5. DISCUSSION

With increasing world population, the demand for food, mainly cereal based protein rich diet is increasing, especially in developing countries. Due to scarcity of productive agricultural land and ever increasing population growth, the developing countries are facing the problem of food crisis and hence, protein-rich pearl millet may be one of the substitutes (Weaver 1994). In such scenario, cultivation of above plant is promising considering protein content and presence of carbohydrate pool.

Crop productivity is severely reduced by environmental stress from both biotic and abiotic factors. Abiotic stress leads to loss of natural resources such as plant biomass, biodiversity, and imparts a great role in global warming. Among the abiotic stresses, salinity is one of the main factors that contribute to desertification of arid lands. Approximately one-third of the irrigated agricultural land is affected by salinity (Munns and Tester 2008).

Soil salinization has become a great challenge for rehabilitation of range lands and plant productivity (Alqarawi et al. 2014). The increasing salinization of arid and semiarid regions of the world is expected to have devastating global effects, resulting in 30% land loss within the next 25 years, and up to 50% by the year 2050 (Wang et al. 2005). Salinity is one of the major environmental factors limiting plant growth and crop productivity in arid and semiarid irrigated area (Koca et al. 2007). Salinity not only reduces yield of plants but also disrupts the ecological balance of the area. Stress tolerance of plants is a complex phenomenon involving changes at morphological, physiological, biochemical and molecular levels and is undoubtedly the most important response of plants to the adverse environmental conditions (Ahmad et al. 2014; Alqarawi et al. 2014). Salinity causes nutritional disorders in plants which lead to deficiencies of several nutrients and drastically increasing Na⁺ levels in the plant cells (Iqbal & Ashraf 2013).

Many plant species especially crop species do not grow and tolerate salinity due to the accumulation of salts especially NaCl which compete with other nutrients and cause specific toxicity (Tester and Davenport, 2003). It is a menace to both agriculture and the soil body. Development of salt tolerant crop varieties and physico-chemical methods for removal of excess salts from agricultural soils has been tried. These approaches have been successful but are costly hence; scientists are looking forward for the alternatives to enhance the crop
production under the salt-affected soils which involve inoculation of salt tolerant fungal endophytes in agricultural crop.

Plants grown in fields are surrounded by various microorganisms like bacteria and fungi that help and improve the plant growth and yield under various stress conditions. The usage of fungal endophytes as a biologically based strategy to alleviate the adverse impact induced by salt is of such alternatives. It acts as growth regulator and mitigates the harmful effects of plants exposed to salt stress. They play a key role in alleviating the toxicity induced by salt stress thus normalizing the uptake mechanism in plants by supplying essential nutrients. In this way, the plant recovers the water balance machinery, enhancing their tolerance capacity, and thereby enduring the salt stress.

Fungal endophytes are the most diverse and unexplored group of microorganisms which makes the symbiotic association with the higher forms of life and produces many beneficial products to the host (Shiomi et al 2006). Fungi are widely known as the source of various bioactive metabolites, the best example is the taxol, anticancer drug, which only occurs in plants (Strobel and Daisy 2003). Endophytic microbes getting more attentions after knowing that they can protect the host plant against pathogen, pest and herbivores (Weber 1981).

Very fewer plants have been reported for its endophytic biodiversity and their capacity for production of bioactive substances. Recently many studies are done on the endophytic diversity, reproduction, taxonomy, ecology of host and its effort on host plant (Arnold 2007).

The present study was conducted to isolate and identify the type of fungal endophytes residing in saline desert of Rann of Kutch, Gujarat, which is extreme geographical area of India. This area is mainly saline desert having unique diversity of flora and fauna. Organisms which survive in extreme environments and present in natural habitats are known as extremophiles. Halophiles are type of extremophiles which survive in high salt containing areas like salt desert, sea and salt lake. It is characterized by low rain fall and discrete vegetation. This region is different from other desert areas as it is situated near sea and low lying level due to which marine water easily enter into it. Therefore, this area becomes mixture of saline, coastal and marshy desert where soil and water both are saline in nature. These properties makes Rann of Kutch special for biodiversity because of having unique
flora and fauna in desert where important endemic and high conservative species are present at international level.

**Diversity of fungal root endophytes**

Microorganisms belonging to a particular niche are specific and its survival depends on the physical as well as on environmental factors (Goswami et al 2014). Different authors have reported about the diversity of endophytes, host ecology and taxonomy (Selosse and Schardl 2007). In this study we report that endophyte community varies with host preferences (Cannon and Simmons 2002; Cohen 2006). Many of the isolated endophytes do not produce spores (Photita et al 2001). Also an occurrence of sterile mycelia did not show any kind of spore formation. Many authors have proved that geographical factor play an important role for species composition (Cannon and Simmons 2002). Species of *Fusarium*, *Trichoderma* and *Penicillium* is already known to be pathogenic for some plant hosts. Most of the pathogenic fungi primarily behave as endophyte with latent pathogenicity (Pereira et al 1993).

Total 65 endophytic fungi were isolated from the roots of pearl millet growing in above extreme region. Pearl millet is very important crop for India, acclimatises well in drought and diverse environments, is easily cultivable, highly nutritious and one of the encouraging crop for arid and semi arid region. Not so much attention was given to this crop in the past and also very little research is done as compared to other cereals. They are good source of proteins and have antioxidant properties. As the pH of the soil changes from normal to alkaline or acidic the number of microbes also changes.

During this study, 76.92% of fungi belong to Ascomycetes followed by 18.46% of sterile mycelia, 3.09% of Deuteromycetes and 1.53% of Zygomycetes. The fungi within roots were well isolated by using various methods and media. Among all the groups of isolated fungi species of *Fusarium* and *Aspergillus* showed deviations in its distribution during samplings. Interesting observation was recorded that population of *Fusaria* and *Aspergilli* were abundant in number compared to other species such as *R. arrhizus*, *Curvularia* sp., *D. rostrata*, *H. fuscoatra* and *E. rubrum* it may due to the heavy and dominant spores of them. Out of 65 fungal isolates 15 distinct species were found after their morphological characterization.
In this study *P. indica* (Verma et al 1998) was also considered as a positive control for every experiments, which is well known root endophytic fungus discovered from Thar Desert of India, belongs to family basidiomycetes (Verma et al 1999; Pham et al 2004) that colonizes the root cortex of many plant species. Like arbuscular mycorrhizal fungi, *P. indica* and *Sebacina vermifera* (Basidiomycota, Sebacinales) has a broad spectrum against soil born fungal pathogens, promotes plant growth and induces resistance against various insects/pests (Varma et al 2013).

