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Influence of Native Endophytes on Early Stages Growth of *Vigna radiata* (Moong) under Salt Stress Condition

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ABSTRACT

Background: Green gram (*Vigna radiata*) also known as moong bean is an annually cultivated in East Asia, Southeast Asia and Indian subcontinent. *V. radiata* is very important source for the protein as in our regular diet and it proved essential amino acid such as phenylalanine, leucine, isoleucine, valine, lysine, arginine, methionine, threonine and tryptophan.

Methods: Here, we studied the influence of seed endophytes on germination and development under salinity stress condition. Seeds were treated with sodium hypochlorite for 30 min under shaking condition at 100 rpm for surface sterilization and treated with 70% ethanol for 2 min and followed five times rinse with autoclaved water. Surface sterilised seeds were homogenised in autoclaved water with the help of mortal-pestle. Homogenised seed solution made serial dilution and spreaded over nutrient agar for endophytic bacterial growth. Seeds were treated with bacteriocide and fungicide to make endophytes free, followed by sown for germination at 0 mM, 50 mM, 100 mM and 150 mM NaCl concentration.

Result: Endophyte free seedlings were more susceptible against salt stress over normal seedlings. Therefore endophyte free seedling shoot and root biomass was 23.5% and 65.7% lower than control seedling biomass at 0mM salt respectively, while root length was 70% lower than control seedling root at 0mM salt concentration. Proline content in shoot and root observed an increase with increase of salt concentration. At 0 mM salt, proline content was 0.00782±0.00043 and 0.00648±0.00017 (µmol/mg) in root of normal and endophyte free seedling respectively, while in shoot, it was non-significant difference. Glycine betaine content found to be increasing upto 100 mM, followed by decreasing at 150 mM in both root and shoot tissue. Glycine betaine content in endophyte free and control seedling shoot was 74.2±2.5 and 96.0±2.73 (µg/200 mg) respectively at 100 mM salt concentration. This result suggests, not only heritable genomic DNA but also endophytes associated with seed are very much important for the seedling growth and development which is also finally helps to combat abiotic stress situation.

Key words: Bacteriocide, Endophyte, Fungicide, Glycine betaine, Proline, *Vigna radiata*.

INTRODUCTION

Vigna radiata also called green gram or moong bean or mash, which is annually cultivated in East Asia, Southeast Asia and Indian subcontinent. It belongs to the legume family and seed is rich source of protein, vitamin B complex, calcium and potassium (Oghbaei and Prakash, 2017). Root associated with rhizobium for the nitrogen fixation, phosphate solublisation, siderophore formation, phytohormone production and help in biotic and abiotic stress in leguminous plants (Gopalakrishnan *et al.* 2015). Apart from rhizobium, others endophytic fungi and bacteria are associated with *Vigna radiata* have been isolated from non- rhizobial root tissue (Pandya *et al.,* 2015). Non-rhizobial endophytes were also bearing similar character as of rhizobium like, phytohormone secretion, nutrient solubilisation, antipathogenic activity *etc*, to induce the plant growth of *V. radiata* (Bhutani *et al.,* 2018). Endophytic microbes (fungi or bacteria) are very important for medicinal and non-medicinal plant, have been reported from most of plant species. The role of endophytes are not limited to help in growth of host, but also known to induce the secondary metabolites within host (Kushwaha *et al.,* 2019). Plant carry their heritable character in genomic DNA from one generation next generation but the influence of seed associated endophytes on seedling growth cannot be avoided (Shahzad *et al.,* 2018).

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Salinity is one of the major threats in crop to decrease in the shoot length, root length and weight of the plants which enormously impacted leading to decrease in crop production (Machado and Serralheiro, 2017). High salt concentration influenced on biochemicaland physiological trait of plant such as ion homeostasis, uptake, osmolyte synthesis, antioxidant enzyme activation, synthesis of antioxidant compounds and polyamines, nitric oxide and hormone modulation (Sharma *et al.,* 2019). The main protective osmolytes are proline, glycine betaine, polyols and sugar, while their accumulation in varying amounts amongst different plant species (Gupta and Huang, 2014). Water

