Chapter-3

Materials and methods

3.1. Isolation of bacterial endophytes from finger millet

Seeds and plant sample different finger millet cultivars i.e., VL-352, VL-348, and PRM-1 were obtained for this study from the VCSGU “University of Horticulture and Forestry” Bharsar, Uttarakhand, (30.30574° N, 78.9924° E). For the isolation of bacterial endophytes healthy plants were chosen. Plant samples surface was cleaned several times with autoclaved distilled water after being first washed in tap water to remove dirt particles. The young finger millet plant's stem, leaves, and roots were separated in order to carry out a further surface sterilization process. Plant samples surfaces were sterilized for one min using 70% ethanol, for 2 min using 2% NaClO on the stem, and leaf surfaces, and for further two min using 2% NaClO on the root surfaces. Plant samples were rinsed 6-7 times using sterilized DW and then dried in LAF. Sterility was evaluated by incubating 100 µl aliquots of the final rinse's sterile water at 28ºC for 15 days on potato dextrose and NA Petri plates. The Petri plates were then checked for the presence or absence of microorganismal growing colony to validate the disinfection technique. After incubation, the surface sterilizing procedure was regarded successful if no growth was seen on the plates, if growth was observed, the complete surface sterilization procedure was repeated. Plant tissue samples of stem, leaves, and root were crushed separately in a sterile mortar and pestle for a homogenous mixture. After allowing it to settle for 20 minutes, the supernatant portion was gradually diluted in 12.5 mM phosphate buffer with a pH of 7.1. Subsequently, 100 µl from each dilution (10-1 and 10-2) was evenly spread over an NA medium, and incubated at 28°C

for 7 days. Endophytic bacteria were isolated, purified based on distinctive colony traits, and preserved on the slant for further research and identification. The above process of endophytic bacteria isolation was performed for all the three finger millet cultivars [19].

3.2. Screening of efficient iron and zinc-solubilizing bacterial endophytes

The method of [119] was used to carry out the selection of potential zinc mobilizing endophytic bacteria for qualitative assessment, Tris minimal medium. The Tris minimal medium was composed of the following ingredients per liter: Tris–HCl (6.06 g), NaCl (4.68 g), KCl (1.49 g), NH_4Cl (1.07 g), Na_2SO_4 (0.43 g), $MgCl_2 \cdot 2H_2O$ (0.2 g), $CaCl_2 \cdot 2H_2O$ (30 mg), and agar (15 g). The pH of the medium was adjusted to 7.0 prior to autoclaving. The agar medium was amended with 0.1% of each type of Zn source: zinc carbonate, zinc phosphate, zinc oxide separately. Similarly for the estimation of iron solubilization potential of bacterial endophytes, modified basal medium containing (g/L) 0.2 g yeast extract, 0.5 g NH_4Cl, 0.1 g $MnSO_4$, 10 g sucrose, and 15 g agar; 2 g $FePO_4$ as iron source. In order to modify the media, bromo phenol blue (BPB), a pH indicator dye, was used. In 70% ethanol, a stock dye solution containing 0.5% of the dye was made, and the pH was set to 6.5 N KOH. To 100 ml of basal agar media, 0.5 ml of the dye from the previously prepared solution was added. The mixture was then autoclaved and plated. All glassware were cleaned three-four times using deionized DW after soaking in 0.1 M HNO_3 for an hour before being used. On tris minimum and modified basal agar medium plates, freshly cultivated endophytic bacteria were spot injected. Subsequently, the petri plates were placed in an incubator set at a temperature of 28±2°C and left to incubate for a period of seven days. In accordance with the

methodology given by [120] the halo zone formed surrounding the bacterial colonies specified the solubilization of zinc/iron, diameter of colony and halo zone (mm) were recorded and SI as was measured.

$$SI\ (mm) = CD\ (mm) + HD/CD\ (mm)$$

Where SI= solubilization index (Iron/Zinc), CD= colony diameter (mm), HD= Halozone diameter.

