CHAPTER: 3

MATERIALS AND METHODS

3.1 Isolation and screening of plant growth promoting rhizobacteria

Rhizosphere soil samples from groundnut fields were collected from four agricultural locations in Saurashtra, Gujarat, including Kotdapitha and Virnagar in Rajkot district, Kalawad in Jamnagar district, and Garani in Amreli district. A total of eighty-four rhizobacterial isolates were obtained from these samples. To assess the plant growth-promoting (PGP) potential, various traits of these isolates were tested. These rhizospheres bacteria are known to enhance plant growth through the production of indole-3-acetic acid (IAA), ammonia, hydrogen cyanide (HCN), gibberellins, siderophores, chitinase, and the solubilization of nutrients such as phosphate, zinc, and potassium, in addition to nitrogen fixation.

3.1.1 Collection of Soil Samples

Soil samples from the rhizosphere of groundnut plants were collected from the agricultural fields located in Kotdapitha, Virnagar (Rajkot district), Kalawad (Jamnagar district), and Garani (Amreli district), Saurashtra, Gujarat. Groundnut plants were uprooted, with shoots cut off, and the roots along with the attached rhizosphere soil were carefully collected and stored in sample bags under aseptic conditions (Gupta et al., 2020). The soil samples were kept at 4°C until further analysis. For bacterial isolation, the samples were serially diluted from 10^{-3} to 10^{-8}, and colonies exhibiting distinct morphological characteristics were isolated.

3.1.2 Primary Isolation

Bacterial isolates were retrieved from the rhizospheres soil samples. To create serial dilutions, 1 g of the soil was suspended in sterile distilled water, and the resulting dilutions were plated onto nutrient agar medium (Hi-Media, Mumbai, India). The plates were incubated at 37°C for 24 hours, after which the bacterial cultures were purified by transferring them to nutrient agar slants at 37°C, following the method outlined by Zhao et

al. (2018). The bacterial isolates were then evaluated for their ability to promote plant growth and development.

3.2 Evaluation of Isolates for Plant Growth-Promoting (PGP) Traits

Table 3.1: Qualitative and Quantitative Analysis (PGP Traits)

Sr No.	**Qualitative**	**Quantitative**
1)	IAA Production	IAA Production
2)	Ammonia Production	Ammonia Production
3)	HCN Production	HCN Production
4)	Phosphate Solubilization	Phosphate Solubilization
5)	Potassium Solubilization	Gibberellins Production
6)	Zinc Solubilization	
7)	Chitin Hydrolysis	
8)	Nitrogen Fixation	
9)	Gibberellins Production	
10)	Siderophore Production	

All the isolates were analyzed for their ability to produce indole acetic acid (IAA), hydrogen cyanide (HCN), and ammonia, as well as their capacity for phosphate, potassium,

and zinc solubilization. They were also tested for nitrogen fixation, chitin breakdown, siderophore production, and gibberellin production to identify the most effective strains for promoting plant growth.

3.2.1 Indole Acetic Acid Production

Indole acetic acid (IAA) production by the bacterial isolates was evaluated using the colorimetric method as described by Gordon and Paleg (1957). The bacterial isolates were cultured in 5 mL nutrient broth (NB) supplemented with 100 mg/mL L-tryptophan and incubated at 37°C for 48 hours. After incubation, the cultures were centrifuged at 10,000 rpm for 5 minutes, and 1 mL of the supernatant was mixed with 2 mL of Salkowski's reagent which consists of 0.5 M ferric chloride ($FeCl_3$) and 35% perchloric acid ($HClO_4$). The mixture was gently shaken and incubated at room temperature for 30 minutes. A pink coloration indicated IAA production, and the intensity of the colour was measured spectrophotometrically at 530 nm.

Preparation of Salkowski's Reagent:

- 500 mL of distilled water
- 300 mL of concentrated $HClO_4$
- 15 mL of 0.5 M $FeCl_3$

Standard Curve:

The standard curve was prepared in the range of 20–200 µg/mL.