soluble proline rise intracellular concentration in response to salinity stress and also serves as reserve of nitrogen source during stress recovery (Hayat *et al.,* 2012). The importance of proline and glycine betaine in *Nicotiana tabacum* has been reported to increase the activity of antioxidant enzymes to decrease the reactive oxygen species for defence response (Hoque *et al.,* 2008). Glycine betaine is an amphoteric quaternary ammonium compound, highly soluble in water and raises their concentration during salt stress condition. Glycine betaine act as osmotic protectant, protect enzymes/proteins, reduce reactive oxygen and protects the photosynthetic system during osmotic or salt stress (Giri, 2011). Both proline and glycine betaine are important osmolyte to protect *Oryza sativa* seedling during salt stress (Hasanuzzaman *et al.,* 2014).

The influence of seed endophytes on seed germination under saline condition has not yet been explored in *Vigna radiata*. In concern with innate endophytes associated with moong seed and their influence on seedling development and growth under salt condition studied *in vitro* condition.

MATERIALS AND METHODS

Fungicide and bacteriocide treatment to seed

Local variety moong - Pusa Baisakhi seeds were purchased from Kattigenahalli market, Bangalore, India. 10 gram seeds were inoculated in autoclaved 100 ml conical flask containing 20 ml distilled water and supplemented with antibiotics streptomycin (0.2 mg/ml), tetracycline (0.2 mg/ml) and fungicide mancozeb 75 WP (1 mg/ml) for overnight at room temperature under shaking condition. Mancozeb was purchased in January, 2019 which was prohibited to be manufactured and sold from May, 2019 by the Ministry of Agriculture and Farmers Welfare in India. The study was carried out in REVA University, Bangalore from the month of February 2019 to April 2021. Initially, different concentration of the antibiotics streptomycin (2 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.1 mg/ml, 0.01 mg/ml) and tetracycline (2 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.1 mg/ml, 0.01 mg/ml) were used, while fungicide (1 mg/ml) remained constant during all time.

Isolation of seed associated bacterial endophytes

Seeds were treated with 20% sodium hypochlorite for 30 min under shaking condition at 100 rpm for surface sterilization and then washed with distilled water under aseptic condition (Kushwaha *et al.,* 2019). Thereafter, seeds were treated with 70% ethanol for 2 min and followed five times rinse with autoclaved water. To check the surface sterilization, last wash water was spread on nutrient agar (NA) and potato dextrose agar (PDA) for bacterial and fungal growth respectively. Surface sterilized seeds were macerated in distilled water with help of autoclaved mortar and pestle under aseptic condition. 10^{-1} to 10^{-9} serial dilution made from macerated seed solution and 100 µl from each dilution were spread on nutrient agar to isolate the seed associated endophytic fungi.

Evaluation of endophyte free seedlings

Fungicide and bacteriocide treated seeds were five times washed with distilled water, followed by macerated in distilled water with help of sterilized mortar and pestle under laminar. 100 µl of macerated seed solution were spread on nutrient agar and potato dextrose agar media to check bacterial growth and the fungal growth respectively. For bacterial growth, NA plates were kept at 37°C for overnight and to check fungal growth PDA plates were kept at 30°C for a week.

Seed germination under salt stress

Bacteriocide and fungicide treated or untreated control seeds were kept on sterilized blotting sheet in petriplate. 10-12 seeds were placed in each plate in three replication. 10 ml of salt solution added and was kept at ambient condition for seedling development. Different salt concentrations of sodium chloride (0 mM, 50 mM, 100 mM, 150 mM) were prepared in distilled water. Seeding growth was observed every day upto 10 days. After 10 days, samples were collected for the fresh biomass, height, proline and glycine betaine estimation in all treatments.

Proline estimation

Proline was estimated according to (Bates *et al.* 1973), approximately 500 mg of plant material (root andshoot) was homogenized into 10 ml of 3% sulphosalicylic acid and filtered with Whatman filter paper #2. 2 ml of filtrate was mixed with 2 ml of acidic ninhydrin and 2ml glacial acetic acid in the test tube. Thereafter, reaction mixture was kept at 100°C in water bath for 1 hour and then reaction terminated in ice. In this reaction mixture 4 ml toluene was added and was mixed vigorously for 15-20 seconds. Upper layer of toluene containing chromophore used to take the absorbance at 520 nm and toluene taken as blank.

