was brought to a neutral value by adding 5NNaOH. The suspension was washed sometimes with double distilled water for desalting purpose. Following desalting, the suspension was centrifuged for 10 minutes at 8000 rpm, and the resulting precipitate was collected for subsequent utilization as colloidal chitin. All pure isolates inoculated on plate with 1% chitin supplemented in N-agar. After 5 days of incubation at room temperature, the zone of clearance due to chitin hydrolysis was recorded as positive chitinase producing bacteria. (Bhatt *et al.*,2020)

3.5.3 Siderophore Production

A qualitative assay of siderophore production was conducted in Chrome Azurole S (CAS) agar medium. CAS medium was prepared and spot inoculate with bacterial isolates and incubated at 37°Cfor 3-5 days. Development of yellow orange halo around the colony was considered siderophore production. (Rana *et al.,*2012)

3.5.4 Phosphate Solubilization

The phosphate solubilization ability of the bacteria was analysed using Pikovskaya agar plate, incubated for 72-96 hours and detecting a distinct clear zone surrounding the colonies (Pikovskaya, 1948). The phosphate solubilizing index (PSI) was calculated using following formula,

$$PSI = \frac{\text{Colony diameter (in mm) + Halo zone diameter(in mm)}}{\text{Colony diameter (in mm)}}$$

3.6 Quantitative characterization of PGPR traits of the isolated rhizobacteria (PGPR)

3.6.1 Ammonia Production

The bacterial isolates were tested for the qualitative production of ammonia 24 hr. old bacterial culture inoculation into a peptone water medium, followed by incubation at 37°C for 48 hours. Subsequently 1.0ml of Nesseler's reagent was added to each tube to detect ammonia production, as indicated by colour development. The development of yellowish-brown colour in the test tube indicates the production of ammonia (Ghavam *et al.,* 2021).

3.6.2 IAA Production

The bacterial culture has been inoculated in the tryptophan broth medium and incubated 37°C for 48 hours after the incubation, centrifuged the sample at 10000rpm for 5 minutes and collect the supernatant into the fresh test tube then added 2.0 ml of 2ml Salkowaski followed by the 30 minutes of incubation at room temperature. Colour change (pink) indicated a high amount of IAA production, it recorded by the absorbance at 530nm (Bhatt *et al.,* 2020)**.**

3.7 Effect of PGPR on Monocot (Cumin, Rice) and Dicot (Groundnut, Mung, Chickpea) by Pot Analysis in the Laboratory Condition

3.7.1Pot Culture Analysis in the Laboratory

For the evaluation of rice, mung, cumin, groundnut and chickpea growth promotion KS2, KC8, KC9 and KC11were tested with the sterile and non-sterile soils in the Microbiological laboratory at Atmiya University. For the testing paper glasses were utilized with the capacity of 50gm of soil. All the treatments arrange in 3 replicas of pots. Treatment was arranged based on a completely randomized design. After 15 days of root length, shoot length, and number of leaves has been evaluated (Nezarat & Gholami, 2009). Then prepared the combinations for the pot culture experiment employing potent bacterial isolates such as (KS2, KC8, KC9, and KC11) with rice, mung, cumin, groundnut and chickpea seeds (Karthick *et al.,* 2008).

a. Control; Cumin seeds soaked with double distilled water
b. Soil + Cumin seed coated with KS2 inoculant
c. Soil + Cumin seed coated with KC8 inoculant
d. Soil + Cumin seed coated with KC9 inoculant
e. Soil + Cumin seed coated with KC11 inoculant
f. Control; Groundnut seeds soaked with double distilled water
g. Soil + Groundnut seed coated with KS2 inoculant
h. Soil + Groundnut seed coated with KC8 inoculant
i. Soil + Groundnut seed coated with KC9 inoculant
j. Soil + Groundnut seed coated with KC11 inoculant
k. Control; Chickpea seeds soaked with double distilled water
l. Soil + Chickpea seed coated with KS2 inoculant

m. Soil + Chickpea seed coated with KC8 inoculant
n. Soil + Chickpea seed coated with KC9 inoculant
o. Soil + Chickpea seed coated with KC11 inoculant
p. Control; Mung seeds soaked with double distilled water
q. Soil + Mung seed coated with KS2 inoculant
r. Soil + Mung seed coated with KC8 inoculant
s. Soil + Mung seed coated with KC9 inoculant
t. Soil + Mung seed coated with KC11 inoculants
u. Control; Rice seeds soaked with double distilled water
v. Soil + Rice seed coated with KS2 inoculant
w. Soil + Rice seed coated with KC8 inoculant
x. Soil + Rice seed coated with KC9 inoculant
y. Soil + Rice seed coated with KC11 inoculant