3.2.2 Ammonia Production

A bacterial culture that had been grown for 24 hours was transferred into 10 mL of peptone broth in test tubes. These test tubes were then incubated at a temperature of 37°C for 48 hours to allow the bacteria to grow and interact with the broth. After the incubation period, 0.2 mL of freshly prepared Nessler's reagent was added to each test tube. The reaction was observed for a color change, and the appearance of a yellow to brown color indicated the presence of ammonia, confirming the production of ammonia..Ammonia was quantitatively determined by measuring its absorbance at 600 nm (Demutskaya and Kalinichenko, 2010).

Preparation of Nessler's Reagent:

- 5 mL of water
- 2 g of potassium iodide
- 3 g of mercury (II) iodide
- Adjust the final volume to 20 mL
- Add 40 g of 30% potassium hydroxide to provide the alkaline base

Standard Curve:

The standard curve was prepared in the range of 10–100 µg/mL.

3.2.3 Nitrogen Fixation

Nitrogen fixation was tested using Jensen and Jackson's method (1973). The bacterial strains were streaked onto Jensen's agar plates and incubated at 30 ± 2°C for 24 hours to observe growth.

3.2.4 Zinc Solubilization

Zinc solubilization ability was assessed using Pikovaskay's medium containing insoluble zinc compounds like zinc oxide (ZnO), zinc sulfide (ZnS), and zinc carbonate ($ZnCO_3$) (Manasa et al., 2019). Bacterial cultures were spot-inoculated onto the medium, and the halo zones around the colonies were measured after 48 hours of incubation at 28°C.

3.2.5 Potassium Solubilization

Potassium solubilization ability was tested using Aleksandrow’s agar medium (Zhang and Kong, 2014). The medium was modified with the addition of bromothymol blue (Raji and Thangavelu, 2021) and adjusted to a pH of 7.2 ± 0.2. A 5 µL of fresh bacterial culture was placed in the center of the plates. The plates were kept at 30°C for 4 days to the bacteria grow. The color of the medium changed from blue to yellow, it showed that the bacteria could solubilize potassium.

3.2.6 Chitin Hydrolysis

Colloidal chitin was prepared by mixing 10 g of crab shell chitin with 150 mL of concentrated hydrochloric acid (HCl) and stirring for 2 hours at 4°C. The solution was washed with chilled water, filtered, and adjusted to neutral pH (pH=7) (Mathivanan et al., 2014). The colloidal chitin was centrifuged at 8000 rpm for 10 minutes, and the pellet was used for the chitinase assay. The bacterial cultures were inoculated onto nutrient agar plates containing 1% colloidal chitin and incubated at room temperature for 5 days. A clear zone around the colonies indicated chitin hydrolysis.

3.2.7 Hydrogen Cyanide (HCN) Production

Hydrogen cyanide (HCN) production was assessed following the method of Alström and Burns (1989). A 100 µL bacterial culture was streaked onto nutrient agar plates containing 4.4 g/L glycine. A Whatman No. 1 filter paper was placed on top of the plate and soaked in an alkaline picrate solution (2% sodium carbonate in 0.5% picric acid). The plates were sealed with parafilm and incubated at 28°C for 4 days. A colour change in the filter paper from yellow to light brown or reddish-brown indicated HCN production.

Quantitative Method for HCN Production:

The production of HCN was quantified by soaking 3 mm strips of Whatman No. 42 filter paper in a picrate alkaline solution, allowing them to dry, and placing them in a test tube containing 5 mL of bacterial culture. The tubes were incubated at 28 ± 2°C for 3–5 days. After incubation, the filter paper strips were placed in fresh tubes with 10 mL of distilled water, vortexed, and the optical density was measured at 515 nm (Patel and Desai, 2015).

Standard Curve:

The standard curve was plotted using potassium cyanide in the range of 10–100 µg/mL.