3.3. Plant growth endorsing potential of iron and zinc solubilizing bacterial endophytes

3.3.1. IAA estimation

The selected endophytic bacteria were further investigated for their potential to produce indole acetic acid (IAA). After 24 hr of cultivation, endophytic isolates were inoculated into L-tryptophan, 1.0 mg/mL supplemented NB, the culture flasks were then incubated in shaking incubator at 150 rpm at 28°C for 48 hr [121]. From each broth flask, 5 mL of culture were taken and centrifuged at 4°C at 6000 rpm for 30 min. After centrifugation one ml of Salkowski's reagent (50 ml of 35% perchloric acid was suspended in a 0.5 M $FeCl_3$ solution) [122] was then added to the 1 ml of supernatant and allowed to incubate in the dark for 30 mins. The appearance of a pink colour was an indicator of indole acetic acid production and uninoculated NB served as the control. At 530 nm the OD (optical density) of the coloured solution was computed using SHIMADZU, UV1800ENG240VSOFT spectrophotometer, to determine the quantity of IAA synthesized by bacterial endophytes. IAA production by endophytes was measured using a standard IAA graph.

3.3.2. HCN production

Qualitative estimation

The procedure given by [123] was utilized to assess endophytes' ability to synthesize hydrogen cyanide. 24 hr grown finger millet endophytic bacteria were inoculated on glycine supplemented (4.4 g/L) NA Petri plates. Filter paper was cut into petri dish-sized pieces and immersed in a solution of picric acid for one min. Following the placement of the filter paper within the lid, parafilm was used to seal the petri dishes, and they were then kept at 28°C for a period of four days. The HCN synthesis was visually evaluated based on the shift in colour of the filter paper from dark yellow to darkish brown. The results were denoted by the letters -, +, ++, and +++ and are listed as negative, weakly positive, moderately positive, and strongly positive respectively. [19].

Quantitative estimation

For the quantitative estimation for the production of HCN by endophytic isolates, glycine (4.4 g/L) supplemented nutrient broth medium was used. Three replications of each isolate were performed, with uninoculated flasks acting as the controls. Autoclaved filter paper stripes were immersed in picrate solution (0.5% picric acid and 2% Na_2CO_3), and hanging them inside each flask, put cotton plugs on the mouth, after that the flask was sealed with parafilm and incubated in shaking incubator at 140 rpm/28°C for 4 days. Sodium picrate strips' colour altered to a brownish substance indicated the production of HCN. The colour was eluted from strips in to the test tube containing 10 ml of distilled water, using a UV/Visible spectrophotometer, the color's absorbance at 625 nm was determined [124].

3.3.3. Ammonia excretion

Ammonia production by endophytic bacteria was evaluated by the method described by [125]. In nutshell, 10 mL of peptone water was supplemented with overnight cultivated bacterial endophytes, test tubes were kept for 48 hr at 28°C. After 48 hr, Nesselar's reagent (0.5 mL) was poured in to each tube. Colour change from brownish to yellowish was considered a positive test for production of ammonia.

3.3.4. Siderophore production

Qualitative estimation

The capability of endophytic isolates to synthesise siderophores that chelates iron was assessed using a modified universal CAS method. Prior to the experiment, all glassware had been rinsed in 3 mol/l HCl and then washed in deionized water to eliminate any iron residue. The CAS reagent was made, in brief 20 ml of a 1 mM $FeCl_3$ (ferric chloride) solution prepared with 10 mM HCl were added to 100 ml of distilled water to dissolve 121 mg of CAS. Under stirring, 20 ml of hexadecyl trimethyl ammonium bromide solution was supplemented with this solution. HDTMA solution was made through mixing 729 mg of HDTMA with 400 ml of DW. Prior to usage, the CAS-HDTMA solution autoclaved separately. For the preparation of CAS agar plates, 900 ml of sterilised Luria Bertani agar medium was combined with 100 ml of CAS reagent. Bacterial isolates were spot inoculated on modified agar plates, and kept for 5-7 days incubation at 28ºC. Occurrence of an orange zone round the bacterial colonies represents the production of a siderophore [126].

