3. MATERIALS AND METHODS

Experimental material

3.1. Plant material

Pennisetrum glaucum L. plants were obtained from the saline desert of little Rann of Kutch, Gujarat, India.

3.2. Microbial cultures

a) Fungal isolates

Fungal root endophytes isolated from the plant roots of P. glaucum and upon subculturing on different media pure colonies were obtained and stored at 4°C for further use.

b) Piriformospora indica

P. indica culture was obtained from the culture collection of Amity Institute of Microbial Technology, Amity University Uttar Pradesh, Noida, U.P. It was subcultured on PDA plates by placing a small disc of fungal inoculum in the center of petriplates and incubated at 30°C for a week. Then this plate was stored at 4°C for further use.

3.3. Chemicals

All the chemicals and different hormones for isolation of fungal isolates, cultivation of fungal endophytes and media preparation for use in tissue culture were obtained from Sigma Aldrich (India) and HiMedia Laboratories (India). Forward and Reverse primers for PCR amplification of ITS region of fungal isolates was obtained from Sigma Aldrich USA. Marker DNA was procured from MBI Fermentas having 1000 base pairs. Taq polymerase and 10mM dNTPs were also used from MBI Fermentas. Agarose was obtained from HiMedia. DNA gel extraction procured from AxyPrep and purification kit of Axygen Biosciences, USA. Chemicals for gel preparation of protein separation was procured from Sigma Aldrich and marker protein of Page Ruler TM Fermentas, USA of 10-100 kDa was utilized having broad range of molecular weight. P. glaucum RAJ 121 seeds obtained from National Seed Corporation, IARI, New Delhi, India. Bio-Zinc granules were obtained from Prathista Industries Limited, Secunderabad.
Other materials

Plastic wares, glass wares, Petriplates, tips, PCR and Eppendorf tubes were purchased from Tarsons and Borosils.

Primers used for ITS region

ITS1 (5’–TCC GTA GGT GAA CCT GCG G-3’)
ITS4 (5’–TCC TCC GCT TAT TGA TAT GC-3’)

3.4. Media preparation

a) Media for fungi isolation

Five different media were selected for the isolation of fungal isolates such as PDA (Potato Dextrose Agar), CDA (Czapek Dox Agar), MEA (Malt extract agar), SDA (Sabouraud dextrose Agar) and RBA (Rose Bengal Agar). The media were prepared as a broth culture in double distilled water and 0.8% of agar was added. Before autoclaving pH was set for each media. Prepared media was then autoclaved at 121°C for 15-20 min at 15psi. Stock solution of antibiotics (Streptomycin and Chloramphenicol) was separately autoclaved. After autoclaving antibiotics were added in the media by filtering it with sterile syringe to inhibit the bacterial culture. 25 ml of media was then poured into the petriplates and stored at 4°C for further use. For details see appendix.

b) Media for plant tissue culture

MMN (Modified Melin-Norkrans) medium is a minimal medium commonly used for the micropropagation of plants. It consists of all the salts in desired amount that is required for regeneration and organogenesis of plantlets. First the concentrated stock solution (10X of 500ml) of all form of ingredients such as macronutrients, micronutrients and Fe-EDTA complex were prepared (Hermann et al 1998). While using, these stock solutions were diluted and mixed. For solidification of medium, agar of 0.8% (w/v) was added to each medium. The medium was then autoclaved at 121°C for 15-20 min at 15psi. After autoclaving 25 ml of medium was then poured into the Petriplates and stored at 4°C for further use.
Materials and Methods

Methods

Site of study
Study site was located in the desert area of little Rann of Kutch, Gujarat, India which extends northern east from Gulf of Kutch and covered an area of 5200 sq k. Geographical distribution of this area is 23°7’ to 23°42’ North longitudes and 70°38’ to 72°45’ East latitude. Soil was darkest brown in color with wide and deep-seated cracks coated with a layer of salt.

3.5. Collection of samples
For sample collection fresh and symptomless thirteen plants of Pennisetum glaucum were selected within approximately 1km region of geographical reference point in the month of July to Sep 2012. P. glaucum plants grew in dark brown alkaline soil (pH 8.7) and they were uprooted for roots sampling and they were carefully packed into sterile plastic bags. The plastic bags were transported to the laboratory and kept at 4°C for the isolation of fungus.

3.6. Isolation and maintenance of fungal root endophytes
Next day plastic bags were brought to the normal room temperature and visual inspection was done to confirm that roots were healthy and free from lesions. From each plant five mature and fresh looking roots were selected randomly and attached soil particles were removed by thorough washing under the running tap water (Selvanathan et al 2011).

a) Surface sterilization of root segments
The complete protocol for endophytic fungi isolation carried out in aseptic condition (Saithong et al 2010).
In laminar air flow hood the outermost layer was peeled off and then the exposed inner part of root cut into small segments of 4 mm length. Root segments were washed with sterile distilled water and then it was sterilized by washing with 75% ethanol (v/v) for 1 min then followed by treatment with 3% mercuric chloride for 3 min. To remove the traces of mercuric chloride again they were washed with 75% ethanol for 1 min and final washing with sterile distilled water.

For the isolation of fungal endophytes surface sterilized roots were further crushed with the help of sterile mortal pestel using Tris-EDTA buffer (10 mM, pH 7). Then the crushed suspension was centrifuged for 10 min at 10,000 rpm. 100 µl of supernatant were spread by
Materials and Methods

using sterile spreader on five different media plates i.e., PDA (Potato Dextrose Agar), CDA (Czapek Dox Agar), MEA (Malt extract agar), SDA (Sabouraud dextrose Agar) and RBA (Rose Bengal Agar). All these media were supplemented with antibiotic Streptomycin and Chloramphenicol both in the amount of 100 mg/L each (Photita et al 2005) to inhibit the growth of bacteria. Paraffin sealed culture plates were kept for incubation under ambient conditions of laboratory (28± 2ºC) for 7-10 days.

b) Culturing and subculturing
After a week of incubation, the emerging hyphal tips of fungi can be observed from the root sample. To obtain a single colony, fungi with distinct morphotypes were transferred to the new media plates by separating a tiny portion of fungal hyphae at its edges (Strobel et al 1996). This separation was based on the morphological appearance and characteristics of isolated fungal cultures. The new colonies were subsequently reisolated and sub-cultured at least ten times on plates until a pure single colony was obtained (Stierle et al 1993).