3.2.8 Phosphate Solubilization Test

All bacterial isolates were screened for their ability to solubilize calcium phosphate using Pikovskaya agar (Pikovskaya, 1948). The isolates were spot-inoculated on Pikovskaya agar

plates and incubated at 28 ± 2°C for 7 days. The formation of a halo zone around the colonies was considered an indicator of phosphate solubilization.

Quantitative Analysis of Phosphate Solubilization

Phosphate solubilization was quantified by measuring the amount of soluble phosphorus released using the chlorostannous reduced molybdophosphoric acid blue method (Bhatt et al., 2015). For this, 1 mL of bacterial culture was inoculated into 100 mL sterile Pikovskaya broth in an Erlenmeyer flask, followed by incubation at 28 ± 2°C for 11 days with shaking at 120 rpm. The broth from each sample was withdrawn on the 3rd, 5th, 7th, and 10th days for measurement of soluble phosphorus. The cultures were centrifuged at 10,000 rpm for 15 minutes, and the supernatant (100 µL) was mixed with 10 mL of chloromolybdic reagent and 5 drops of chlorostannous acid reagent. After mixing, the final volume was adjusted to 50 mL with distilled water. The blue colour developed was measured spectrophotometrically at 660 nm.

Standard Curve:

The standard curve was plotted using tri-calcium phosphate (TCP) in the range of 10–50 µg/mL.

Gibberellin (GA) Production

Bacterial isolates were evaluated for their ability to produce gibberellin (GA). The bacterial culture was grown NB media supplemented with 1 mM L-tryptophan and incubated at 37°C for 24 hours at 150 rpm. After incubation, the cultures were centrifuged at 10,000 rpm for 5 minutes, and the cell-free supernatant was collected for gibberellin estimation.

GA production was determined using the Folin-Ciocalteu reagent (Abou-Aly et al., 2019). To the 1 mL of bacterial culture supernatant, 1 mL of Folin-Ciocalteu reagent and 1 mL of concentrated HCl was added, and the mixture was boiled in a water bath for 5 minutes. After cooling, the greenish-blue color produced was measured at 760 nm.

Standard Curve:

A standard curve was prepared with gibberellin (GA) in the range of 10–100 mg/mL.

Siderophore Production

Siderophore production by bacterial strains was tested using the universal CAS assay (Schwyn and Neilands, 1987). All glassware was first rinsed with 3 mol/L HCl to eliminate iron contamination, followed by washing with deionized water (Cabaj and Kosakowska, 2007). Both qualitative and quantitative methods were used for the determination of siderophore production by the bacterial isolates. For both methods, the CAS reagent was prepared as follows:

Preparation of CAS-HDTMA solution:

To prepare the CAS-HDTMA solution for siderophore detection assays, 0.1 g of Chrome Azurol S (CAS) was dissolved in 50 mL of distilled water, stirring until the dye was fully dissolved. The pH of the CAS solution was adjusted to 6.8 using a buffer if needed. Next, 0.1 g of Hexadecyltrimethylammonium bromide (HDTMA) was dissolved in 50 mL of distilled water, ensuring it was fully dissolved by stirring. The CAS and HDTMA solutions were combined in a clean beaker at a 1:1 ratio, adjusting as necessary for specific experimental requirements, and stirred gently for thorough mixing. The pH of the combined solution was checked to ensure it remained around 6.8, and adjustments were made with a buffer if required. The solution was then ready for use and stored in a dark bottle at 4°C to prevent dye degradation, remaining stable for several weeks when properly stored.

Qualitative method

This assay was performed according to the modified method given by Hu and Xu (2011). CAS agar plates were prepared by mixing 100 ml CAS reagent in 900 ml sterilized LB agar medium. Four bacterial strains were spot-inoculated on each plate. An un-inoculated plate was taken as control. After inoculation, plates were incubated at 28 ºC for 5–7 days and observed for the formation of an orange zone around the bacterial colonies (Louden et al., 2011).