These fifteen species of isolated fungi and *P. indica* were then further screened based on their salt tolerance level. Only *A. terreus*, *E. rubrum* and *P. indica* showed the salt tolerance at higher level upto 1.5% of NaCl and none of them showed growth on 2% of NaCl.

**Biochemical characteristics of fungal endophytes**

All 15 distinct fungal isolates were screened based on their carbon sources, various plant growth promoting traits and extracellular enzymatic assays. All the isolates utilized the glucose as the main carbon source. Some isolates like *P. indica*, *A. terreus*, *S. rostrata* and *E. rubrum* which showed the zone of hydrolysis in all the given sources of carbon such as glucose, sucrose, xylose, cellubiose and starch.

Different plant growth promoting traits of fungal endophytes were also evaluated such as phosphate solubilization, indole acetic acid, HCN, siderophore and ammonia production. Unlike bacteria, it has been reported that fungi are more efficient in solubilizing phosphate (Nahas 1996). Phosphate solubilization is very important process to enhance the soil fertility (Rodriguez et al 2006). In our study qualitatively 13 fungi out of 15 isolates exhibited clear zone of solubilization around the fungal growth. *E. rubrum* showed maximum solubilization index (1.56) followed by *G. thapsina* (1.52) and then *P. indica* and *A. terreus* (1.34) (Fig 4.3, Table 4.6). The mechanism behind the solubilization of phosphate by phosphate solubilizing fungi could be the production of organic acids (Kpomblekou and Tabatabai 1994; Kim et al 1997). Quantitative estimation showed that *E. rubrum* was found to be maximum phosphahate solubilizer 69.53 µg/ml then *Aspergillus* sp. 68.73µg/ml on 10th day of incubation (Fig 4.4). This study was confirmed by recent report where *Aspergillus* genus is best known for its phosphate solubilization activity which includes species such as *A. terreus, A niger, A. flavus A. carbonum, A. fumigatus, A. wentii and A. nidulans* and these are reported from
rhizospheric zone of soyabean, maize, chilli and acidic lateritic soils and also compost (Prerna et al 1997). A. terreus was also examined for its plant growth promoting effects and found it as a potassium secreting fungus and enhanced the nutrient uptake in Okra (Abelmoscus esculantus) grown in K-deficient soil (Prajapati 2013).

Fungal endophytes are also well known for its production of phytohormones such as indole acetic acid and gibberellic acid which has the ability to enhance the plant growth in stressed condition. For IAA production intensity of red color was observed which varied among all the distinct fungal endophytes. E. rubrum (35.84±0.29) µg/ml has showed the maximum IAA production followed by P. indica (34.29±1.45) µg/ml and then H. fuscoatra (33.82±1) µg/ml (Fig 4.5). In another report Eurotium sp. also found to increase the production of IAA which ranges from 24 µmol ml⁻¹ under stress condition and enhanced the microbial metabolism and plant growth (Bianco et al 2006; Camerini et al 2008) which supports our result.

Strong HCN production was observed by fungal isolates G. thapsina, A. terreus and E. rubrum (Fig 4.6, Table 4.7). Iron is one of the essential elements for the growth of living organisms. Under stressed condition where iron is limiting element PGPF produces small low molecular weight substances known as siderophores to competitively sequester the ferric ion (Whipps 2001). Fungal siderophores is having lower affinity than bacterial siderophores but they easily sequester and provide the iron to the plants (Loper and Henkels 1999). Siderophore production was detected both on medium as well as broth. E. rubrum (1.4±0.2) cm showed the color changes from blue to orange zone on CAS medium (Fig 4.7, Table 4.8). In broth the maximum siderophile production was showed by A. terreus, H. fuscoatra, S. rostrata, E. rubrum and Curvularia sp. (Fig 4.8). Color changes from brown to yellow were also observed for ammonia production by fungal endophytes such as H. fuscoatra, P. indica, A. terreus and E. rubrum (Table 4.9). They were also characterized based on their growth on different source of carbon containing media showed in Fig 4.2 and Table 4.5 as they form the zone of hydrolysis. To our knowledge this is the first report on root endophytic fungi biodiversity on the basis of secretion of extracellular enzymes, phosphate solubilization and IAA production from above extreme region of India.
**Discussion**

**Enzymatic assay**
All the 15 isolates were screened for extracellular enzyme activity such as amylase, protease, cellulase, laccase and lipase. Isolates were grown on specific medium (Fig 4.9). Many reports are there for biodiversity of endophytes, host ecology, taxonomy (Selosse and Schardl 2007). Endophytes are known to produce natural bioactive products because of their capability to grow in unusual and stressful environmental condition (Strobel and Daisy 2003).

The stability of fungal amylase is more than that of bacterial amylase (Duochuan et al 1997). Table 4.10 reveals that *F. semitectum* showed the maximum zone (2±0.01) cm of hydrolysis for amylase activity followed by *E. rubrum* (1.9±0.53) cm. Twelve endophytic fungi were positive for amylase and cellulase activity, 8 isolates have shown positive results for protease activity 12 fungal isolates were positive for lipase activity and 5 for laccase activity.