3.8 Biochemical Characterization of Selected Potent Bacterial Isolates

3.8.1 Indole Test

Tryptophan broth was utilized to assess the presence of tryptophanase enzyme in a bacterial strain. The bacterial culture was introduced in to the broth medium, and the tubes were then incubated at 37°C for 48 hours. Following incubation, Kovac's reagent was added to the tubes. The detection of a positive indole test was confirmed by the formation of cherry red ring in the reagent layer on top of the medium (Aneja, 2005).

3.8.2 Methyl-Red Test

The bacterial culture was introduced into a nutrient broth medium containing peptone, 5.0 g; glucose, 5.0 g; NaCl, 5.0 g; distilled water, 1000 ml; pH, 7.0 and tubes were incubated at 37°C for 2 days. A positive result was indicated by the appearance of bright red color by the addition of 2- 3 drops of methyl red reagent into each tube (Ludvigsen *et al.,* 2018).

3.8.3 Voges-Proskauer Test

The bacterial isolates were introduced into broth, followed by an incubation period at 37°C for 48hrs. Subsequently, 10-12 drops of 5% α-naphthol and 6-8 drops of aqueous KOH solution added to each tube. The mixture was shaken well to facilitate the formation of cherry red ring which indicate positive result (Ludvigsen *et al.,* 2018).

3.8.4 Utilization of Citrate Test

Simmons citrate broth was employed by examine the ability to use citrate from organisms. The pure bacterial isolate was streak on to the slant and then incubated at 37°C for a duration of 48 hours. Results were recorded after observing the growth on slant and alkaline reaction. Bacterial growth would be visualized on to the slant surface and the medium colour will be changed from green to blue. (Vaughn *et al.,*1950).

3.8.5 Starch Hydrolysis

Bacterial strain was grown onto the surface of the starch agar plate following a 24 hrs of incubation, the surface of the plate was submerged with iodine to detect the presence or absence of starch in the area surrounding the bacterial growth by revealed as a clearing zone around the isolated culture (Evangelista *et al.,*2017).

3.8.6 Catalase Activity

This test assesses the capacity to breakdown hydrogen peroxide by secretion of catalase enzyme. Place the one drop of 3% hydrogen peroxide on the sterile glass slide followed by bacterial colony and assorted well. The presence of air bubbles indicates the positive result. (Kesaulya *et al.,* 2021).

3.8.7 Motility Test

Employed to identify the capability of bacterial movement away from the inoculation line. The bacterial colony was inoculated into the soft agar medium through a needle and all the tubes were incubated at 37°C for 48 hours, to observe the migration (Kesaulya *et al.,* 2021).

3.8.8 Carbohydrate Fermentation

Phenol red broth containing specific carbohydrate source for media completion. Mature bacterial culture was inoculated into the tubes and followed by incubation period for 2 days at 37°C. After the completion of incubation period acid production that indicates lowering the pH of the test medium, that detected by the color change of the pH indicator by change the colour red to yellow and to determine the gas production durum's tubes were utilized (Hassan *et al.,*2018).

3.9 Molecular Identification of Selected Potent Bacterial Isolates

3.9.1 16S RNA Sequencing

For the molecular identification of mature bacterial isolates of KS2, KC8, KC9 and KC11 were outsourced at Gene Explore Diagnostics & Research Centre Pvt. Ltd. Ahmedabad, Gujarat for 16S RNA sequencing. As per the std protocol of Sanger's method, DNA sequencing reaction of PCR amplicon was carried out with 357F & 1391R primers using BDT v3.1 Cycle Sequencing Kit on ABI 3500xl Genetic Analyzer. The partial 16s rRNA sequence was used to carry out BLAST with the database of NCBI GenBank database. Based on the maximum identity score first ten sequences were selected and aligned using multiple sequence the alignment software programs (Dipanwita *et al.,* 2015).