3.3 Identification of Potent PGPR Strains

Three isolates exhibiting significant plant growth-promoting (PGP) traits were selected for identification through both morphological and molecular techniques. The Gram staining

and KOH (potassium hydroxide) methods were used for initial morphological classification and to determine the Gram characteristics, while the species-level identification was achieved using the 16S rRNA gene sequencing method. These findings emphasize the potential use of these isolates as effective agents in agriculture for enhancing plant growth and health, suggesting avenues for future research and practical applications.

3.3.1 Gram Staining and KOH Method

The morphological identification was carried out by examining bacterial colonies. A smear of a young culture from a solid medium was placed onto a clean glass slide, heat-fixed, and stained using the Gram staining procedure. The slide was first stained with crystal violet for one minute and then rinsed with tap water. Iodine was applied for one minute, followed by decolorization using 95% ethanol for a few seconds. After rinsing again, the slide was counterstained with safranin. Once dried, the slide was observed under an oil immersion lens. The isolates were classified as Gram-negative bacteria, exhibiting various forms including rod-shaped, spiral, twisted, and vibroid shapes, as reported by Gandhimaniyan et al. (2020).

The KOH test was also performed to distinguish bacteria based on their cell wall composition. In this test, potassium hydroxide breaks down the thin peptidoglycan layer of Gram-negative bacterial cell walls, which aids in differentiating between Gram-positive and Gram-negative bacteria.

3.3.2 Molecular Identification of PGPR Isolate by 16S rRNA Sequencing

DNA was extracted from the overnight culture of RG8, RG12, and RGKP3 isolates. Its quality was assessed by electrophoresis on a 1% agarose gel, which showed a single band of high-molecular-weight DNA. The gene fragment was amplified by PCR, producing a discrete amplicon visible on the gel. The PCR amplicon was purified using column purification to remove contaminants. Sequencing of the purified PCR product was carried out with primer27F using the BDT v3.1 Cycle Sequencing Kit on an ABI 3730xl Genetic Analyzer. The gene sequence was then compared to the NCBI GenBank database through a BLAST search to identify the closest match. The top ten sequences with the highest identity scores were selected for alignment using multiple sequence alignment software.

The obtained sequences were compared with those available in the GenBank database, and a phylogenetic tree was constructed based on 16S rRNA gene sequences from the isolated strains and related species. The sequences were aligned and integrated using MEGA X software. A total of 100 closely similar nucleotide sequences were analysed to determine the closest match.

3.4 Synthesis of Zinc oxide Nanoparticles (ZnO NPs) Using Chemical Method

Zinc oxide nanoparticles were synthesized using the sol-gel technique as described by Gnanasangeetha and Sarala Thambavani (2011). The synthesis procedure and characterization of the nanoparticles were thoroughly investigated.

Zinc acetate (0.02 M) was dissolved in 100 mL distilled water and stirred continuously at 180°C for 30–40 minutes, and 2 M NaOH solution was added dropwise until white coloration was observed. The mixture was then quenched in an ice bath for 30 minutes. The white pellet was collected by centrifugating the solution at 8000 rpm for 10 minutes. The particles were characterized using various techniques.

3.4.1 Characterization of Synthesized ZnO

The ZnO NPs synthesized chemically were characterized using a range of techniques, including UV-visible spectrophotometry (Shimadzu UV-1800), X-ray diffraction (XRD), scanning electron microscopy (SEM), and transmission electron microscopy (TEM, JEOL JEM 2100 TEM HR LaB6 version) at the Central University of Gujarat, Gandhinagar. These methods helped to confirm the formation and dispersion of the ZnO NPs, providing insight into their structural and optical properties.

3.5 Bacterial Growth Curve

The liquid medium NB was prepared and autoclaved. The bacterial strain was inoculated into the media in a culture tube. The culture was incubated at an appropriate temperature in a shaking incubator. At specific intervals, an aliquot or smaller portion of the culture was removed. The sample's optical density was determined using a spectrophotometer set to 600 nm. Readings continued to be taken until the bacteria reached the stationary phase.