Like this 64 distinct single colonies of fungal isolates were obtained and they were numbered as BBMF-1, BBMF-2.....BBMF-64 (Bhuj Bajra Manjita’s Fungus). They were periodically transferred and preserved on agar slants at 4ºC for further use.

3.7. Screening of fungal endophytes on morphological basis

a) Identification of fungal endophytes
Morphological identification of fungal endophytes was based on the characteristic morphology of fungal colony or hyphae, nature of growth, pigmentation and color of spores, type of fruiting body, spores arrangement and reproductive structures. The isolates were identified on the basis of colony and morphological characteristics: nature of growth, spore color, pigmentation, the fruiting body and arrangements of spores (Barnett and Hunter 1998).

b) Macroscopic characteristics
This identification was mainly depend upon the visual appearance or observation of fungal endophytes on agar plates which includes growth pattern, appearance and texture, color and pigmentation of colony on both sides top and reverse of culture plates (Kornerup and Wancher 1978).
c) **Microscopic characteristics**

In this small portion of fungal hyphae were mounted on glass slides stained with specific stains and observed under microscope. The tentative identification was based on the different morphological characteristics were observed like presence or absence of macro-, meso- and micro-conidia, their shape and size, presence/absence of chlamydospores, color and arrangement of spores, septate or aseptate hyphae etc (Leslie and Summerell 2006). Some fungal endophytes did not produce the spores and it was considered as a sterile form of fungi (Suryanarayanan et al 2000). The fungal cultures were sent to Fungal Identification Service, Mycology and Plant Pathology Group Agharkar Research Institute, G G Agarkar Road, Pune for the confirmation.

d) **Staining and photomicrography of fungal endophytes**

Lactophenol cotton blue stain was specifically used for fungal isolates by making semi permanent slide of fungi. Placed a single drop of lactophenol cotton blue stain on clean and dried slide. With the help of nichrome wire loop lifted the tiny hyphae of fungus at its edges from fresh culture plates and mixed it with stain on glass slide. Gently teased it with sterilized needle. Placed the cover glass over and care has to be taken to avoid the trapping of air bubbles. Slides were focussed and observed under 40X of compound microscope (Motic BA310). Photomicrographs were taken by using a camera attached with compound microscope operated with an image analyzer programme.

3.8. **Screening of fungal endophytes on salt tolerance basis**

For salt tolerance test a disc of 5mm of different fungal inocula were placed aseptically with the help of sterile forcep at the centre of petriplates of 0.5% agar medium containing different concentrations of NaCl i.e., 0.25, 0.5, 0.75, 1, 1.5 and 2% respectively. For control plates fungal inocula were placed into an agar medium without any salt concentrations. The experiments were repeated 3 times for each treatment and kept for incubation at 25±2°C under dark condition for 7 days.
3.9. Screening of fungal endophytes on biochemical basis

3.9.1. Carbohydrate utilization test
Fungi are heterotrophs they depend upon the carbon source which was provided to them artificially. The assimilation of different carbon sources were checked by inoculating the 4mm agar blocks of fungal endophytes from 14 day old culture plate to the basal media plates. These plates were supplemented with five different sources of carbon i.e., sucrose, cellulbiose, xylan, starch and glucose separately in the quantity of 10g/L. To easily visualise the carbon hydrolysis media plates also contained 0.01% of dye Coomossie brilliant blue. Three replicates were made of each experiment. After inoculation the plates were kept for incubation at 28ºC for a week.

3.9.2. Plant growth promoting traits of fungal endophytes

a) Phosphate solubilization (Qualitative)
The screening of phosphate solubilization of fungal endophytes were done on Pikovskaya’s agar medium supplemented with different dyes i.e., bromo phenol blue (SubbaRao 1982) and rose bengal (Nopparat et al 2007) each at a concentration of 0.003% for easy visualization. On one week of incubation at 28ºC the fungal growth showed clear zone of hydrolysis and phosphate solubization index (SI) was calculated (Edi-Premono et al 1996).

\[
SI = \frac{\text{Colony diameter} + \text{Halo zone diameter}}{\text{Colony diameter}}
\]

b) Phosphate solubilization (Quantitative)
Quantitative method for detection of phosphate solubilization of fungal endophytes was performed in broth culture (Jackson 1973). 5 mm of two plugs of fresh fungal agar disc were suspended aseptically in 50 ml of Pikovskya’s broth in 200ml of conical flasks. Cultures were kept on incubation at 28ºC for ten days on constant shaking condition. On second, fourth, sixth, eighth and tenth days of incubation two ml of fungal culture were centrifuged for 15 min at 10,000 rpm. After centrifugation supernatant of fungal culture were mixed with chloromolybdic acid in the ratio of 1:10 and their volume made up to 40ml with distilled water. To this mixture one ml of chlorostannous acid was mixed and total volume was adjusted up to 50 ml with distilled water. Blue colour developed and absorbance was
recorded at 600 nm of wavelength in spectrophotometer. The amount of total soluble phosphate was calibrated from the standard curve of KH$_2$PO$_4$.

c) **Indole acetic acid production**

For indole production the fungal isolates were inoculated and grown in 25ml of Czapek liquid medium (pH 6.5) with or without tryptophan. Culture filtrate of fungal endophytes was filtered by Whatman filter paper no. 1. 1ml of culture filtrate were mixed with 2ml Salkowski reagent and again incubated for short duration of 20 min in dark (Brick et al 2004). The absorbance reading was recorded at 530nm using UV-VIS spectrophotometer. The indole content was calculated using standard curve of IAA.