Quantitative estimation

Quantitative estimation of siderophore production was done by the method described by [126]. For this, 1ml LB broth was taken in 1.5 ml centrifuge tube and autoclaved, bacteria isolate (10^8 CFU/ml) were inoculated in LB broth, and incubate for 48 hr, and

then centrifuged for 10 mins at 10,000 rpm. For each isolate, tubes were taken in triplicates, along with a control (uninoculated LB broth tube). 0.5 ml of each bacterial culture's was added to 0.5 ml of the CAS reagent, kept at room temperature for 20 mins, the optical density at 630 nm was measured. Using the formula below, percent siderophore units (psu), a unit of siderophore synthesized by endophytes, were calculated by using the formula mentioned below [127]:

$$(PSU)\ \%\ \text{siderophore unit} = Ar\text{-}As/Ar \times 100$$

Ar denotes the reference absorbance of the uninoculated broth and CAS solution, and As denotes the sample absorbance of the supernatant of the sample and CAS solution at 630 nm (Arora & Verma 2017).

3.3.5. Phosphate solubilization

The ability of endophytic bacteria to solubilize phosphate was evaluated using NBRI-BPB agar medium [128]. NBRI-BPB growth medium comprising bromophenol blue, contains (g/L) 10 glucose, 5 $Ca_3(PO_4)$, 5 $MgCl_2.6H_2O$, 0.25 $MgSO_4.7H_2O$, 0.2 KCl, 0.1 $(NH_4)2SO_4$, 15 agar, 0.025 BPB. Bacterial isolates grown for 24 hours were applied as spots on 'NBRI-BPB' agar medium and subsequently incubated at 28 ºC for a period of 7 days. Petri dishes were screened for the formation of a halo zone surrounding the colony, which was a sign of P solubilizing ability of bacterial endophytic isolates. Based on the halo zone size and colony diameter, the solubilization index (SI) was calculated according to the method described by [120].

$$SI\ (mm) = CD\ (mm) + HD/CD\ (mm)$$

Where SI= solubilization index (Iron/Zinc), CD= colony diameter (mm), HD= Halozone diameter.

3.3.6. Organic acid production

Using an agar plate assay, all selected isolates were checked for synthesizing organic acid based on the change in pH. Each bacterial isolate was spot inoculated onto 'Czapek-Dox' agar dishes having the following ingredients (g/L): 30.0 sucrose, 0.01 $FeSO_4.7H_2O$, 0.5 KCl, 1.0 KH_2PO_4, 0.5 $MgSO_4$, 2.0 $NaNO_3$, 15.0 agar, 1000 L/L triton X-100, and bromocresol green (a pH indicator), pH 6.0, The culture dishes were kept in an incubator at a temperature of 28°C for a duration of 4-7 days. After incubation, the Petri plates were examined for the appearance of yellow zones around the bacterial colonies, an indication of the production of organic acids [129].

3.4. Measurement of extracellular enzyme

3.4.1. Lipase synthesis

For lipase activity estimation, the endophytic isolates were cultured on a peptone agar medium comprising (gL-1): peptone 10.0, NaCl 5.0, $CaCl_2$ H_2O 0.1, agar 16.0, pH 6.0, supplemented with 1% tween twenty 20 individually sterilised (v/v). After incubation, the presence of a precipitate surrounding the colony caused by the creation of calcium salts of the lauric acid released by the enzyme demonstrated that the positive lipase activity [130].

3.4.2. Protease synthesis

Protease activity was assessed by using skimmed milk agar containing g/L glucose 1.0, NB 8.0, agar 18.0, pH 7.4. 15.0 ml of skim milk that had been separately sterilised was added. freshly grown isolates were streak inoculated on milk agar using a sterile loop and allowed to grow for 24 hr at 28 ºC. After incubation, clear halos were seen surrounding the colonies when 2.0 ml of HCl at 0.1 mol l^{-1} were poured on to the Petri plates was an indicator of protease production by endophytic bacteria [131].