3.6 Preparation of bacterial culture

A fresh bacterial culture was prepared for mass cultivation by inoculating it into an Erlenmeyer flask containing 250 mL of NB. The flask was incubated at 37°C for one to two days on an orbital shaker. The resulting culture was then applied to groundnut seeds and used for seed priming.

3.7 Seed Germination and Vigor Index

To assess the effect of PGPRs on groundnut seed germination, the seeds were surface sterilized using 2% mercury chloride ($HgCl_2$) and subsequently soaked in different treatments for eight hours. Control seeds were kept immersed in distilled water. Previously prepared ZnO NP and PGPR concentrations were applied by spraying with a hand sprayer. Measurements of the vigor index, seedling length, and germination percentage were taken on the fifth-day following inoculation. The vigor index is an essential measure for predicting the performance of groundnut seeds, influencing germination rates and seedling establishment (Biradarpatil et al., 2019). Germination percentage, seedling length, and vigor index were calculated using the following formulas:

$$Germination\ percentage = \frac{Total\ NO.of\ germinated\ seeds \times 100}{Total\ number\ of\ seeds}$$

$$Seedling\ length\ = \frac{Sum\ of\ Seeds\ length\ of\ five\ seeds}{Total\ number\ of\ seeds}$$

$$Vigor\ index = (Germination\ \%\ \times seedling\ length\ )$$

3.8 Optimization of synthesized ZnO NPs on plant growth

Various concentrations of ZnO NPs (100–800 ppm) were inoculated with RG12 (*Bacillus haynesii*) to determine the optimal concentration for enhancing plant growth and development. In the experiment, peanut seeds were soaked in 100 mL of sterilized nutrient broth medium, as described by Gnanasangeetha and Sarala (2013). The seed treatment trials

were carried out in the laboratory in three different sets. Each set of trials aimed to assess the synergistic effects of ZnO NPs and PGPR on seed germination, vigor index and plant growth. The pots were watered to their full capacity to ensure consistent moisture levels. To minimize the impact of soil variability, efforts were made to use homogeneous soil across all pots. These lab trials provided a controlled environment to evaluate how different concentrations of ZnO NPs, in combination with PGPR, influenced seed germination and subsequent plant growth.

3.8.1 Pot experiment

The nutrient broth medium was inoculated with a single isolated colony of *Bacillus haynesii*, and the optical density (OD) of the supernatant (RG12 culture) was measured at 660 nm. ZnO NPs and the bacterial culture were then used to coat the groundnut seedlings. Three seedlings were planted per pot, each containing a mixture of autoclaved soil and sand in a 3:1 ratio, with 1 mL of the bacterial inoculum applied to each seedling (Zand et al., 2020). The treatments were applied as follows: the control group consisted of untreated plants; the ZnO NPs treatment included only the ZnO NPs at concentrations ranging from 100 to 800 ppm; the PGPR + NPs treatment involved the combination of *Bacillus haynesii* with ZnO NPs at 100 to 800 ppm. Treatments that did not include either PGPR or ZnO NPs were selected as control.

3.8.2 Measurements of Physiochemical and Biochemical Parameters

Plant samples were collected for physicochemical and biochemical analysis on the 30th day after sowing. The plants were thoroughly washed with deionized water after harvest, and the growth of their shoots and roots was measured using an appropriate scale. Additionally, the pre-harvested plants were dried on filter paper to remove any excess surface moisture and stored for further analysis.

a) Physicochemical Parameters:

The plant roots were carefully collected for further analysis to evaluate their properties. A detailed study was conducted to examine the primary physical and chemical parameters of

the roots. These parameters, which provide essential insights into the plants health and soil interactions, are summarized below.

Root Length and shoot length

The plant components were placed using a meter scale to measure the lengths of the roots and shoots.

Leaf number, number of branches, and number of roots

The number of roots, branches, and leaves in each sample was manually counted.

Fresh weight

An uprooted plant was used to record the fresh weight of the plant pieces. Before weighing, the plant components were sterilized of contaminants contained between two layers of filter paper using distilled water.