d) **HCN production**

All the dominant fungal endophytes were screened for production of HCN (hydrogen cyanide) by Lorck method (2004). For this PDA medium plates were used which were amended with glycine of 4.4g/L and on which fungal isolates were inoculated. Whatman filter paper no.1 which was soaked in a solution of 2% sodium carbonate in 0.5% picric acid were placed on the lid of plate and covered and sealed with parafilm. The control plates without the inoculation of fungal isolates. All plates were kept upside for incubation at 28ºC for 3-4 days. Results were interpreted based on the color change from yellow to light or dark brown of filter paper.

e) **Siderophore production (Qualitative)**

Siderophore plate assay were screened by CAS (chrome azurol sulfonate) method (Schwyn and Neilands 1987). Fungal endophytes were spot inoculated on agar plate and incubated at 28ºC for 3-4 days. The result was interpreted on the basis of color change from intense blue to yellow orange halo around the fungal growth due to movement of ferric ion to siderophore.

f) **Siderophore production (Quantitative)**

Quantitative estimation of siderophore production was carried out in Fries’s broth (Coleman 1995). Fungal inocula were inoculated and incubated for 28 days. On 7, 14, 21 and 28 days of incubation, one ml of culture filtrate were mixed with 2 ml of 3% ferric chloride solution. Yellow to dark brown color was developed, which indicates the presence of siderophores content and absorbance were recorded at 260 nm (Logeshwaran et al 2009).
Materials and Methods

g) Ammonia production
For production of ammonia freshly grown fungal isolates were grown in 10ml of Peptone base water for 3-4 days at 28°C. After incubation 0.5 ml of Nessler’s reagent was mixed in culture filtrate. Formation of brown to yellow color was indication of ammonia production (Cappuccino and Sherman 2005).

3.9.3. Detection of extracellular enzyme production
Qualitative extracellular enzyme assays were performed with 15 dominant fungal root endophytes out of 64 of total fungal isolates. Screening was done by growing small inocula on specific media for particular enzymes (Hankin and Anagnostakis 1975). Evaluation of fungal endophytes to produce the extracellular enzyme was determined by placing the 5mm mycelia plugs from 15 day old culture plate and kept on basic agar media supplemented with different dissolved/suspended substrates in adequate amount and incubated them at 25±2°C for 3-6 days. After incubation clear zone around the growth of fungal isolates were observed. All experiments were done in the replication of three by considering the mean±standard deviation.

a) Amylase
Amylolytic activity was detected by growing the fungal isolates on GYP (Glucose Yeast Peptone) medium of pH 6 which contained the substrate i.e., 0.2% starch. After incubation for 3-5 days of fungal growth the culture plates were completely flooded with 1% Iodine solution. Reddish zone of hydrolysis were observed in positive plates.

b) Cellulase
For cellulolytic activity fungal isolates were allowed to grown on GYP (Glucose Yeast Peptone) which contained the 0.5% CMC (carboxy-methylcellulose). On 5-7th day of incubation the culture plates were washed with 0.2% congo red and then destained for 10 min with 1 M of NaCl (sodium chloride). Visualization of yellow zone of hydrolysis around the fungal isolates on red media which indicated the cellulytic activity.

c) Laccase
In this GYP (Glucose Yeast Peptone) agar medium plates of pH 6 were supplemented with 0.005% α napthol. Fungal disc were inoculated on this and then incubated for 5 days. Positive
tests were considered when medium were turned into blue from colorless due to oxidation of α napthol by enzyme.

d) Lipase
Lipolytic activity was detected by growing the fungal isolates on Peptone agar medium which is supplemented with separately sterilized 1% Tween 20. After incubation clear precipitation was observed around the fungal isolates because of formation of calcium salt of lauric acid.

e) Protease
For proteolytic activity Yeast Nitrogen based (YNB) medium supplemented with 0.5% glucose, 0.5% casein having pH 7. Fungal endophytes were inoculated and were incubated for a week. After proper incubation mycelium was treated with 1M HCl which formed the clear visible zone around the fungal growth.

3.10. Interaction of root endophytic fungi with *P. indica*
The type of interaction between isolated fungal endophytes and *P. indica* were checked by dual culture test. A 5mm of fungal agar disc from fresh culture plate was aseptically placed on YMP (Yeast Malt Peptone) agar medium on one side of plate and on another side same size of agar disc of *P. indica* were inoculated and incubated at 28ºC for 10 days. The control plates with only disc of *P. indica* no fungal endophytes were inoculated. The zone of inhibition which was the distance between fungal root endophytes and the area covered by *P. indica* and results were recorded at 3rd, 5th, 7th and 9th day of incubation.

3.11. Interaction of *P. indica* with seeds under different salt stress on agar plate

3.11.1. Surface sterilization of *P. glaucum* seeds
Prior to surface sterilization the seeds of *P. glaucum* were soaked in distilled water for 12h in dark at room temperature. Whole procedure was performed in aseptic condition. Soaked seeds were immersed in 20% ethanol in sterile jam bottle for few seconds. Then it was decanted off and the seeds were washed 3-4 times to remove the traces of alcohol. After that seeds were suspended in 1% sodium hypochlorite at least for 5 min under constant shaking condition. Seeds were washed with sterile distilled water for five times and then treated with 70% ethanol for 30sec under constant agitation. Alcohol was decanted and washed for 5 times with distilled water. Seeds were quickly treated with 5% sodium hypochlorite. Lastly
seeds were washed with sterile distilled water for 5 times to remove the last traces of sodium hypochlorite.