3.10 Compatibility Test

To develop the microbial consortia, the compatibility of the isolates assessed. The microorganism used in the experiment were tested for their compatibility using nutrient agar medium. This involved streaking dual inoculants on nutrient agar medium to determine compatibility among the microorganisms, plates were incubated at 28℃ for 3-4 days(Kavya Y *et al.,* 2020).

3.11 Development of Consortia

The effect of pure isolate was studied for monocot and dicot plants. Seeds were soaked for Overnight in the 24hours old culture of each isolate. Ten soaked seeds were introduced in the pots containing soil. Pots were used for each particular treatment as replicates and four pots containing infested soil planted with sterilized seeds served as controls. In addition, non-infected soil served as controls. The germinated seedlings were planted in a pot containing autoclaved soil, with one plant in every pot. After ten days, 2 mL suspensions of the pure isolates (1.0×10^8 CFU/mL) and water were poured into individual pots.

a. Control Simple soil + Cumin seeds
b. Soil + KS2 + KC8 + Cumin seeds
c. Soil + KS2 + KC9 + Cumin seeds
d. Soil + KS2 + KC11+ Cumin seeds
e. Soil + KC8 + KC9 + Cumin seeds

f. Soil + KC8 + KC11 + Cumin seeds
g. Soil + KC9 + KC11 + Cumin seeds
h. Control Simple soil +Groundnut seeds
i. Soil + KS2 + KC8 +Groundnut seeds
j. Soil + KS2 + KC9 + Groundnut seeds
k. Soil + KS2 + KC11+ Groundnut seeds
l. Soil + KC8 + KC9 + Groundnut seeds
m. Soil + KC8 + KC11 + Groundnut seeds
n. Soil + KC9 + KC11 + Groundnut seeds
o. Control Simple soil + Chickpea seeds
p. Soil + KS2 + KC8 + Chickpea seeds
q. Soil + KS2 + KC9 + Chickpea seeds
r. Soil + KS2 + KC11+ Chickpea seeds
s. Soil + KC8 + KC9 + Chickpea seeds
t. Soil + KC8 + KC11 + Chickpea seeds
u. Soil + KC9 + KC11 + Chickpea seeds
v. Control Simple soil + Mung seeds
w. Soil + KS2 + KC8 + Mung seeds
x. Soil + KS2 + KC9 + Mung seeds
y. Soil + KS2 + KC11+ Mung seeds
z. Soil + KC8 + KC9 + Mung seeds
aa. Soil + KC8 + KC11 + Mung seeds
bb. Soil + KC9 + KC11 + Mung seeds
cc. Control Simple soil + Rice seeds
dd. Soil + KS2 + KC8 + Rice seeds
ee. Soil + KS2 + KC9 + Rice seeds
ff. Soil + KS2 + KC11+ Rice seeds
gg. Soil + KC8 + KC9 + Rice seeds
hh. Soil + KC8 + KC11 + Rice seeds
ii. Soil + KC9 + KC11 + Rice seeds

3.12 Isolation of fungal pathogen from infected soil and plant samples

For the isolation of pathogenic fungal strain in the Potato Dextrose Agar medium was employed. Dilution suspension was prepared by taking 1.0ml of suspension sample in a test tube by adding 9.0ml of sterile D/W for every sample. 10^{-3}, 10^{-4} and 10^{-5} dilutions were employed to isolate fungi culture from infected plant samples in order to avoid over-crowding of the fungal colonies in plates. Similarly collected infected plant samples from the agricultural field and surface sterilized by 2% of $HgCl_2$ solution then crushed the samples by using sterile glass rod with 10 ml of distilled water. Then 1.0ml serially diluted sample was added in 1% streptomycin-containing potato dextrose agar medium petri plates and to maintain the triplicates. Then plates were placed in the incubator for 7 days at 28˚C. At higher dilutions, fungal colonies are easily isolated as because they format well-dispersed surface hyphae colonies (Rathna *et al.,* 2016).

3.13 Microscopic identification of fungal pathogens

Lacto phenol Cotton Blue staining method has been performed with the usages of inoculating needle and Bunsen burner. Small portion of grown fungal culture was transferred into the lacto-phenol cotton blue containing glass slide with placing to the cover-slip. Then stained fungal species were observed under the light microscope (40X) for morphological identification (Aneja 2002). While for the bacterial identification gram staining methods has been performed for all the selected bacterial isolates.