Roughly 4000 bioactive compounds can be obtained from fungus like *Aspergillus, Penicillium* and *Acremonium* species (Onifade 2007). Cellulase producing fungus mainly reside in terrestrial region which can be used in paper and pulp industry (Eriksson 1993) while extracellular laccase enzyme secreted by marine fungi (Bucher et al 2004) helps in the degradation of lignin. Lipase activity of fungus shows that cholesterol can be used as a source of energy (Maria et al 2005). In 2006, Cohen et al found that *Colletotrichum gloeosporioides* is the best producer of alkaline lipase and hydrolase and wide range of oils. From salty marshy places marine fungi secrete protease and lipase activity (Gessner 1979). Enzymes of fungal origin are more stable than plants and animal origin, as they can be easily helpful in food processing, beverages, lather and textile industries (Maria et al 2005). Fungal endophytes for production of extracellular enzyme and bioactive metabolites like antibiotics, anticancer drugs and taxol should be screened further for eco-friendly improvement of life on earth.

In all screening tests only five fungal endophytes i.e., *G. thapsina, H. fuscoatra, S. rostrata*, *A. terreus* and *E. rubrum* and positive control *P.indica* had showed the positive results.
Effect of fungal endophytes on *P. indica* growth

Interaction studies were done between distinct isolated fungal endophytes and *P. indica* to check whether they possess synergistic or antagonistic relationship with each other. *In vitro* assay as well as microscopic examinations were done where alteration of *P. indica* mycelia were observed that ranges from small to large (Table 4.12). The interactive results revealed that none of the fungal isolates showed mutual interaction with known root fungal endophyte i.e., *P. indica* and radial growth was also reduced drastically (Fig 4.10) (Table 4.13). This assay also indicated that in nature *P. indica* interacts with diverse group of fungi and their interactions varies from positive to negative association. This may be due to production of certain chemicals, completion for nutrients or direct antagonism (Akhtar et al 2010).

*In vitro* and *in vivo* interactive studies

Fungal endophytes and *P. indica* were interacted with surface sterilized seeds of *P. glaucum* *in vitro*. On 15th day of incubation plant growth and root colonization of treated as well control were observed and found some endophytes enhanced the seed germination (Table 4.16). Out of 15 isolates only two (*A. terreus* and *E. rubrum*) along with *P. indica* has shown the positive impacts on plant growth so they were considered for further *in vivo* experiments (Table 4.17).

Salinization affects the plants

High salt levels in soil and irrigated water are the big threat for agricultural production especially in arid and semi arid areas. Excess ions present in the rhizospheric zone injure the plant roots and its gradual accumulation in shoots simultaneously damaging the plant metabolism which results in stunted growth and low yield. Salinity can affect the plants directly or indirectly. Direct effects of salinity on plants are (a) diminution in the osmotic potential of soil which reduces the available water and creates the drought in plant therefore to overcome this situation plants maintain the internal osmotic potentials to prevent the water movement from roots to the soil (Jahromi et al 2008) (b) the excessive toxicity of Na⁺ and Cl⁻ ions disrupt the structure of enzymes, macromolecules, plasma membrane and cell organelles and hamper the process of photosynthesis, protein synthesis and respiration (Feng et al 2002) and (c) causes nutrient imbalance in plants by nutrient uptake or transport to the plant shoot which leads to ion deficiencies (Adiku et al 2001). So to deal with this adverse climatic condition plants have also evolved the complex defence mechanism by which toxicity of...
Discussion

NaCl and less water potential in soil could be minimized, a root-endophytic fungus *P. indica*, improve the plant resistance from leaf and root diseases and alleviate the salt stress tolerance in barley (Waller et al 2005).

**Beneficial impact of fungi on plants**

Moreover mutualistic association of plants with endophytic and mycorrhizal fungi enhance the salt tolerance and reduce the losses in yield in depreciation. We have seen the example of *P. indica* which protects the bajra plant from high stress caused by salinity upto 300 mM of NaCl. But the mechanism behind this tolerance is not known. In order to confirm the better impact of *P. indica* to establish the salt tolerance in plants we have also studied the biochemical markers in salt stress like metabolic and antioxidative enzymatic activities. We also found that *P. indica* colonized bajra plants showed high biomass than control plants. This enhancement in biomass implies that endophytic fungi *P. indica* having mycorrhizae like plant growth promoting activity. Similar results were observed by Waller et al 2005. Other example of *P. indica* was also seen in barley where its association and colonization enhanced the uptake of nutrients like nitrogen and phosphorous as same results were also seen in other case by mycorrhiza (Sherameti et al 2005). This observation supports our data.

**Effect on different growth parameters under various salt levels**

   a) **Germination**

Emergence of seedling was greatly affected at different level of salinity. Salinity also alters the normal equilibrium in physiological process of plants which finally leads to death (Poljakaff-Mayber 1975). Rate of germination decreases with increasing concentration of salt was also observed by Maouomicale and Licandro (2002) and Chiroma et al (2007). Number of germinated seeds were observed after equal time intervals and found that *P. indica* treated seeds germinated in high rate at different levels of salt compared than control of respective salt levels Table 4.14 and Table 4.15. Reduction in germination rate and death of seedlings were observed and it may be due to combined effect of specific ion toxicity and osmotic stress (Haung and Redmann 1995). High salt concentration reduces the water potential in the selective medium which affects the absorption of water by germinating seeds and inhibits the germination (Maas and Nieman 1978).
In present study some of fungal endophytes were isolated and showed beneficial effects on pearl millet plants that is important cereal crop grown in semiarid tropics. Observations and results (Table 4.18 and Fig 4.13) of current study suggested that seed of pearl millet treated with fungal endophytes enhanced the seed germination and vigor of seedlings. Highest seed germination of $68\pm0.8\%$ and $901\pm1.6$ of seedling vigor was offered by \textit{P. indica} at higher salt concentration. This is the first report on seed germination and seedling vigor induced by the fungi \textit{P. indica}. This fungus showed the considerable and significant influence on pearl millet plants.