3.11.2. Preparation of *P. indica* culture and seed treatment
Culture of *P. indica* were freshly grown in YMP broth at 25-28°C for 10 days under constant shaking condition at 70 rpm for one week. The filtrate of *P. indica* was used for seed treatment. For this the colony forming unit of each fungi were of $10^8$ unit/ml and 25-30 surface sterilized seeds were inoculated in each fungal suspension under sterile condition. The inoculated fungal suspensions were kept for 2-3h for better attachment of microprapagules. The control seeds were kept in sterile distilled water for same time.

3.11.3. Seed germination on agar plate containing salt
For seed germination *P. indica* coated seeds were inoculated in 0.8% agar medium containing different concentrations of NaCl i.e., 0.25, 0.5, 0.75 and 1%. 10 seeds were placed aseptically with the help of sterile forcep in salt containing plates at equidistance. For control plates seeds were inoculated into an agar medium without any salt concentrations. The experiments were repeated 3 times for each treatment and kept for incubation at 25±2°C under continuous fluorescent light (1000 Lux) for 72h. Seed germination was observed for different time intervals i.e. 24, 48 and 72h.

3.11.4. Root colonization
For root colonization, on 15th day of sowing, 10 root samples were taken randomly from colonised as well as uncolonized plants. Roots were cut into the small segments (1-2 cm) and washed with water to remove the adhering agar. Then treatment was done with 10% KOH solution followed by acidification with 1M HCl and then stained with lactophenol blue (Dickson and Smith 1998). Root colonization was observed under light microscope (40X) (Varma et al 1999). It was done just to confirm the presence of identical spores or hyphae in the roots in order to avoid any misinterpretation.
3.12. Plant tissue culture

   a) Surface sterilization of *P. glaucum* seeds
   Same as method described in 3.11.1.

   b) Germination of seeds
   Water agar was prepared by suspending 05% agar in the distilled water. Bajra seeds were placed on water agar in Petriplates. Efforts were made to keep distance between the seeds. These Petriplates were kept in dark for 24-48 h at 25°C.

   c) Interaction of fungal endophytes with seeds
   Interaction of *P. glaucum* seeds with the different fungal cultures were conducted in MMN 1/10 medium (Hermann et al 1998). Fully grown fresh plates of fungal isolates served as inocula from each plate one circular disc of 3mm was cut with the help of sterile pipette tip and placed on the centre of MMN 1/10 containing Petriplates. Then two surface sterilized seeds were aseptically placed on either sides of fungal disc by using sterile forcep on each plate. Precautions were taken to maintained the distance of about 2cm on either side from fungal disc. Control plates were also maintained in which only surface sterilized seeds were kept. Experiments were done in five replicates. Inoculated plates were incubated in plant tissue culture under continuing light intensity at 1000 Lux at 25 ±2°C for 15-20 days. After 2-3 days of incubation, seed germination and microbial growth was observed.

   d) Root colonization
   Same as method described in 3.11.4.

3.13. Green house experiments

3.13.1. Analysis of different characteristics of soil
Physical and chemical characteristics of soil obtained from the Little Rann of Kutch, Gujarat was done to perform the further experiments.

3.13.2. Preparation of selected fungal broth culture for pot trials
For pot experiment the broth culture of selected fungal root endophytes (*Aspergillus terreus* and *Eurotium rubrum*) were used for seed treatment. To obtain fungal suspension of *P. indica*...
and different fungal agar discs of equal size from freshly grown culture plates were inoculated into the 50ml PD broth in 150 ml of beaker. The inoculated bottles were kept for incubation at 28±2°C on shaking condition of 70 rpm for 10 days. After incubation the biomass was separated from the culture filtrate with the help of sterile Whatmann filter paper no 1 under sterile condition. Morphology of spores and mycelia were checked under the microscope to eliminate any contamination.

3.13.3. Interaction of selected fungal endophytes with seeds under different salt stress

a) Surface sterilization of seeds
Same as method described in 3.11.1.

b) Seed treatment
Same as method described in 3.11.2.

c) Experimental design
The experiment was performed in biohardening chamber during the growing season of pearl millet (May- Oct) under the provided optimum conditions of temperature, humidity and light. The 4X5 factorial experiments were designed with four endophytic fungal conditions which included inoculated or uninoculated plants with three different endophytic fungi such as *P. indica* taken as a positive control. Isolated fungi were *Aspergillus terreus* and *Eurotium rubrum* which were combined with five different NaCl concentrations (0, 50, 100, 200 and 300 mM NaCl). Therefore there were total twenty treatments combinations which were arranged in complete randomized block design having three replications of each. 10 fungal coated seeds were placed in each pot at equidistance and water was sprinkled.

Treatment with NaCl was started after the 30 days of plant growth of pearl millet followed by protocol given by Sheng et al. (2008). To avoid the effect of salts on the establishment of different endophytic fungi as well as osmotic shock on the plant roots. NaCl was added gradually to the soil by successive addition of 50ml of prescribed concentration of NaCl solution for 7 days in each pot. Therefore total volume of NaCl became 350ml of each concentration in each pot which brought EC of saturated soil to 0.18, 2.1, 5.3, 9.2 and 11.1 dSm\(^{-1}\) in the following 0, 50, 100, 200 and 300mM NaCl treatments respectively. Leaching from soil was prevented by maintaining the level of water in soil. The plants were regularly
irrigated twice in a week with the autoclaved water and EC of the treated soil extracts were measured by using a soil conductivity meter (Decibel DB-1401) and its value was adjusted once in every 15 days with corresponding NaCl solution.

d) Root colonization
For root colonization of pot trials, it was checked on 30th day of sowing, and rest of the method is same as 3.11.4.