3.14 Pathogenicity test

Fusarium sp. was cultured on potato dextrose agar, following incubation period 7 days at 28℃. Spores were collected in sterile tap water, and the concentration of spore suspension was quantified using a Neubauer counting chamber and adjusted to 1×10^6 spores/ml. Experiment was carried out by infested into agricultural soil at *in-vivo* condition. *Fusarium sp.* was prepared by growing each fungus on potato dextrose medium in for 7 days at 25◦C. Soil invasion was achieved by mixing the fungal inoculum with the soil at rate of 1.5% (w/w) in agricultural soil pots ($25 \times 25 \times 30$ cm^3) and spray water regularly for five days before planting (Shaban & Bramawy, 2011).

3.15 *In*-vitro Antifungal and Antagonistic Activity of Bacterial Isolates against Soil borne Fungal Pathogen *Fusarium sp.*

The antifungal activity was done by agar well diffusion method. The antagonistic activity of the individual bacterial strains KS2, KC8, KC9, and KC11 were initially evaluated *in-vitro*, in confrontation assays against *Fusarium sp.* Dual culture method has been performed for the determination of the antagonistic activity of selected potent bacterial isolatesagainst fungal pathogens *Fusarium sp.* on Potato Dextrose Agar medium. Bacterial isolates were line inoculated between the fungal pathogen and placed on PDA medium in the same petri dish, which is basically 4.0cm away from each other individually. All the culture-inoculated petri plates were incubated at 28±2°C for six days. After the completion of the incubation period plates were observed for antagonistic activity of potent bacterialisolatesagainst soil-bornefungal pathogens and bacterial isolates. The index of antagonism percentage of growth inhibition of soil-borne fungal isolates was determined by the method of Watanabe (1984).

3.16 Ultra Structural Study of Hyphal Cell

100 microliters *Fusarium sp*. (OQ654012) spore suspensions was spread evenly on the surface of PDA plate contain *Bacillus* sp. (KC9) cells and 0.85% NaCl used as control. Plates were placed at 28°C for 5 to 6 days. Then *Fusarium* sp. hyphae were harvested for SEM analysis. SEM analysis performed by outsourced at Department of Microbiology, Junagadh Agriculture University, Junagadh, Gujarat.

3.17 Study the Effect of Seed inoculation with Plant Growth-Promoting Rhizobacteria (KC9 & KC11) by employing Bio-priming Method

3.17.1 Inoculum Preparation

The PGPR strain (KS2, KC8, KC9 and KC11) were inoculated in 100 Erlenmeyer flask containing 50 ml nutrient broth, flasks were incubated at 37°C for 2 days. During inoculation, the viable cell suspension count was 33 x 10^8 CFU/ml (Samy *et al.,* 2008).

3.17.2 Seed Treatment

PGPR strain was employed in monocot (cumin, mung, rice) and dicot (groundnut and chickpea) seed treatments. Seeds were purchased from the local market of Rajkot region. All the seeds were surface sterilized with 0.02% of sodium hypochlorite solution for about 2-3 mins then rinsed thoroughly with double distilled water. For seeds inoculation, dipped all the

seeds into the bacterial (10^8cfu ml$^-$) suspension for 2 mins and considered as treated seeds while only seeds soaked with double distilled water as control non treated (Nezarat & Gholami, 2009).

3.17.3 Bioassay of Seed Germination

A seed germination assay was carried out by employing the paper towel method (Niranjan *et al.,* 2004). 5-5 seeds were selected for each treatment designed for the distribution of the experiment employing a completely randomized design with three replicas, as the petri dish is an experimental component, incubated in moist chamber at 28°C for 7 days. Observed and counted the germinated seed numbers such as groundnut and chickpea. After incubation, germination index and relative seed germination of seedlings root length and shoot length were assessed by employing formula (Fahsi *et al.,* 2021).

$$\textbf{Seed Germination Index} = \frac{\textbf{number of germinated seed}}{\textbf{Total Number of seeds}} \times \mathbf{100}$$

$$\textbf{Relative Seed Germination} = \frac{\textbf{number of germinated seed in given treatment}}{\textbf{Control (number of seeds)}} \times \mathbf{100}$$