Seeds of pearl millet treated with fungal endophytes increased the fresh and dry weight of plants. Few reports are there on increase biomass of many crops like tomato, sorghum, maize rice and wheat when treated with endophytic fungi (Gutierrez- Zamora and Martinez – Romero 2001). Microbes help to solubilize the nutrients by producing siderophores and phosphorous and phytohormones like cytokinines, auxins, abscisic acid and gibberellins (Sturtz et al 1997). Treatment of seeds is only one feasible technology because of economic constraints in crop production. Alternative method is the use of biotic and abiotic inducers, having plant growth promoting effects and also induces the resistance to pests and diseases. Preferable method is to use the endophytes which decrease the application of chemical fertilizers due to cost and its contribution in agricultural system.

b) Growth attributes

We have measured the shoot and root lengths, fresh and dry weight of colonized plants grown in normal as well as in salt containing soils and compared the observed data with uninoculated plants grown in same conditions. We observed that colonized plants showed significant increase in growth rates than uncolonized plants (Table 4.19 and 4.20). We also tested the plant growth promoting activity of \textit{P. indica} on bajra plants by increasing the availability of essential minerals and nutrients such as phosphate, iron etc. Here we reported that \textit{P. indica} significantly enhanced the growth of bajra plants under different level of salt stress same results were also observed by Kumar et al (2011). Upon colonization bajra plants can survive and withstand even at higher salt concentrations i.e., 300mM NaCl and showed significant enhancement in growth rate than uncolonized plants. Different growth parameters like shoot and root length were also observed to be higher in colonized plants than uncolonized plants. Length of roots are important parameters to indicate the effect of salt
stress as roots are directly attached with soil and help to absorb the water from soil (Akram and Ashraf 2011).

We observed that root colonization by *P. indica* and endophytic fungi in the roots of pearl millet decreases with increasing the salinity levels. Earlier reports suggested that salt addition in soil inhibits the growth of hyphae of *G. intraradices*, which also decreases the frequency of mycorrhizal colonization (Jahromi et al 2008). Inspite of decreasing its colonization in roots there was high biomass of roots and shoots due to *P. Indica* and fungal endophytes colonization which indicates that promotional effects of fungus on plant growth were sometime independent of percentage of root colonization.

Study was also done on fenugreek plants where its growth is reduced in presence of salinity and this study was confirmed by observing the nutritional imbalance in plants because of salinity (Grattan and Grieve 1999). They also observed that under salinity plants inoculated with mycorrhiza had increased the growth and biomass than non- mycorrhizal plants. Results were also there on many plant species (Shokri and Maadi 2009; Latef and Chaoxing 2011). Better growth and high biomass in colonized plants which indicated the enhanced tolerance level of plants against salt stress and that is manifested by increasing the nutrient uptake and maintaining the ionic ratios than uncolonized plants.

Symbiosis with fungal endophytes is key element to help the plants to survive in adverse climatic conditions (Augé et al 1992). Reports are there on maize plants which was colonized with *G. mosseae* showed higher shoot and dry biomass than non colonized plants under salt stress, which implies that colonized plants enhance showed better growth than non colonized plants and this was in accordance with many studies done on tomato in green house conditions (Al-Karaki and Hammad 2001), cotton (Feng and Zhang 2003), barley (Mohammad et al 2003), and maize (Feng et al 2000b).

In our study pearl millet colonized with endophytes showed significant enhancement in shoot length, root length and leaf number under salinity. Fresh and dry weight of plant biomass was also increased significantly at all salt concentrations. AM fungi inoculated plant *Theobroma cacao* showed significant increment (Chulan and Martin 1992). Therefore, overall response is significantly higher in colonized plants than uncolonized. Cantrell and Linderman (2001) also found that AM fungi improved the plant growth under different salt stress condition. In 2004
Giri and Mukerji reported that under salinity *Sesbania aegyptiaca* and *Sesbania grandiflora* showed increase in mycorrhizal dependency with plant age. Plants need endophytic fungi for acclimatization as well as continued uptake of nutrient during successive stages of growth (Giri and Mukerji 2004). It is impossible to compare and determine the mycorrhizal dependency of plants under salinity. It is also found in bajra plants that mycorrhizal dependency is higher in saline condition compared to plants growing without salt treatments which suggested that its dependency is important factor. This study also found that AM fungi increase the phosphorous uptake which is one of the important mechanisms for plant tolerance against salinity (Rabie and Almadini 2005).

c) Ion uptake

Na\(^+\) concentrations in shoots and roots increased proportionally to the NaCl level added in soil. It was also observed that contents of Na\(^+\) were much higher in uncolonized plants than colonized plants which indicates that symbiotic association can prevent the Na\(^+\) uptake on some extent when this became toxic (Allen and Cunningham 1983). In 2011 Hammer et al also found that the presence of endophytic fungi in plants selectively prevents the uptake of Na\(^+\) from soil. The ratio of Na\(^+\) shoot: root continuously increasing in non colonized plants than colonized. These findings proved that fungi activate the regulatory effect on Na\(^+\) translocation in aerial parts, therefore maintain the ratio of K\(^+\): Na\(^+\) and Ca\(^{2+}\): Na\(^+\) in shoots.

Our studies exhibited that treatment with NaCl reduces the K\(^+\) level which is antagonistic to Na\(^+\) in plant cells. As both Na\(^+\) and K\(^+\) has almost same physiological properties, and this is inevitable that competition exists between Na\(^+\) and K\(^+\) in many process. Notably Na\(^+\) always competes with K\(^+\) at entry site of root plasma membrane to enter into the symplast (Grattan and Grieve 1999). Hence Na\(^+\) present in cytoplasm competes with K\(^+\) for its binding sites and therefore inhibits the different metabolic process which mainly depends on the concentration of K\(^+\) in plants.

The requirement of potassium in plants is: 1) To balance the charge in cytoplasm- which counteracts the excess negative charges on nucleic acid and proteins 2) To activate the important enzymatic reactions which is used to form the pyruvate 3) To maintain the osmotic pressure of cell turgor and vacuoles (Maathuis 2009). On other hand Na\(^+\) interferes the acquisition of K\(^+\) by roots and also alters the integrity of root membranes and its selectivity.
(Grattan and Grieve 1999). Maintaining the high ratio of K\(^+\): Na\(^+\) in cytosol is crucial to increase the salt stress tolerance of plants (Maathuis and Amtmann 1999). In present study concentration of K\(^+\) decreased as concentrations of NaCl increased which brings the K\(^+\) deficiency in plants. This is the basic reason for plant growth reduction as this result was also observed in *T. foenum-graecum* plants (Grattan and Grieve 1999). It was also observed in *G. intraradices* inoculated plants which results in higher K\(^+\) concentrations in foliar part and roots compared to uninoculated plants which showed the ability of mycorrhiza to prevent the K\(^+\) homeostasis disruption.