3.13.4. Analysis of growth attributes
After 60 days of incubation plants were observed for shoot and root length (cm), fresh and dry weight of shoot and root (gm) were recorded for each treatments.

a) Length of shoot and root (cm)
For length measurement ten plants were selected randomly from each treatment. The shoot and root were separated from each other and then measured with the help of standard scale from the base of selected part of the plant to the tip of the part in centimetres. All calculations were done in mean±standard deviation.

b) Fresh and dry weight of shoots and roots (gm)
The fresh and dry weights were recorded of randomly selected parts of plants i.e., shoot and roots. The fresh weight were recorded on 60th day of plant on weighing balance and for dry weight the parts were dried in hot air oven at 70°C for overnight and next day weighed on balance in grams.

3.14. Analysis of seed germination and vigor index
Seed germination was observed which was considered as a emergence of radical and plumule of each treatment. Vigor index (Abdulbaki and Anderson 1973) was calculated on 7th day by formula.

Vigor index (VI) = Mean of root length + Mean of shoot length X % Germination
3.15. Analysis of physiological characteristics

3.15.1. Relative water content of leaves

Determination of RWC was done on medial leaves of differently treated plants. 1g of fresh leaves were dipped in 50ml of distilled water and kept for 4hr. After this the turgid leaf samples were oven dried at 70°C for 48h before determining the dry weight. The RWC value was calculated by the equation given by (Sairam et al 2002).

\[ \text{RWC (\%)} = \left( \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}} \right) \times 100 \]

Where, FW is the fresh weight, DW is the dry weight, and TW is the turgid weight.

3.15.2. Chlorophyll estimation

One gm of fresh leaves was washed; blotted dried and then homogenized in 10ml of chilled 80% acetone. Acetone extract was centrifuged at 15,000 rpm for 15min at 4°C. Supernatant was taken out in fresh tube and its volume raised to 10ml by 80% acetone. The extract was measured at absorbance of 645 nm to 663 nm, respectively for Chl a and Chl b. Content of Chl a, b and total Chl (mg/g fresh weight) was calculated using the equation given by Wellburn (1994).

\[
\begin{align*}
\text{Chlorophyll a (mg/g FW)} &= [12.21 (A_{663}) - 2.81(A_{645})] \\
\text{Chlorophyll b (mg/g FW)} &= [20.13 (A_{645}) - 5.03 (A_{663})] \\
\text{Total Chlorophyll (mg/g FW)} &= [17.3 (A_{645}) + 7.18 (A_{663})]
\end{align*}
\]

where,

\[ A_{663} = \text{Absorbance at 663 nm} \]
\[ A_{645} = \text{Absorbance at 645 nm} \]
3.16. Analysis of biochemical characteristics

3.16.1. Lipid peroxidation

The extent of lipid peroxidation was determined in relation with thiobarbituric acid reactive substances (TBARS) content (Heath and Packer 1968). Products of lipid peroxidation are fatty acid hydroperoxides and malondialdehyde when react with thiobarbituric acid produced red colored complex called as thiobarbituric acid reactive substance that showed the absorbance at 532nm and acted as an indicator of in vivo peroxidation of lipid in many plants. To determine the LPO 0.5 g of fresh plant leaves of treated and control plants homogenized in 10ml of 0.1% trichloroacetic acid (TCA). This homogenate sample was then centrifuged for 15min at 15000g. After centrifugation the resulted supernatant was considered to estimate the content of TBARS. 1ml of supernatant was mixed with 4.0ml of 0.5% thiobarbituric acid (TBA) which is prepared in 20% TCA. The mixture was then heated for 30min at 95°C and kept for cooling on ice bath. Again centrifuged it for 10min at 10000g, absorbance of supernatant was determined at 532nm and 600nm. The value of nonspecific absorption was recorded at 600nm and then subtracted from the absorbance value taken at 532nm. The TBARS content can be calculated by its extinction coefficient of 155 mM⁻¹ cm⁻¹.

\[
TBARS \ (n \ mol \ g^{-1} \ FW) = \frac{(A_{532} - A_{600}) \times V \times 1000}{155 \times W}
\]

Where,
- \(A_{532}\) = Absorbance at 532 nm
- \(A_{600}\) = Absorbance at 600 nm
- \(V\) = Extraction volume
- \(W\) = Fresh weight of volume

3.16.2. Proline estimation

Proline was determined by Bates et al 1973. Approximately 0.1 g of fresh weight of leaves of treated plants were homogenised in 4ml of 3% sulphosalicyclic acid in mortar and pestle. Extract was centrifuged at 10000 rpm for 10 min at 4°C. Two ml of supernatant was mixed with 2ml of acid ninhydrin and 2 ml of glacial acetic acid. The mixture was boiled in water bath at 100°C for 1 hour. After boiling the tubes were kept in an ice bath to terminate the
reaction for 5 min. Six ml of toluene was slowly added to the chilled reaction mixture and it was vigorously mixed on cyclomixer for 20 sec. In tube two phases of solutions were appeared at room temperature. The upper phase of reactant chromophore which contained toluene was aspirated in separate tube and then absorbance were recorded at 520 nm against toluene as a blank. Concentrations of proline of each treatment were determined by standard curve of standard L-proline and it was expressed in mg/FW.

3.17. Analysis of nutrient content

3.17.1. Total carbohydrate content
For the carbohydrate content determination by Yemm and Willis (1954) method was used. All the reactions were carried out in ice. 200 mg of fresh leaves were collected and subjected to homogenization with 95% chilled sulphuric acid. 1 ml of test sample was reacted with 2 ml of chilled anthrone reagent at 100°C for 10 min. Tubes were cooled at room temperature and absorbance was measured at 630 nm.

3.17.2. Total soluble protein content
Concentration of total protein in plant leaves was estimated by Bradford method (1976). 0.5 g of fresh leaves were collected and homogenised in 0.2 M of phosphate buffer pH 7.5. Homogenised sample was centrifuged at 4°C for 15 min. Supernatant was collected and mixed with equal volume of 10% TCA and vortexed. Again centrifuged the sample for 10 min at 6000 rpm at 4°C. The resultant supernatant was discarded and pellets of different sample were washed twice with double distilled water. Dissolved the pellets in 0.1 N of NaOH. This solution was ready for soluble protein estimation. From this solution of different treatments 0.1 ml of aliquots were allowed to mix with 5 ml of Bradford reagent. Vortexed the sample and kept for incubation for 10 min and taken the absorbance at 595 nm.