3.4.3. Phytase synthesis

The modified phytase-screening medium (PSM) [132] was used to inoculate the endophytic isolates. The PSM contains Na-phytate: 5 g, sucrose: 10 g, $(NH_4)_2SO_4$: 2 g, tryptone: 3 g, yeast extract: 2 g, KCl: 0.5 g, $MgSO_4$: 0.5 g, $MnSO_4$: 0.01 g, and the pH was set to 7.0. Petri dishes were kept at 28°C for three days, the bacterial colony that produced a clearing zone was chosen and was determined to be positive for phytase synthesis [133].

3.4.4. Amylase synthesis

Amylase synthesis was estimated according the method defined by [134]. The colonies were inoculated into starch agar medium plates, and incubated at 28ºC for 48 hr. Plates were flooded with iodine, after 48 h to observe the clear zone formation around the colonies, an indicator of hydrolytic activity or amylase production by endophytic bacteria.

3.4.5. Gelatinase synthesis

For the estimation of the production of gelatinase, freshly grown endophytic isolates were stab-inoculated into gelatin agar tubes (semisolid with 7.5g/L agar). The cultures were stored in a refrigerator at 4°C after 48 hr of incubation until the bottom resolidified. After cooling, the medium will still be liquid if gelatin was hydrolysed. The medium will resolidify while being refrigerated if gelatin did not get hydrolysed [135].

3.4.6. Tannase synthesis

To assess the synthesis of the enzyme tannase, tannic acid was utilised as the source of carbon in the tannic acid agar medium. For this, tannic acid was sterilised using a nitro cellulose membrane filter, and the plates were incubated at 28°C for a period of five days before being saturated with 0.01M $FeCl_3$. This reaction with metabolites produced the brown colour, which is a sign that tannase is being produced [136].

3.4.7. Urease synthesis

The endophytic isolates were inoculated onto urea agar slants having (g/L) urea: 20.0, NaCl :5.0, KH_2PO_4: 2.0, peptone :1.0, dextrose :1.0, phenol red :0.012, agar :15.0. inoculated tubes were kept at 37°C for 24 hr in order to check for changes in the reddish pink colour that signify positive urease activity [137].

3.5. Environmental stability of zinc solubilizers

3.5.1. Effect of pH

The endophytic isolates were also tested on different pH range, i.e., 5.0, 6.0, 7.0, 8.0, 9.0, 10.0. pH of the NA medium was adjusted using 1N HCL and 1N NaOH, and Petri plates were inoculated with 24 hr grown culture, incubated for 48 hr at 28ºC. Growth of the isolates were recorded as +, ++, +++, and ++++ after incubation [138].

3.5.2. Effect of different NaCl concentration

A 24-hr old bacterial culture was inoculated on NA medium plates with 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, and 10% NaCl, to determine the salt tolerance potential of endophytic isolates. After 24-48 hr, at 28°C of incubation, the bacterial growth was assessed and noted as +, ++, +++, and ++++ [139].

3.5.3. Effect of temperature

For determining the effect of varied temperature on the growth of endophytes, freshly grown (24 hr) endophytic bacteria were streaked on NA plates and then kept at different temperature i.e., 5, 15, 25, 30, 35, 40, and 50°C. The growth of the cultures was evaluated and recorded as +, ++, +++, and ++++ after incubation of 24-48 hr at 28°C [140].

3.6. Identification of selected iron and zinc solubilizers

Endophytic isolates were identified using colony morphology as well as biochemical and molecular characterization. Standard protocols were used for morphological and biochemical characterization [141].

3.6.1. Morphological characterization of the endophytes

3.6.1.1. Colony characters

Individual colony characteristics of the bacterial isolates, such as cell shape, colour, elevation, margin, and texture, were detected on NA media within three days after incubation.