Glycine betaine estimation

Glycine betaine was estimated according to Grieve and Grattan, 1983, approximately 500mg of shoot or root tissues was grinded in 5 ml distilled water and kept in shaking condition at 25°C for 24 hours. The extract was mixed with sulphuric acid and potassium tri-iodide and then kept on ice for 90 mins. The reaction was terminated by adding chilled 1,2-dichloro ethane and kept for phase separation at room temperature for 2 hours. After phase separation, lower organic layer was taken for absorbance at 365 nm. Glycine betaine content in root and shoot tissue was estimated with the help of standard glycine betaine.

Statistical analysis

For each salt treatment, three biological and three technical replicates were considered for statistical analysis. The statistical significant differences between two samples

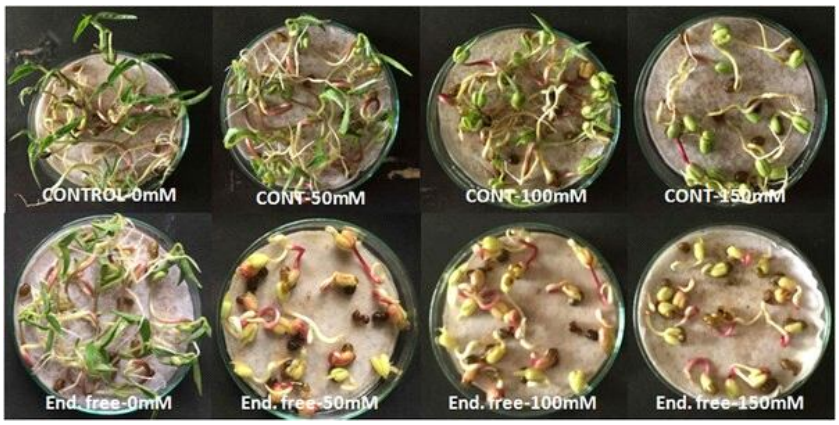
were tested by unpaired Student's t-test though online software (http://www.socscistatistics.com/tests/studentttest/Default2.aspx). Each treatment was followed with six biological replicates. Asterisks indicate a significant difference from the control (Student's t-test; **P<0.05, ***P<0.01). Cont, Control; EF, Endophyte free.

RESULTS AND DISCUSSION

Influence of endophyte on plant biomass under salt stress

Endophytes are recognized as hidden world within plant and their importance for the host benefit are reported by numerous researchers. Endophytes help to plant through secretion of phytohormone hormone, phosphate solublization, nitrogen fixation, sidrophore production and also help during abiotic and biotic stresses (Khandel *et al.,* 2017). The morphology of normal seedling observed better than the endophyte free seedling at each salt concentration (Fig 1). At 0 mM control, the control seedling biomass and endophyte free seedling biomass was 331±7.11 and 253.6±8.02 mg/plant respectively (Fig 2). Plant biomass was decreasing with increasing salt concentration in both normal and endophyte free seedling. At 50 mM, control and endophyte free seedling biomass was decreased by 11.6% and 14.3% respectively (Fig 2a,b). At 100 mM, control seedling biomass was decreased by 23.2% while in case of endophyte free seedling decreased by 17.2% than their respective control (Fig 2a,b). However, at 100 mM control seedling and endophyte free seedling biomass was decreased by 28% and 31.2% respectively to their respective control (Fig 2a,b). In both seedling, salt treatment was influencing in similar fashion but normal seedling was having more biomass at each salt concentration than endophyte free seedling (Fig 2a,b). Previously, 22 endophytic bacteria were isolated from *Vigna radiata* roots and some of bacteria were retaining plant growth promoting activity (Bhutani *et al.,* 2018). Among abiotic stress, salt stress is very common; alleviating salt concentration in soil day by day as well as saline affecting area increasing worldwide is a big problem (Shrivastava and Kumar, 2015). Therefore, the colonization endophyte *Serendipita indica* (formerly *Piriformospora indica*) with Arabidopsis helps to grow under salinity stress (Lanza *et al.,* 2019).

Recently, 25 endophytic microbes were reported from the root of *Vigna radiata* and some of them showed growth promoting characteristic (Gujar *et al.* 2019). When other sources are compared for the endophytes, the seed is one heritable source which carried from one generation to the



CONT- Control, End. free- Endophyte free.