3.17.3. Total reducing sugar content
For total reducing sugar estimation DNSA method was followed. Fresh leaves were collected and homogenized with methanol in mortar pestel. 1 ml of homogenized sample was mixed with 2 ml of DNSA solution and thoroughly mixed. Kept it for incubation at 100°C for 10 min and let the sample cooled and added the 2 ml of distilled water to it. Again mixed well the sample mixture and taken the absorbance reading at 540 nm.
3.17.4. Total amino acid content
Free amino acid was estimated by the method described by Yemm and Cocking (1955). Fresh leaves were collected and homogenized with 70% ethanol. 100µl of ethanolic extract of plant sample was mixed with 1ml of 80% ethanol, 1ml of 0.2M citrate buffer pH 5 and 2ml of acetonic ninhydrin solution. Above mixture was incubated for 15min at 100°C in a water bath. The mixture was immediately cooled under running tap water for 5 min and added the 8ml of distilled water to it. The resulted color of the sample became purple and absorbance was taken at 570nm and calibrated the content of amino acid by using glycine as standard compound.

3.18. Analysis of antioxidants content

3.18.1. Methanolic extract
Fresh leaves were oven dried. One g of leaf sample was homogenised in 10 ml of methanol (1:10g/ml). Centrifuged the mixture at 10,000 rpm for 10 min at 4°C. Supernatant collected and again centrifuged the pellet for 10 min. Recollected the supernatant, pooled and kept it at 4°C for further use. All experiments were performed in replication of three.

3.18.2. Total phenol content
Total phenolic concentration was determined by Folin-Ciocalteu colorimetric method (McDonald et al 2001). 0.5 ml methanolic plant extract was mixed with 5 ml Folin Ciocalteu reagent followed by neutralization with Na₂CO₃. The mixtures were incubated for 15 min and absorbance was recorded at 765 nm.

3.18.3. Total flavonoid content
Flavonoid concentration was determined by Aluminium chloride colorimetric method (Chang et al 2002). 0.5ml of plant extract was mixed with 1.5 ml of methanol followed by treatment with 10% AlCl₃ and mixed with potassium acetate and diluted with distilled water. The mixture was incubated at room temperature for 30 min and the absorbance was measured at 415 nm.
3.19. Analysis of enzymatic activities

3.19.1. Preparation of enzyme extract

Enzyme extracts were prepared by homogenizing the leaves in liquid nitrogen of treated and control plants of 90 day old and dissolved in the 100mM buffer of sodium phosphate of pH 7.4 consisting of 0.1mM EDTA, Triton–X100 of 0.5% v/v and 1% (w/v) of PVP. The prepared homogenate was then subjected to centrifuge for 20 min at 15000rpm. Supernatant was used to measure the specific activities of different antioxidative enzymes influenced by different salt stress conditions of treated as well as control plants. Stored the enzyme extract at -20ºC to analyse further.

3.19.2. SOD activity

This enzymatic assay was based on the formation of blue colored compound formazone by using nitroblue tetrazolium and O$_2^-$ radical, which showed absorbance at 560 nm, and SOD enzyme decreased absorbance at this nanometer by 50% due to enzyme which reduces the O$_2^-$ radical formation (Dhindsa et al 1981). 0.05 ml of enzyme extracts were then reacted with 2.5ml of reaction mixture which contained 50 mM of phosphate buffer (pH 7.8), 13.33 mM of methionine, 0.1 mM of EDTA, 75! M of nitroblue tetrazolium chloride (NBT), 50 mM of sodium carbonate, and 0.95 ml of distilled water.

This reaction was started by addition of 2 1 M riboflavin and tubes were placed under light of 20W of florescent lamps for the period of 15min. The complete reaction mixture solution with no enzymes gave the maximum color formation that was control. To stop the reaction switched off the lights and put the tubes in dark. Nonirriadiated reaction mixture with enzymes referred as blank. Then absorbance was measured at 560nm.

\[
\text{SOD Activity (mg}^{-1}\text{ protein h}^{-1}) = \frac{\% \text{ Reduction in color}}{\text{Dilution}} = \frac{\text{between blank}}{60} \times \text{factor} \times \text{Incubation time} \times \text{mg protein in sample}
\]

Characterization of Fungal Root Endophytes for Saline Stress (Bhuj, Gujarat)

62
3.19.3. CAT activity
This assay was based on H$_2$O$_2$ absorbance at 240nm under UV range. Decrement in absorbance was measured for given period of time suggested by Aebi (1984). Three ml of reaction mixture which consist of potassium phosphate buffer of 50mM (pH 7.0), 50ul of enzyme extract, 12.5 mM hydrogen peroxide and volume made up to 3 ml by addition of distilled water. Addition of H$_2$O$_2$ started the reaction and absorbance decreased with time and readings were recorded for 5 min after every 30 sec. Activity of enzyme was calculated by the decomposition of H$_2$O$_2$. The initial and final concentration of H$_2$O$_2$ was computed by comparing it with the standard curve of known concentration of H$_2$O$_2$. Calculation of enzyme activity was done by reduction in the concentration of hydrogen peroxide by the equation.

\[
\text{Quantity of reduced H}_2\text{O}_2 \text{ (min}^{-1}\text{mg}^{-1}\text{protein)} = \text{Initial reading} - \text{Final reading}
\]

3.20. DPPH radical scavenging activity
Determination of free radical scavenging assay of different plant extracts and positive control i.e., ascorbic acid were measured in context with radical scavenging ability or hydrogen donating capacity with the help of using DPPH stable radical by the given method of Susanti et al (2007).