Dry weight

Plant samples were oven-dried at 70°C after a consistent increase in weight, and their dry weight was then measured.

b) Biochemical Parameters

Upon completing the analysis of physical parameters, a biochemical assessment was performed to measure proline, total sugar, protein content, flavonoid content, carotenoid, and chlorophyll content.

Chlorophyll Content

Chlorophyll and Carotenoid were estimated using the Arnon (1949) method. Fresh leaves (0.1 g) were mixed with 5 ml of 80% (w/v) acetone. The homogenized mixture was centrifuged at 2000 rpm for 5 minutes to separate the suspension. The supernatant was used to determine the chlorophyll concentration. OD readings were taken at 645 nm (chlorophyll a), 663 nm (chlorophyll b), and 480 nm (carotenoid), with 80% acetone serving as the blank.

Chlorophyll a = 12.7 x A_{663} -2.69 x A_{645}

Chlorophyll b = 22.9 x A_{645} -4.68 x A_{663}

Total chlorophyll = (12.7 x A_{663}) + (22.9 x A_{645})

Carotenoid Content

Fresh leaves (0.1 g) were mixed with 5 ml of 80% (w/v) acetone. The homogenized mixture was centrifuged at 2000 rpm for 5 minutes to separate the suspension. The OD of the solution was measured at 480 nm to determine carotenoid content, using 80% acetone as the blank.

$$\boldsymbol{Carotenoid}\ (100mg\ plant\ leaves) = \frac{4 \times dilution\ total\ sample\ volume \times Absorbance\ (OD)}{Weight\ of\ the\ sample(plant\ leaves)}$$

Flavonoid Content

The flavonoid content was measured using the method described by Zhishen et al. (1999). The 80% methanol homogenate of the leaves was centrifuged at 3000 rpm for 10 minutes. To prepare the aluminium chloride ($AlCl_3$) reagent, 133 mg of crystalline $AlCl_3$ and 400 mg of crystalline sodium acetate were dissolved in 100 mL of 80% methanol. Two millilitres of the supernatant were mixed with 400 µL of water and 1 mL of the $AlCl_3$ reagent. After thorough mixing, the absorbance was measured at 430 nm against a blank.

Proline Content

The proline content in the leaves was determined following the method described by Bates et al. (1973). Using a mortar and pestle, the leaves were homogenized in 5 mL of 3% sulfosalicylic acid. The extracts were centrifuged for 15 minutes at 3000 rpm. In the test tube, 2 mL of supernatant was added, followed by 2 mL of glacial acetic acid and 2 mL of ninhydrin reagent. For 1 hour, the reaction mixture was incubated at 100°C. After cooling the reaction mixture, 4 ml of toluene was added in it, producing a brick-red colour. The mixture was rapidly stirred until the toluene layer separated. The top layer of the reaction

mixture was removed, and the absorbance of the supernatant was measured at 520 nm with toluene as a blank.

$$\boldsymbol{Proline\ content\ (mg/g)} = \frac{\boldsymbol{K\ value} \times \boldsymbol{dilution\ factor} \times \boldsymbol{Absorbance\ (OD)}}{\boldsymbol{Weight\ of\ the\ sample}}$$

where K value =19.6

Sugar Content

The sugar content in plant leaves was assessed using glucose as the reference standard. In a clean mortar and pestle, fresh plant material (0.5 g) was homogenized in 10 mL of distilled water and centrifuged at 3000 rpm for 15 minutes. In 0.1 mL of supernatant, 1 mL of 0.5% (v/v) phenol was added. The samples were incubated at room temperature for 1 hour, and later 5 mL of saturated sulfuric acid (H_2SO_4) was added. The absorbance of each sample was recorded at 420 nm against a blank as the reference (Nawaz and Bano, 2020).