High amount of Na\(^+\) creates several metabolic and osmotic problems such as reduction in photosynthesis and protein synthesis in plants. Transport of sodium is unidirectional in nature which results in the accumulation of Na in leaf tissue and shoots of plant. Inoculated plants accumulate the salts in roots which prevent the transportation of Na to shoot and this detrimental effect of salts is decreases by endophytic fungi. It was suggested that Na is accumulated in the intraradical hyphae of fungi (Cantrell and Linderman 2001). The most detrimental nature of Na is that it competes with K for its binding sites which are part of various cellular functions. It was proved that under salinity colonized plants accumulate more K to maintain the K/Na ratio therefore prevent the disruption of enzymatic activity and protein synthesis inhibition.

Uptake of ions is one of the forms of osmotic adjustment in saline conditions and that leads to many problems like reduction in leaf function, ions imbalance and toxicity (Yildirim et al 2009). Salinity affects the uptake rate of nutrients which results in deficiency symptoms. Excessive Na\(^+\) accumulation causes wide range of metabolic and osmotic problems in plants (Hoai et al 2003).

In present study sodium content in leaves was increased 2-3 times than control in respective salinity i.e., 50, 100, 200 and 300 mM (Table 4.27) which indicates that plants of pearl millet did not have capacity to inhibit the movement of sodium in photosynthetic part. But when plants were colonized by endophytes this ratio was found to be decreased. Potassium is important macronutrient which plays major role in osmo-regulation, stomatal behaviour, cell expansion, enzyme activity and neutralization of negatively charged ions and maintains the membrane polarization.
d) Photosynthetic pigment

Salinity is the major example of abiotic stress which affects the growth, development and yield of plants. Under salt stress different physiological parameters related to plant growth, photosynthetic apparatus and production of various metabolites which is stress induced gets impaired. Photosynthesis is required for plant growth and development and drastically affected by various environmental stresses. In current study we mainly focussed on chlorophyll and carotenoid pigments as they play important role in process of photosynthesis and phyto-protection.

During salt stress high sodium causes ion toxicity which can degrade the chlorophyll and carotenoids. Several reports are there where the concentrations of chlorophyll a and b were reduced in many crops like under salinity cabbage (Brassica oleracea capitata L.), wheat (Triticum aestivum L.), sunflower (Heliantus annuus L.), and sugarcane. (Ashraf and Harris 2013, Gomathi and Rakkiyapan 2011) the extent of decrease in pigment content is mainly depend on the level of salt tolerance in plant species.

In many plant species (pea, wheat and sunflower etc) the content of chlorophyll is acts as potential biochemical indicator for salt tolerance though it is not applicable for all type of species (Ashraf and Harris 2013). In one report of sugarcane plant (Gomathi and Rakkiyapan 2011) studied that at many growth stages of plant salinity causes marked decrease in the content of Chl and carotenoid in comparison to the salt tolerant varieties which exhibit the stability of membranes and pigment contents. We also measured different photosynthetic pigments like Chl a, Chl b, and carotenoids of plants at various salt concentrations such as 50, 100, 200 and 300 mM in bajra inoculated with E. rubrum. The content of these pigments were significantly enhanced in colonized plants compared with control plants grown under same conditions of higher salt stress. It was previously reported that chlorophyll content was also found to be increase in apple tree on 15th day of inoculation by Eurotium sp. (Miliute and Buzaitc 2011). Hence E. rubrum protects the plants against environmental stress. Various stressful environments like salinity, extreme temperatures and aridity could affect the components of photosynthetic apparatus like membrane integrity and capacity of photosynthesis (Demmig-Adams and Adams 1992).
To study the response of plants under environmental stress the fundamental characteristics is to analyse the growth parameters of plants. Salt induces many changes such as closing of stomata, reduction in photosynthesis, induction of photoinhibition and disruption of nutrient balance by altering its availability, transportation and partitioning of different nutrients (Jouyban 2012). Therefore immediate results of salt stress were seen in form of decrease in body mass, stunted growth and reduction in chlorophyll concentration. Reduction of chlorophyll content was reported in plants such as Bean (Beinsan et al 2003) Poulownia imperialis (Astorga et al 2010) and Carthamus tinctorius (Siddiqi et al 2009). The reason behind this reduction was the increment of enzymes which causes destruction called as chlorophyllase. To explore the contribution of fungal endophytes and P. indica in plants for its protection against the salt stress, we have inoculated the bajra plants with fungi.

There are many reasons which decrease the chlorophyll content under salinity in plant tissues. One reason might be antagonistic effect showed by Na on the absorption of Mg (Alam 1994). In 2002 Giri et al observed that endophytes increases the absorption of Mg in colonized plants and suppresses the harmful effect of Na under salinity.

Leaf senescence is progressed by yellowing due to degradation of chlorophyll and loss in photosynthetic activity (De Michele et al 2009). This is also quantified by reduction in the chlorophyll concentration and enhancement in membrane permeability (Lutts et al 1996). Concentration of chlorophyll is severely retarded in non colonized plants as compared to colonized plants which indicates the high leaf senescence in non colonized plants.