Methanolic extracts of fresh leaves were prepared by homogenising the leaf sample in methanol. 2ml of each of methanolic extract of treated and control samples were taken at different concentrations i.e.10, 30, 60, 90, 120, 150, 180, 210 and 240 ug/ml were mixed with 3ml of 0.004% of freshly prepared DPPH solution prepared in methanol. Sample reactions were incubated for 30min and absorbance was recorded at 515 nm. Indication of scavenging ability of given extracts were observed when color was changed from purple to yellow which shows the degree of DPPH decolorization and calculated by given formula.

\[
\text{% inhibition of DPPH} = \frac{(\text{Absorbance of DPPH} - \text{Absorbance of sample extracts})}{\text{(Absorbance of DPPH)}} \times 100
\]

Percentage inhibition by DPPH was plotted against different concentrations of each extracts.
3.21. Estimation of different ions uptake in leaves

a) Measurement of cationic and anionic ions
The ions were evaluated by the flame photometery method where 60mg of dry weight of leaves were subjected at 800°C to converted into ash form.

b) Sample preparation
Leaves of *P. indica* and fungal endophytes inoculated or non inoculated (control) under different salt concentrations (2\textsuperscript{nd} and 3\textsuperscript{rd} leaf from top) were taken and kept it for dry in hot air oven at 60°C for overnight. 60mg of dried leaves sample 5ml of HCL and 3-4 drops of HClO\textsubscript{4} were added drop wise. Mixed the sample and evaporated it at 60-70°C. To this mixture equal volume i.e., 2ml of HCl and HNO\textsubscript{3} were added and mixed it. Samples were heated at 60-70°C for evaporation. To this 1ml of HCl solution was added again it kept for evaporation at 60-70°C. Then samples were dissolved in the 2ml of distilled water. The color of sample was transparent and finally subjected to the process of flame photometery to identify the ions. Data evaluated was used to determine the rate of net ion transfer in leaves according to the given equation by Salim and Pitman (1983). It was then determined in mg/kg unit of dry weight.

3.22. Molecular identification of selected fungal endophytes

3.22.1. Genomic DNA extraction
The genomic DNA was isolated only of those fungal root endophytes which has promoted the growth of *P. glaucum* plant in green house condition. Two fungal endophytes were selected as plant growth promoting and subjected to molecular identification. After 10 days of incubation with the help of filtration process, fungal broth culture were filtered through sterilized Whatman no. 2 filter paper and fungal mycelia were separated from filtrate.

For this fungal broth cultures were prepared by inoculating the 3 fungal disc of equal size in PD broth and incubated at 25±2°C, 70 rpm for 10 days on shaker. The fungal mycelia were kept for freeze drying process for half a day. Genomic DNA of fungal endophytes was extracted by CTAB (Cetyl trimethyl ammonium bromide) method (Ausubel et al 1994). 500mg of freeze dried mycelia were then robustly crushed in chilled liquid nitrogen by using mortel pestel until a fine powder is obtained. 10 ml of extraction buffer (pH- 8) were used to
lyse the fungal cells mixed thoroughly and kept for 30 min incubation at 65°C on constant shaking.

After 30 min the lysate was then extracted by adding the equal volume of mixture of chloroform: isoamyl alcohol (24:1) and subjected to centrifugation at 10,000g for 12 min at 4°C. The three different layers were obtained from which aqueous phase was obtained and transferred to new sterile tube and genomic DNA got precipitated as chilled isopropanol was added in double the volume of aqueous phase and again centrifuged at 10,000g for 10 min at 4°C. After centrifugation pellet was obtained and washed with 70% ethanol atleast two times, air dried it and dissolved the genomic DNA in 25 µl of sterile distilled water. DNA concentrations of different fungal isolates were quantified by taken the absorbance at 260 and 280nm by using spectrophotometer.

3.22.2. PCR amplification of rDNA
To amplify the PCR product of rDNA of fungal isolates PCR reactions were carried out by using universal ITS primers specific for fungal amplifications like ITS1 (5’–TCC GTA GGT GAA CCT GCG G-3’), that hybridizes the end of DNA and ITS4 (5’–TCC TCC GCT TAT TGA TAT GC-3’) hybridizes at the starting (White et al 1990). PCR reactions contained total volume of 25ul where isolated fungal genomic DNA act as template of 100ng 1ul, 10X PCR Buffer of 2.5ul, 2mM deoxyribonucleoside triphosphates dNTPs 2µl, 25mM MgCl2 of 1.5µl, 1U Taq DNA pol (0.5µl) and 1.5µl of 10pM each of forward and reverse primers.

All the PCR reactions were performed on ice in Thermal Cycler (Bio-Rad Inc. Limited, USA). Different PCR steps having specific conditions: Initial denaturation of template DNA at 94°C for 3min, which followed by at 94°C for 2min, 59°C for 1min, 72°C for 2min and final extension at 72°C for 8 min. Total number of cycles for PCR was 35.5μl of above amplified PCR product was then separated on 1.0% agarose gel by electrophoresis using 0.5X TBE buffer pH 8. Gel was run for 90min at 80V with the help of using running buffer of 0.5X TBE. After complete run of gel it was stained with 1% ethidium bromide for atleast 45min and then photographs were taken under UV illumination.
a) Purification of PCR amplified product
The observed gel under UV illumination amplified DNA bands of 500-700bp was cut and then purified by using DNA gel extraction and purification kit. DNA Purity was again checked on agarose gel.

b) Sequencing of purified PCR product
Extracted and purified amplified product of PCR of different fungal isolates was sent to GSBTM, Gujarat for DNA sequencing.

3.22.3. Construction of phylogenetic tree
To analyse the phylogenetic tree of different fungal isolates additional related DNA sequences were searched for comparisons from NCBI DNA sequence database by using BLAST. Alignment of various sequences was done by CLUSTAL W and phylogenetic tree of fungal isolates were constructed by using MEGA 4.0 software and by UPGMA method (Tamura et al 2007).