Protein Content

The protein content of the leaves was measured using Bradford's method. The leaves were homogenized in 10 mL of 0.1 M phosphate buffer using a mortar and pestle. The extract was then centrifuged at 10,000 rpm for 15 minutes, and the supernatant was collected for the estimation of total soluble protein. To 1 mL of the diluted supernatant, 0.1 M phosphate buffer was added, followed by 5 mL of Bradford's reagent. The mixture was thoroughly mixed, and the absorbance of the resulting blue-colored complex was measured at 595 nm (Bonjoch and Tamayo, 2001). The total soluble protein concentration was determined using a standard curve, which was prepared with BSA (bovine serum albumin) in the concentration range of 20-100 mg/g.

3.9 Optimization of synthesized ZnO NPs on plant growth with 3 potent strains

Seed treatment

Nutrient broth medium was inoculated with single isolated colonies of *Pseudomonas*

songnenensis, *Bacillus haynesii*, and *Priestia megaterium*. Following centrifugation at 3000 rpm for 10 minutes, the OD of the supernatant was measured at 660 nm.

ZnO NPs and the bacterial cultures were then used to coat the groundnut seedlings. Three seedlings were placed in pots containing a 3:1 mixture of autoclaved soil and sand, with each seedling receiving 1 mL of bacterial inoculum.

The treatments were applied as follows: the control group consisted of untreated plants; one group received only 400 ppm ZnO NPs; another group received only zinc salt; additional treatments included *Pseudomonas songnenensis* (B8) with 400 ppm ZnO NPs, *Bacillus haynesii* (RG12) with 400 ppm ZnO NPs, *Priestia megaterium* (RGKP3) with 400 ppm ZnO NPs, and individual treatments with *Pseudomonas songnenensis* (B8), *Bacillus haynesii* (RG12), or *Priestia megaterium* (RGKP3) only. These treatments were carried out according to the protocol described by Zand et al. (2020).

3.9.1 Physical and Biochemical Parameters (Data Recording and Related Procedures)

1) Physical parameters

The plants were harvested after 30 days, and the fresh and dry weights were calculated. Measures of physical growth, such as the number of leaves, branches, and roots, fresh and dry weight, as well as the lengths of the shoots and roots, were also noted. These measurements are crucial for understanding plant growth and evaluating the effectiveness of different environmental factors or cultivation practices

2) Biochemical parameters

The biochemical assessment was carried out after evaluating the physical parameters in plant studies. This assessment focused on measuring key biochemical components, including proline, total sugar, protein content, flavonoid levels, and the concentrations of carotenoid and chlorophyll. These measurements are vital for understanding the physiological and biochemical status of plants, particularly in response to different environmental conditions.

The chlorophyll content was determined using the Arnon method (Arnon, 1949), which involves measuring OD at three distinct wavelengths: 645 nm for chlorophyll a, 663 nm for chlorophyll b, and 480 nm for carotenoids. Additionally, the flavonoid content was quantified following the method of Zhishen et al. (1999), with absorbance measured at 430 nm against a blank. Proline content in the leaves was assessed using the method outlined by Bates et al. (1973). Furthermore, reducing sugars were quantified using the Dubois method (Dubois et al., 1956), a colorimetric assay that forms a colored complex with phenol and sulfuric acid. The protein content of the leaves was measured using Bradford's method (Bradford, 1976), with a standard curve generated using Bovine Serum Albumin (BSA) in the concentration range of 20-100 mg/g.

The study was designed to investigate SSR expression across multiple branches using a completely randomized design (CRD) to ensure unbiased distribution of treatments and minimize external variability. Branches were sampled and evaluated for SSR expression under controlled conditions, with data collected in triplicate for each branch to ensure reliability and minimize error. A one-way ANOVA was performed on the collected data to partition the total variance and assess significant differences in SSR expression between branches, with the F-statistic used to evaluate the significance. To further compare means, Duncan's Multiple Range Test (DMRT) at a 1% probability level ($\alpha = 0.01$) was applied, grouping means into subsets where no significant differences existed, as indicated by shared letters in the results. Distinct letters signified significant differences, aiding the interpretation of variation. Statistical analyses were conducted using [specify software/tool, e.g., SPSS, R, or SAS], and graphical representations were generated to facilitate visualization of the data.