3.6.1.2. Gram staining

Gram staining is a widely used differential staining technique employed to differentiate and identify bacteria into two major groups: gram-negative and gram-positive. A heat-fixed bacterial smear was prepared by carefully spreading a bacterial sample onto a glass slide, ensuring an even distribution of the bacteria. Then, the slide was flooded with crystal violet after one min, the excess dye is rinsed off using distilled water. The next step involves flooding the slide with iodine for 30 s, the slide was then washed again with water. The third step entails treating the slide with a decolorizing agent, typically alcohol or acetone. After decolorization, the slide is rinsed again with water. Finally, a counterstain safranin, is applied to the slide and allow to stand for about one min. Rinse the slide gently with water to remove excess counterstain. Gently blot the slide with a paper towel or allow it to air dry. Finally, observe the stained slide under a microscope using oil immersion.

3.6.1.3. Motility test

Bacterial isolates' motility was evaluated using a motility medium. Semisolid nutritional glucose media in test tubes were stabbed with a loopful of the 48-hr-old culture and

incubated at 28°C for 24 hr. Diffused expansion into media away from the stab line was an indication of motility by endophytic bacteria.

3.6.2. Biochemical characterization of the endophytes

3.6.2.1. Oxidase test

A small amount of the bacterial culture or a colony picked from an agar plate is transferred onto the slide using a sterile loop. The bacteria culture was completely covered with, an oxidase reagent, dimethyl-p-phenylenediamine dihydrochloride. If the area of application on the slide turned dark blue or purple within 10-20 s, then colour change indicates positive result, On the other hand, if no colour change is observed within the specified time, it indicated a negative result.

3.6.2.2. Catalase test

On a clean glass slides a loopful cultures were placed, and then few drops of 3% hydrogen peroxide were poured over them. Positive results are indicated by the oxygen effervescence that appears surrounding the culture on the slide within a few seconds.

3.6.2.3. H_2S production

Sulphide indole motility (SIM) agar tubes were stab inoculated with endophytic cultures, and incubated for 48 hr at 28 ℃. Following incubation, observation was done. The presence of black colour is a result of H_2S synthesis and it indicates a positive result.

3.6.2.4. Indole

A pure culture of the bacterium being tested was inoculated into a tube containing tryptone broth, which provides a source of tryptophan. Inoculated tubes were then

incubated at 28°C for 48 hr. After incubation, 1ml of Kovac's reagent (which contains p-dimethylaminobenzaldehyde) was added to the tubes. The culture tubes were observed for the appearance of a deep red colour in the upper layer of the broth. A positive result was indicated by the presence of a red colour, which indicates the production of indole. A negative result was indicated by the absence of a red colour.

3.6.2.5. MRVP (Methyl Red-Voges Proskaeur) test

MRVP tests were used to distinguish between those producing acetoin, a neutral product, and those producing acids. The 48-hr grown endophytic isolates were added to 10 ml of MR and VP broth separately, and kept at 28±2 ºC for 48 hr. Following the incubation period, methyl red was added to each set of tubes, followed by addition of 12 drops of VP reagent 1 and 2-3 drops of reagent 2 in the other sets of tubes. After shaking the tubes, colour of each set of tubes was then checked for changes. Appearance of red indicates a positive test, while the yellow colour formation indicates a negative test. A positive VP test is indicated by the formation of crimson to ruby pink colour in VP tubes, while a negative test is indicated by no change in colour.

3.6.2.6. Citrate utilization test

The bacterial isolates were streaked individually in Simmons citrate agar slants and incubated for 48 hr. A positive result is indicated by a change in the colour of the medium from green to blue, indicating citrate utilization, while negative result is indicated by no colour change on the slant.