3.23. Gel electrophoresis of leaf protein treated with P. indica under salt stress
Influence of salt stress on plant proteins of pearl millet was studied by using SDS-PAGE techniques.

3.23.1. Protein extraction
All the steps for protein extraction were carried out on ice. Fresh plant leaves were collected and same day prepared for protein extraction. 200 mg of plant leaves were weighed and then homogenized in prechilled motar and pestle with 1ml of extraction buffer containing 50 mM of Tris-HCl pH 7.4; 2 mM of MgCl₂, 1 mM of EDTA; 2 mM of DTT; 2.5 mM PMSF, 0.1% of Triton-X 100. The homogenized solution was centrifuged for 10 min at 12000 rpm at 4°C. Collected the supernatant and again centrifuged for 10 min at 12000 rpm at 4°C. The clear solution of supernatant was collected and stored at -20°C or same day used for quantification of protein.
3.23.2. Protein estimation
Different protein sample was used to quantify by Bradford's method (Bradford 1976). 100µl of protein samples were mixed with 2.5ml of Bradford reagent and kept for incubation for 5-10 min. Similarly 0.1ml of standard solution of BSA stock (1mg/ml) were also mixed with 2.5ml of Bradford reagent and incubated for 5-10 minutes. After incubation absorbance of different test and standard sample was recorded at 595nm against blank.

3.23.3. Electrophoresis analysis
Non-denaturing, discontinues gel electrophoresis was performed to separate the proteins by the method of Davis (1964). SDS-PAGE was done by Laemmli method (1970) which employed the 10% resolving gel and 5% stacking gel. The samples were prepared (80µl protein sample + 20µl SDS loading dye + 1µl β-mercaptoethanol). The protein samples and equal amount of marker protein were loaded in the wells and power is supplied 27-30 mA omni PAGE Mini Wide vertical unit (Cleaver Scientific, UK) for 2h. The protein bands were stained with Coomassie brilliant blue R-250 (CBB) solution for 2h agitated slowly on orbital shaker platform, then destained the stained bands with destainer until the background became clear with stain-free blue bands was obtained. Molecular weight of different protein bands of samples were calibrated and compared with 10-100 kDa protein ladder in the electrophorogram.

3.24. Rootonic with Bio-zinc singly or dual treatment on *P. glaucum* seed

3.24.1. Soil treatment
Sandy clay soil of pH range 6.3 to 8.7 was obtained from Noida Uttar Pradesh, India mixed with mature farm yard manure (1:1). Autoclaved soil was mixed with Bio-Zinc in the amount of 0.01g which was obtained from Prathista Industries Ltd. Hyderabad, India was thoroughly mixed with 100g of soil.

3.24.2. Preparation of Rootonic and pathogenic fungus *F. solani*

a) Cultivation of *P. indica*
Axenic culture of *P. indica* was incubated in jam bottle containing 100ml of Hill and Kaefer autoclaved medium on shaking condition of 70 rpm at 25-28°C for 10 days and then biomass was separated by using sterile Buckner funnel and nylon cloth (350nm) from the
Materials and Methods

culture filtrate under sterile condition. Morphology of spores and mycelia were checked under the microscope to eliminate any contamination.

b) Mixing with talcum powder
25 gm of fresh weight of culture biomass was mixed with 1ml of carboxyl methyl cellulose (CMC 0.1mg/ml) in 100ml of broth. Finally 25ml of above prepared CMC suspension was added to the 75gm of sterilized talcum powder (magnesium sulphite act as a carrier). CMC was used as an adhesive so that inoculums get sticks to the powder.

c) Cultivation of F. solani
_Fusarium solani_ culture were also grown in jam bottle consist of autoclaved Potato Dextrose Broth at 25-28°C for 10 days under constant shaking condition at 70 rpm. Axenic cultures were filtered separately under sterile condition. Morphology of the spores and mycelia were checked under the microscope (Motic BA310) to eliminate contamination if any.

3.24.3. Surface sterilization of seeds
Same as method described in 3.11.1.

3.24.4. Seed pelleting and sowing
This formulation was pelleted on seeds surface of pearl millet. The formulation contained the colony forming unit value of $10^8$ with 20% of moisture and organic Bio- Zinc (a product of Prathista Industries Pvt. Ltd., Secunderabad) mixed at the rate of 0.01% of total w/v. 0.01% of jaggery solution was sprinked on the seeds before pelleting. The pelleted substance was kept for overnight in shade for better attachment of the micropropagules. Seventy seeds were placed in each pot. All experiments were done in triplicates.

a) Experimental design
Eight treatments were performed in triplicate manner:

b) **Green house experiment**

The experiment was performed in the environmentally controlled green house at 25±2°C with 60% of moisture in light intensity of 10,000 Lux. 100 kg sandy loamy soil mixed with peat in equal proportion filled in big cemented pots, size 92X35X32. Seventy pellated seeds were placed in each pot at equidistance and water was sprinkled. Experiments were done in triplicates. On every alternate day seeds were checked for its germination simultaneously moisture level was maintained around 60%.

3.24.5. **Analysis of growth parameters**

Each pot having 70 plants of pearl millet were analysed for different growth parameters like shoot length, number of leaves, fresh and dry weight of shoot of treated as well as control plants were measured after 30 days.

a) **Root colonization**

Same as method described in 3.11.4.

3.24.6. **Analysis of biochemical parameters**

a) **Chlorophyll estimation**

Same as method described in 3.15.2.

b) **Total phenol content**

Same as method described in 3.18.2.

c) **Total flavonoid content**

Same as method described in 3.18.3.

d) **Statistical analysis**

All data are represented as mean ± SD for at least three replicates. Analysis of variance (ANOVA) method was employed for carrying out statistical analysis of triplicate data and mean values were compared with Least Significant Difference (LSD) test at significance level of 1% Snedecor and Cochran (1980).