3.18 Development of Consortia for Biocontrol Agent employing KC9 & KC11

The impact of pure isolate was investigated concerning wilt disease. Seeds were soaked for Overnight in the 24 hours old culture of each isolate. The soil was infested with *F. oxysporum* (25 CFU/g soil) and irrigated. Ten soaked seeds were placed in the pots containing soil infected with the pathogen. Pots were used for each particular treatment as replicates and four pots containing infested soil planted with sterilized seeds served as controls. In addition, four pots with each treatment and non-infected soil served as controls. The percentage of germination and wilt were monitored 10 days from showing, respectively. The cultivar seeds were selected, disinfected by soaking in 3% sodium hypochlorite for 5 min, and then washed three times with sterile D/W. After disinfestation, the seeds were germinated in Petri dishes with wet sterile filter papers at 26°C with 16 hrs of light per day. The germinated seedlings were planted in a pot containing autoclaved soil, with one plant in every pot. After ten days, 2 mL suspensions of the pure isolates (1.0×10^8 CFU/mL) and water were poured into individual pots. After inoculation for ten days, the 2 mL spore suspensions (1.0×10^8 spores/mL) of *F. oxysporum* were poured into each pot. Each treatment was performed in

replicates according to the criteria for disease resistance pattern proposed by (Hilal*et al.,* 2016).

3.18.1 *In-vitro* Laboratory Experiment for Pot Culture Analysis

The mature culture isolates were inoculated in 100ml of nutrient broth and kept in an orbital incubator shaker at 37°C for 7 days. After the completion of the incubation period, collect the 5.0ml culture inoculum has been introduced into every pot of monocot and dicot seeds. 5-5 seeds of cumin have been taken for every pot experiment were soaked it for over-night. Then prepared the combinations for the pot culture experiment (Karthick *et. al.,* 2008).

a. Control Simple soil + Cumin seeds
b. Control Simple soil + *Fusarium* sp. + Cumin seeds
c. Soil + KC9 + Cumin seeds
d. Control Simple soil + KC9 + *Fusarium* sp. + Cumin seeds
e. Soil + KC11 + Cumin seeds
f. Control Simple soil + KC11 + *Fusarium* sp. + Cumin seeds
g. Soil + KC9 + KC11 + Cumin seeds
h. Control Simple soil + KC9 + KC11 +*Fusarium* sp. + Cumin seeds
i. Control Simple soil + Groundnut seeds
j. Control Simple soil + *Fusarium* sp. + Groundnut seeds
k. Soil + KC9 + Groundnut seeds
l. Control Simple soil + KC9 + *Fusarium* sp. + Groundnut seeds
m. Soil + KC11 + Groundnut seeds
n. Control Simple soil + KC11 + *Fusarium* sp. + Groundnut seeds
o. Soil + KC9 + KC11 + Groundnut seeds
p. Control Simple soil + KC9 + KC11 +*Fusarium* sp. + Groundnut seeds
q. Control Simple soil + Chickpea seeds
r. Control Simple soil + *Fusarium* sp. + Chickpea seeds
s. Soil + KC9 + Chickpea seeds
t. Control Simple soil + KC9 + *Fusarium* sp. + Chickpea seeds
u. Soil + KC11 + Chickpea seeds
v. Control Simple soil + KC11 + *Fusarium* sp. + Chickpea seeds
w. Soil + KC9 + KC11 + Chickpea seeds

x. Control Simple soil + KC9 + KC11 +*Fusarium* sp. + Chickpea seeds
y. Control Simple soil + Mung seeds
z. Control Simple soil +*Fusarium* sp. + Mung seeds
aa. Soil + KC9 + Mung seeds
bb. Control Simple soil + KC9 + *Fusarium* sp. + Mung seeds
cc. Soil + KC11 + Mung seeds
dd. Control Simple soil + KC11 + *Fusarium* sp. + Mung seeds
ee. Soil + KC9 + KC11 + Mung seeds
ff. Control Simple soil + KC9 + KC11 +*Fusarium* sp. + Mung seeds
gg. Control Simple soil + Rice seeds
hh. Control Simple soil + *Fusarium* sp. + Rice seeds
ii. Soil + KC9 + Rice seeds
jj. Control Simple soil + KC9 + *Fusarium* sp. + Rice seeds
kk. Soil + KC11 + Rice seeds
ll. Control Simple soil + KC11 + *Fusarium* sp. + Rice seeds
mm. Soil + KC9 + KC11 + Rice seeds
nn. Control Simple soil + KC9 + KC11 +*Fusarium* sp. + Rice seeds