3.6.2.7. Nitrate reduction test

The 48-hr old bacterial isolates were inoculated into NB, supplemented with 1% sodium nitrate, and then incubated for 4 days at 28°C. In the culture tubes, one ml of the sulphanilic acid reagent and one ml of the -napthylamine reagent were introduced, and the colour shift was noticed. Nitrate reductase activity was detected by the development of a pink colour.

3.6.2.8. Substrate utilization

Basal agar medium was used to examine the carbon and nitrogen utilisation capabilities, medium comprising (g/L) 2.64, $(NH_4)_2SO_4$; 2.38, KH_2PO_4; 5.65, K_2HPO_4; 1, $MgSO_4$; 6.4, $CuSO_4$; 15, agar; 1.1, mg $FeSO_4$; 7.9, mg $MnCl_2$; 1.5, mg $ZnSO_4$. The media contained 1% of each of the following carbon sources for carbon utilization: lactose, glucose, galactose, fructose, malt extract, inositol, mannitol, maltose, arabinose, rhamnose, ribose, sucrose, trehalose, mannose, sorbitol, and dextrin. In the aforementioned medium, nitrogen (N) source utilisation was examined by introducing 2.64 g/L of several N sources, each, along with 10 g/L of glucose as a carbon source. Prior autoclaving, pH adjusted to 7.0, and After 24-48 hr of 28°C of cultivation, endophytic growth on cultural plates was noted as +, ++, +++, and ++++.

3.6.3. Molecular characterization of endophytic bacteria

Genomic DNA from the selected bacterial endophytes was isolated using the HiPurA™ Bacterial Genomic DNA Miniprep Purification kit by following the manufacturer's protocol. For 16S gene amplification, the universal primers 16F27 -5'-AGAGTTTGATCMTGGCTCAG -3' and R-1492R -5'-ACGG(CT)TACCTTGTTACGACTT -3' were used. The amplified products have been resolved on a 1% agarose gel and configured with a gel documentation technique

(GelDoc UVPGelDoc-Ite imager). Amplified products were sent to future biotech labs in Lucknow in order to identify the bacterial culture to its closest species based on 16S rDNA sequencing using the same primers. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 16SrRNA-F and 16SrRNA-R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 16S rRNA gene was generated from forward and reverse sequence data using aligner software. After uploading the DNA sequences to NCBI Genbank, accession numbers were obtained. Phylogenetic and molecular studies were performed using the MEGA 11 programme [142]. Bacterial sequences from the EZbiocloud database were compared to the type bacterial nucleotide sequences using the CLUSTAL W tool, described by [143]. The Kimura 2-parameter model was used using the maximum likelihood approach for delving into evolutionary history [144]. The evolution of the species under study is represented by the consensus bootstrapping tree created after 1000 iterations. Furthermore, utilising the BLAST (Blastn) search programme, the sequences of the chosen isolates were examined and added to the GenBank database (NCBI).

Based on maximum identity and habitat, the most appropriate comparable sequences from the NCBI database were selected and loaded into MEGA version 11.0. By combining the neighbor-joining method with bootstrap analysis using 1000 iterations, we were able to create a rooted phylogenetic tree that intuitively showed the genetic distances between various bacterial strains.

3.7. Effect of bacterial endophytes on Seed germination

The selected bacterial endophytes were also checked for their effect on finger millet seed germination. The finger millet seeds of all three varieties were surface sterilized,

seeds were treated with harvested bacterial cells, mixed with sterile DW (5 ml) for 15 min. The experiment was carried out in Petri plates having sterile filter paper (Whatman No 1), and 0.7% agar. The Petri plates were kept in plant growth chamber (24 °C) and seed germination data was recorded. This experiment was caried out to assess the efficacy of endophytes on seed germination. The control seeds were treated with sterile distilled water only [145]. The percentage germination was computed after the incubation by using formula mentioned below:

Germination % = number of seed germinated / total number of seeds x 100

Along with % germination, the plumule and radical length were also measured [146].