Salt stress specifically affects the leaf senescence by increasing the accumulation of highly toxic ions like Na$^+$ and Cl$^-$ or decreases the K$^+$ and Ca$^{2+}$ (Yeo et al 1991). Colonized plants decrease the accumulation of Na$^+$ ions than uncolonized plants. The harmful effect of salinity on chlorophyll concentration is mitigated by applying potassium fertilization (Bohra et al 1995). High concentration of chlorophyll is attained in colonized plants of T. foenum-graecum by improving the uptake of K$^+$ and Ca$^{2+}$. 
e) **Antioxidants**

Antioxidant compounds such as phenol and flavonoid are important constituent of plants because of presence of hydroxyl groups which having the ability to scavenge (Das and Pereira 1990). It is one of the considerable attentions for researchers due to its antioxidant properties. Plants rich in phenolic compounds are in great demand in food industry as it retards the oxidative degradation of lipids also improves the quality and maintains the nutritional value of food. Mechanisms behind their action are to scavenge or chelate the radicals (Kessler et al 2003). A phenols compound terminates the free radical (Shahidi and Wanasundara 1992).

f) **Nutrient content**

With increasing salt levels carbohydrate contents increase as starch hydrolytic activity of enzymes increases (Bartels et al 2005). Sugar is the important molecule which influences the many physiological responses in plant genes which regulates the photosynthetic process, metabolism and many defensive responses (Crowe et al 2002). Under environmental stress carbohydrate accumulated in plants to regulate and adjust the osmotic potential (Dhanapackiam and Ilyas 2010). Several literatures reported that sugar accumulation enhanced under salt stressed condition in tomato (Amini and Ehsanpour 2005) and barley (Bagheri and Sadeghipour 2009). This is due to starch degradation and synthesis of sugar by non photosynthetic pathways etc.

With salinity potassium ions also removed by plant roots that causes physiological imbalance as potassium is important for protein synthesis. Loss of potassium ions diminished the plant growth and development (Chen et al 2007). If stress prolonged, protein synthesis gets affected and finally decline (Caplan et al 1990). But some researchers suggested that protein content increases with salinity for e.g., cotton (Jiang et al 2005), *Pancratium maritimum* (Khedr et al 2003).

g) **DPPH**

Free radicals play an important role in broad range of pathological manifestations. Antioxidant compounds which fights against the free radicals and protect the organisms from many diseases. They scavenge the reactive oxygen species and also protect the defence
mechanisms driven by antioxidant (Umamaheswari and Chatterjee 2008). In present study, methanol extracts of plant leaves and its derived fractions with varying concentrations were used to determine the antioxidant activity by using DPPH radical scavenging assay. DPPH assay is widely using method which determines the capacity of free radical scavenging in plant extract. It is stable, rapid, easy and very sensitive method to check the antioxidative activity of any specific compounds present in plant extracts (Koleva et al 2002). This assay is based on reduction of methanolic solution of DPPH in presence of antioxidants and final product formed is non radical DPPH-H.

As free radical DPPH were reduced by plant extract the color changes from purple to light yellow at varying degree which depends upon the presence of antioxidative compounds. Range of discoloration depicts the scavenging capacity of plant extract. In current study varying concentration of each of treated plant extracts were tested and highest capacity for neutralization of DPPH radicals was showed by *P. indica* and moderate activity was seen in *E. rubrum*.

h) **SOD and CAT**

A strong correlation exists between antioxidant defence system and the level of salt tolerance was reported in plants (Garratt et al 2002). Under stress condition antioxidant system prevent the plant by forming ROS (Harinasut et al 2003). Reports are also there on high SOD activity present in salt tolerant plant (Benavides et al 2000). Present study showed that salinity increases the SOD activity in shoots not only in uncolonized plants but also in colonized plants. It could be concluding that SOD activity is useful in salt tolerance. Furthermore, in colonized plants SOD activity is enhanced as compared to non colonized one because symbiotic relationship increases the plant tolerance capacity grown in semi arid regions by enhancing the activity of antioxidant enzymes (Alguacil et al 2003). This enhancement leads to increase the plant capacity to scavenge the superoxide radicals. As SOD activity is increased it also increases the levels of \( \text{H}_2\text{O}_2 \) resulting increases thereby the enzymatic capacity for decomposition (Hernandez and Almansa 2002). Enzymes which act as hydrogen peroxide scavengers like peroxidase, catalase, glutathione reductase and ascorbate peroxidase.
The important function of CAT is to regulate the growth of fungi intra-radically. CAT scavenges the H$_2$O$_2$ which is one of the efficient mechanisms that attenuates the enhancement of defence responses in plants (Wu et al 1997). During senescence activity of CAT is decreased that causes increase in peroxide levels (Becana and others 1986).

Under salinity there is induction and activation of antioxidative enzymes which detoxify the ROS and protect the interactions of plants and fungi (Alguacil et al 2003). Increasing activity of antioxidant enzymes reduce the oxidative burst i.e., excess production of ROS and hence P. indica colonized plants create the high oxidative defence mechanism. That is why there is generation of high amount of antioxidative enzymes in P. indica colonized plants than uninoculated. Treatment with fungal endophytes also reported to increase the CAT activity in Olea europaea which is grown in semi-arid conditions. It is well known that CAT helps in the H$_2$O$_2$ decomposition in peroxisomes. Its activity increases with salinity in both endophytic as well as in non endophytic plants which indicate that high amount of H$_2$O$_2$ is produced under salinity in peroxisomes.

i) Lipid Peroxidation
Under salinity ROS accumulated which damages the cellular membrane (Hernández et al 1995) that is toxic to many living cells and results in oxidative damages to lipids, proteins and DNA. However, ROS behave as signalling molecule to response the stress (Apel and Hirt 2004). In present study salinity also affects the lipid peroxidation level and TBARS content and this content increases with salinity. Salt stress causes ion leakage that indicates the damage of membrane integrity which is due to formation of ROS during respiration and leads to oxidation of lipid membrane. Same results were also reported by many findings (Lechno et al 1997; Gomez et al 1999). Many reports suggested that some endophytic fungi having broad host range can easily confer the effective tolerance against ROS under salinity (Rodriguez et al 2008). Salinity induces many changes like lipid peroxidation which was significantly reduced in endophyte treated plants.

Preventing the lipid peroxidation and maintaining the integrity of membrane is the key determinant process for salt tolerance in plants (Garg and Manchanda 2009). In our study lipid peroxidation in leaves occurs in both colonized and uncolonized plants under salinity which increases the leakage of electrolytes and injurious to the plasma membrane of leaves.
Discussion

Alteration in lipid peroxidation acts as an indicator for extent of oxidative damage in salinity (Borsani et al 2001).