3.19 Effect of Seedling Germination with and without Treatment of PGPR strain on monocot and dicot seeds

3.19.1 Collection of treated monocot and dicot Seedlings

The monocot and dicot plant leaves were collected from the pots for further analysis. Plant seedlings were grown for 15 days. Plants were analysed for growth parameters. Different morphological parameters were studied like root length, shoot length and fresh and dry weight of leaves, total free amino acids, overall phenolic compound and total flavonoid content.

3.19.2 Total Chlorophyll Content

The total chlorophyll content, content of chlorophyll a and chlorophyll b were examined by using Arnon (1949). One gram of groundnut and chickpea leaves were taken, then finely cut and gently chopped in clean mortar pestle with chilled 80% acetone (v/v).For the centrifugation, adjust the volume 25 ml with 80% acetone, then centrifuged for 15 minutes at 3000 rpm. The clear suspension was transfer in a fresh tube and the OD was recorded at 645

nm and 663 nm. The blank was taken as 80% acetone (v/v). The content of chlorophyll a and chlorophyll b was calculated by the following formula (Patel *et al.,* 2023).

Where,

Chlorophyll "a" (µg/ml) = (12.7 ×OD at 663 nm) – (2.69 ×OD at 645 nm)

Chlorophyll "b" (µg/ml) = (22.9 ×OD at 645 nm) – (2.69 ×OD at 663 nm)

Total Chlorophyll (µg/ml) = (20.2×OD at 645 nm) + (8.02 ×OD at 663 nm)

3.19.3 Relative water Content (RWC)

The percentage of relative water content (RWC) was measured using the protocol proposed by Patel *et al.,* (2023). The plant leaves were collected randomly, and measure the fresh weight then the leaves were immersed in a tube with water and put in a fridge to incubate for 24 h. The leaves were blotted to dry and measured for weight when fully turgid. The leaves were dried for 24 h at 72°C in an oven. Lastly, the dry weight was recorded, and the percentage of relative water content was measured by the following formula

$$\boldsymbol{RWC}(\%) = \frac{\boldsymbol{Fresh\ weight - Dry\ Weight}}{\boldsymbol{Fully\ turgid\ Weight - Dry\ Weight}} \times \mathbf{100}$$

3.19.4 Total Free Amino Acids

The plant tissue extract was diluted to 1 ml with distilled water and ninhydrin reagent was introduced. The test tubes were then place in boiling water-bath for 20 minutes. The test tubes were cooled and 5.0ml of diluent was added in each tube and the absorbance was measured at 570 nm (Patel *et al.,* 2023).

3.19.5 Total Phenolic Content

The total phenolic content was analysed by using Folin- Ciocalteu (FC) reagent using the method of Ainworth and Gillespie. The seed sample 0.5 g was homogenized with 500 µl of 95% chilled methanol, after the sample was incubated at room temperature for 48 hours. After centrifugation for 5 minutes, the supernatant was transferred to a fresh tube and the TPC was estimated by adding 100 µl FC reagent. The mixture was vortexes and adding the 800 µl Na_2CO.The sample was incubated for 1 hour and after taking the absorbance at 760nm

using a spectrophotometer. The Gallic acid standard curve was prepared and the result were presented as mg GAE/100g (Naz *et al.,*2023).

3.19.6 Total Flavonoid Content

The total flavonoid content of seeds was determined by the aluminium chloride method. First prepare the sample. Take the 5 ml sample and then added to 2% $Alcl_3$ 5 ml in each tube, incubated the tubes for 20 minutes. After the incubation, taken the absorbance at 415 nm. Quercetin standard curve was employed and the results are expressed in mg QE/100g (Brrar *et al.,*2023).

3.20 Statistical Analysis

The experiments were conducted in triplicates and the results have estimated using mean ± standard deviation using statistical approach determine the effect of bacterial isolate on monocot and dicot seedling germination and biochemical components of the plants.