3.8. Plant experiment

Based on their ability to solubilize iron and zinc as well as their capacity to promote growth of plant, the two endophytic bacteria EC3B-22 and EC3B-23 were chosen for pot experimental work. Sterile soil was filled in four-litre earthen pots and used for pot experiments. In all clay pots, the FM seeds were planted at the same depth (1 cm below the top soil) and primed with 3 ml of the bacterial culture solution containing 10^8–10^9 CFU/ml. The sources of zinc and iron utilised were zinc carbonate ($ZnCO_3$) and ferric phosphate ($FePO_4$), respectively. In a pot experiment, finger millet's (*Eleusine coracana*) dried shoot and root biomass, shoot and root length, and the effects of the bacterial endophytes on iron and zinc absorption were examined [147].

For zinc uptake one percent $ZnCO_3$, was added in each pot except the control treatment. The experiment was consisted of following treatments:

i) Control treatment (uninoculated),

ii) ZC (Zinc carbonate),

iii) ZC + EC3B-22, and

iv) ZC + EC3B-23

For iron uptake ferric phosphate 1% $FePO_4$, was used in the following treatments:

i) Control (uninoculated),

ii) Ferric phosphate (FC),

iii) FC + EC3B-22, and

iv) FC + EC3B-23

The method suggested by [19] was followed to surface sterilise the finger millet seeds before usage. Two most promising iron and zinc solubilizing bacterial endophytes namely EC3B-22 and EC3B-23 were used as bioinoculants for the pot experiment. Prior to planting, the seeds were inoculated with bacterial inoculants by dipping the seeds in test bacterial suspension for one hr. To prepare the bacterial inoculum, cells from overnight grown cultures in NB were collected via centrifugation for 10 mins at 10,000 g and using 0.85% (w/v) NaCl solution the cells were resuspended, and the optical density was adjusted to 1.0 absorbance at 540 nm, or 10^8–10^9 cells/mL. Crop was regularly irrigated using tap water to maintain optimum moisture and harvested after 90 days of planting. Plant growth metrics were measured, including grain, shoot, and root iron and zinc content, shoot and root lengths, fresh and dried plant weights. To determine the plant dry matter, after being separated, shoots and roots were dried in an oven at 65°C until a consistent weight was achieved.

3.8.1. Growth parameters

Plants were uprooted carefully and cleansed with distilled water after harvesting. The root and shoot length and dry mass were then noted and manually measured. Roots and shoot were then oven-dried at 80°C until the constant weight was reached to determine their relative dry weights.

3.8.2. Analysis of nutrients

Using a clean mortar-pestle, dried Ragi gains were crushed into an amorphous powder, and 100 mg were then poured into a 150 mL conical flask along with 10 mL HNO3 [(9: 4 ratios with perchloric acid (HClO4)]. Using a hot plate, flasks were heated to 350°C, where the crushed seed material was digested until it was entirely colourless. The digested extract was transferred into a volumetric flask, and its ultimate volume was set to 100 mL using distilled water. Using an atomic absorption spectrophotometer (AAS 4141, Electronics Corporation of India Ltd) the iron and zinc concentration of these samples was determined [96,117].

In order to prepare seed extract for the determination of nitrogen content, 0.5 g of dry finger millet seeds were firstly digested with concentrated H_2SO_4, and then $HCLO_4$, as explained by [148]. In accordance with [149], Employing the molybdovanadate reagent method, the phosphorus content of the extract was measured colorimetrically, and using a flame photometer, the concentration of potassium in the acid digest was measured directly.

3.9. Statistical analysis

All experimental sets employed a fully randomised block design (CRBD) to perform inoculation of bacteria on 'Ragi' plants in order to examine the impacts of endophytes.

DMRTs (Duncan's multiple range tests) were used to examine the variation between the means of different parameters at $P < 0.05$. Using Duncan's means analysis and ANOVA, the plant growth parameter was examined. SPSS (Version 22) was used to perform statistical analysis on the data, and Graphs were made using Microsoft Excel (Windows 11) and origin 2023 software.