We found that colonized plants reduce the lipid peroxidation levels than control plants. Desaturation of lipids is important component in plants to tolerate the salt stress. Many studies showed that salt stress induce the enhancement in lipid peroxidation (Yang et al 2004). This suggested that plants tolerance is associated with activation of antioxidant enzymes (Sekmen et al 2007). In present study we have observed the activity of antioxidant enzymes in leaves of bajra plants. The process of lipid peroxidation is synchronically increases with salt levels which was also observed in plants such as Tomato (Neumann, 2001) Wheat (Hala et al 2005), and Purslane (Yazici et al 2007).

However, the levels of MDA concentrations and electrolyte leakage in colonized plants are much lower than noncolonized plants accordance with similar findings (Feng et al 2002; Garg and Manchanda 2009). It was also found that under salinity presence of endophytes in roots was able to protect the cell membrane of shoots from injury and simultaneously maintain the integrity and stability of membrane by increasing the level of phosphorous and also maintain the high Ca$^{2+}$: Na$^{+}$ ratio. In colonized plants leakage of electrolytes decreased that was related with phosphorous which induced the changes in the levels of phospholipids of membrane and also in properties of permeability (Graham et al 1981).

j) Proline

When plants are exposed to salt stress condition it started to accumulate the chains of metabolites like amino acids. Proline is an important amino acid which acts as beneficial role in many plants under salinity. Apart from this role it also plays as metal chelator, antioxidative defence compounds and also signalling molecule. Stressful environments leads to the overproduction of proline molecule in plants that imparts the stress tolerance level through maintaining the cell turgor and osmotic balance, by membrane stabilization therefore prevent the leakage of electrolytes and also maintain the level of reactive oxygen species which lie in the normal range and finally prevent the oxidative burst in plant cell (Hayat et al 2012). In current study we have measured that accumulation of proline which was significantly higher in colonized plants as compared to noncolonized plants during salinity.
Discussion

Accumulation of proline is one of the sensitive indexes of physiology which is generated in plants in response to salts and other kind of stresses. To survive in harsh stress conditions plants have to survive by adjusting its osmotic potential in leaf is important and this requires balancing the osmosis intra-cellulary. During salinity there is accumulation of organic solutes (soluble sugar and proline) and inorganic ions which maintain the osmotic adjustment. Hence \textit{P. indica} inoculated plants showed better growth by increasing the proline accumulation in leaves under salt stress as compared to uninoculated plants. This high proline concentrations in leaves maintain the high water potential in leaves during stress and also protect the plants against damage inducing oxidative stress. As stress enhanced the ROS generation in plants which cause damages by disturbing the lipids, nucleic acids and proteins (Lee et al 2001).

Proline is key osmoprotectant that get accumulated in plants against salinity stress (Delauney and Verma 1993). In barley (\textit{Hordeum vulgare} L.) proline reduces the osmotic pressure which generated due to salinity (Ueda et al 2007). Increased proline content observed in cotton (Desingh et al 2007), \textit{Paulownia imperialis} (Astorga et al 2010) and wheat (Khan et al 2009) were reported. Many studies are there on proline accumulation under salt stress (Munns and Tester 2008), plays an important protective role to osmotic potential induced by salt (Chen et al 2007; Hoque et al 2008). Proline was best studied in plants exposed to abiotic stress like salinity and drought. Its concentration was best determined by simple, fast and accurate method which depends upon the reaction between proline and acid ninhydrin (Bates et al 1973).

In present study the colonized bajra plant with \textit{P. indica} induced mechanism for better protection against salinity causing oxidative damage than uncolonized plants. At 300mM NaCl proteins disappeared because it got denatured at high salt concentration. Many workers also reported that synthesis of depressed protein its acceleration and degradation in plants at high salt level. \textit{P. indica} colonized bajra plants showed increase in accumulation of proline. Bajra plants under salinity accumulate the proline as protective and nontoxic osmolyte (Parida et al 2002). Non colonized plants found to be rich in proline content than colonized plant accordance with the findings reported by Rabie (2005). The other mechanism is also applied by endophytes for stress tolerance is that increasing the acid and alkaline phosphatase activities and Mg as well as N contents in \textit{V. faba} plants (Rabie and Almadini 2005).
**Discussion**

*E. rubrum, A. terreus* and *P. indica* inoculated plants survived well in different salt treatments which suggested that these fungi help to maintain the osmotic pressure. However, detail study is needed to understand the mechanism for maintaining the osmotic pressure. Our pot trials also support the idea of testing these fungi with bajra plants in saline field conditions.

**Molecular identification**

Analysis of amplified ITS-DNA region of isolated fungal isolates showed inter-specific and intra-specific variations among *Eurotium* and *Aspergillus* species. The amplified products of ITS region of these two plant growth promoting species isolated from salty desert of Rann of Kutch which ranged from 500-600bp. Identification of fungi became more reliable and specific when both classical and molecular methods are combined together (Hyde and Soytong 2008; Than et al 2008). In present study phylogenetic tree were constructed through blast searches from MEGA4 software.

**Influence of *P. indica* on protein profile**

Under saline stress the response of plants to protein accumulation depends on the plant species and cultivar. In present study soluble proteins from leaf extract of *P. glaucum* plants when treated with *P. indica* under different concentrations of NaCl i.e., 50mM, 100mM, 200mM and 300mM were subjected for SDS PAGE. This work was done mainly to compare the pattern of soluble protein accumulation of colonized as well as noncolonized *P. glaucum* plants under salt stress. These patterns represent the level of salt stress tolerance. The above study reveals that salt stress on plants degrades protein and this is the first study of *P. indica* on protein pattern of *P. glaucum*. The breaking up of polypeptides results in appearance of new smaller peptides (Rani 2011). Plants have a multitude of mechanisms which help them to survive and propagate under salt stress. These mechanisms enhanced when plants are colonized with plant growth promoting fungi or abuscular mycorrhizal fungi. In current study *P. indica* prevents the plant protein degradation as compared to non colonized plants under salt stress. Salt stress proteins are believed to prevent protein denaturation. Repair of salt damaged/denatured proteins are essential for both survival and recovery from salt stress. Pretreatment of seeds could enhance salt tolerance in grains. Enhanced salt tolerance across different concentration limits can be exploited for extending cultivation of *P. glaucum* beyond traditional areas where these varieties are being grown.
**Effect of Rootonic and Bio-Zinc on *P. glaucum* plant infected with *F. solani***

In the present study, we have used *P. glaucum* plants to show maximum resistibility against the pathogenecity of *F. solani* upon treatment with the combination of Rootonic and Bio-Zinc as compared to other treatments and control. *F. solani* is one of the biotic stresses which affect the plant growth, development and yield. Here we have measured the important parameters related to plant growth, photosynthetic pigments and induction of metabolites during stress condition.

Zinc plays various important roles in plant metabolism by increasing the enzyme activity, synthesis of cytochrome, ribosomal stabilization (Tisdale et al 1985), carbohydrate metabolism, membrane stability (Disante et al 2010), protein synthesis, pollen formation and auxin synthesis (Brennan 2005). Zinc deficiency found in most of the regions of the world having high pH and high amount of CaCO₃ and phosphate which can fix the free zinc in soil and decrease its availability for plants (Alloway 2004, 2008).

Deficiency of zinc in plants reflects in the development of abnormalities with visible symptoms like chlorosis, stunted growth, smaller leaves and spikelet sterility. It adversely affects the quality of grains and increases the susceptibility to fungal infection (Cakmak 2000). Zinc also affects the water uptake capacity in plants (Tavallali et al 2010). This deficiency can be corrected by application of zinc fertilizers but overdose of fertilizer can become toxic and harmful for plants. Traditionally, the requirement of zinc is being met by the application of inorganic fertilizer like zinc sulphate etc. Addition of Rootonic or Bio-Zinc alone did not make significant impact on seed germination. However, combined application of Rootonic and Bio-Zinc improves the seed germination. Combination of Bio-Zinc and Rootonic enhanced seed germination over *F. solani* alone treatment by 31.02% followed by treatment with Rootonic and Bio-Zinc.

Zinc is important in seed germination for protein synthesis and resistance against pathogens (Siddiqui et al 2002) and on other side *Fusarium* sp. is highly pathogenic and cause different diseases i.e., seed rot, wilt and seedling blight in number of crops (Karim 2005) and therefore, deteriorates the quality of seed germination.
Effect on growth parameters

Analysis of different parameters of plant growth is an important property to study the plants behaviour against biotic stress i.e., pathogenicity of *F. solani*, because it induces stomatal closening, reduce the capacity of photosynthesis and drastically alter the nutrient balance in plants. Thus, its immediate negative effect on plant is stunted growth, lower biomass and decline in chlorophyll concentration. Among all diseases, fungal diseases are mainly responsible for low crop productivity. *P. glaucum* is mainly affected by seed borne pathogens which cause reduction at initial and foliage stage of germination. *F. semitectum, A. flavus* and *A. niger* deteriorates the quantity and quality of floral parts which in turn decrease the grain yield at maturity level (Williams and McDonald 1983).

Pathologists suggested that global yield losses of *P. glaucum* is 45, 9, 32, 3% respectively, due to downy mildew, smuts, striga, rusts, respectively. The survival of *Fusarium* sp. chlyamydospores in soils is up to many years even there is no host and it is difficult to eradicate (Haware et al 1996).

Photosynthetic pigment content

Photosynthesis is an important process for growth and overall development of green plants and can be drastically affected by pathogenicity of *F. solani*. In this study we mainly focussed on pigments of chlorophyll which plays a crucial role in photosynthesis and phytoprotection. The concentrations of chl a, chl b and total chlorophyll of all treated plants were measured and it was found that Rootonic and Bio-Zinc treated plants increased the pigment content than other treatments.

It was observed that this novel combination increases the content of photosynthetic pigments (Chl a, Chl b and total chlorophyll) compared to other treatments and control (Fig 4.29). The major impact of pathogenicity of *F. solani* is the degradation of photosynthetic pigments because of chlorosis. As a result color of leaves becomes brownish in color and stunted growth (Williams and McDonald 1983). The concentrations of pigments are found to be maximum in plants treated with the combination of Rootonic and Bio-Zinc than others.
Total phenolic and flavonoid content
Polyphenols or phenolics are one of the important secondary metabolites that present ubiquitously in plants and its products contains high amount of antioxidants (Razali et al 2008). Previous work documented that phenolic compounds are the categories of antioxidant agents which help in the termination of free radicals (Shahidi and Wanasundara 1992). On other side flavonoid also showed the antioxidant activity due to their scavenging and chelating process for free radicals (Prasad et al 2013). Antioxidant mainly contains the hydroxyl group that’s why it is used as free radical scavengers in plants (Das and Pereira 1990).

For preparation of plant extract, methanol was used instead of ethanol because methanol is most effective in dissolving the active compounds i.e., saponins, tannins and anthocyanins in plant cells (Tiwari et al 2011). Antioxidant activity of plant extract is mainly depending upon the types of solvent used because every compound has different polarity with different rates of antioxidant potential (Kumaran 2007). Most of the parts of plants are rich in phenolic acids and its demand increases in food industry because it retards oxidative lipid degradation and enhance the nutritional and quality of food (Kähkönen et al 1999).

Flavonoids help in the modification of eicosanoid biosynthesis and have antiviral and anti-carcinogenic properties (Duarte-Alameida et al 2007). Phenolic compound has its health benefits as it fights against cancer. Feluric acid is bound phenolic form and it is present in *P. glaucum* which emphasizes its major role as an anti-carcinogenic (Xiaonan et al 2011). Phenolic compounds play an important role in defense mechanism against microbial pathogens and predators based on their toxicity and repellence to microbes and insects. These compounds are also reported as phytoalexins, phytoanticipins and modulator of plant defence genes and pathogenicity (Mazid et al 2011). It is one of the important precursors for lignin synthesis (Lewis and Yamamoto 1990) which acts as a barrier and increase the resistance against *F. solani* in host plants. Incorporation of phenolic compounds is an effective management to control *Fusarium* sp. in plants. (Mazid et al 2011) reported accumulation of phenols is important for plant resistance against pathogens.