Fig 1: Image of control and endophyte free seedling growth under different salt stress condition after 5 days of incubation.

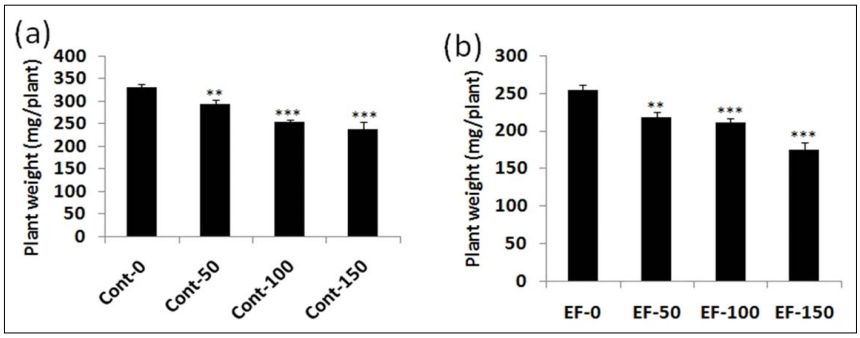


Fig 2: Effect of salt on fresh biomass (a) control seedling plant biomass at different salt concentration (b) endophyte free seedling biomass at different salt concentration.

next (Shahzad *et al.* 2018). Previous study showed that through direct and indirect mechanism endophytes help in germination and promotion of plant growth (Walitang *et al.* 2019). In present study, biomass was decreasing with increasing salt concentration, but biomass decreasing rate observed higher in endophyte free seedling as compared to control. Of course, in control seedling endophyte was present and might be helping plant to resist in salt condition. Rather than the plant surfaces the internal tissues of plant provide a protective environment for the endophytes where temperature, osmotic pressure and UV radiation does not affect the surviving of the bacteria in such extreme environmental conditions (Thamiz Vendan and Balachandar, 2021). Seed endophyte bacterial community also depends on the genotype of plant and salt stress trigger the dominant of important class of bacteria to help under stress condition (Walitang *et al.,* 2018).

Influence of endophyte on shoot and root length under salt stress

Salt stress is negatively influenced on morphology, physiology, plant biomass and yield (Gupta *et al.* 2014). But the application of endophytic bacteria has been recognized for adaptation under salt stress condition (Vaishnav *et al.,* 2019). Shoot and root length of control seedling was higher than endophyte free seedling at each salt treatment (Fig 3). Shoot length of control seedling and endophyte free was 5.5±0.5 and 1.6±0.15 (cm/plant) at 0 mM salt concentration (Fig 3a,b). As the salt concentration was increasing, the shoot length was decreasing by 45.4, 78.8 and 89.6% at 50, 100 and 150 mM respectively in control seedling (Fig 3a). In case of endophyte free seedling, shoot length was decreasing by 14.1, 42.7 and 71.3% at 50, 100 and 150 mM salt concentration respectively (Fig 3b). With increasing the salt concentration, shoot length decreasing rate was higher in control seedling plant than endophyte free seedling (Fig 3a,b).

Root length was higher in control seedling as compared with endophyte free at every salt concentration. At 0 mM, root length of control seedling and endophyte free seedling were 10.8±0.72 and 3.73±0.25 cm/plant respectively, *i.e.* endophyte free seedling length was 65.7% less than control seedling (Fig 3b,c). In control seedling, root length was 24, 45.9 and 57.4% decreased at 50, 100 and 150 mM respectively compared with their 0 mM control (Fig 3c). However, root length of endophyte free seedling was decreased by 22.2, 33.8, 80.3% at 50, 100 and 150 mM respectively than their control plant (Fig 3d). At 100 mM, endophyte free seedling was not able to grow properly and their root length was very less than control seedling root length. In present finding, the control seedling was retaining higher root length, shoot length and plant biomass as compared with endophyte free seedling at 0mM and higher salt. Previously reported that, the application of bacterial endophyte *Gordonia terrae* KMP456-M40 has been enhanced the root length of mangrove seedlings and the biomass of salt-stressed rice under axenic conditions up to 65% and 62%, respectively (Soldan *et al.* 2019). The highest

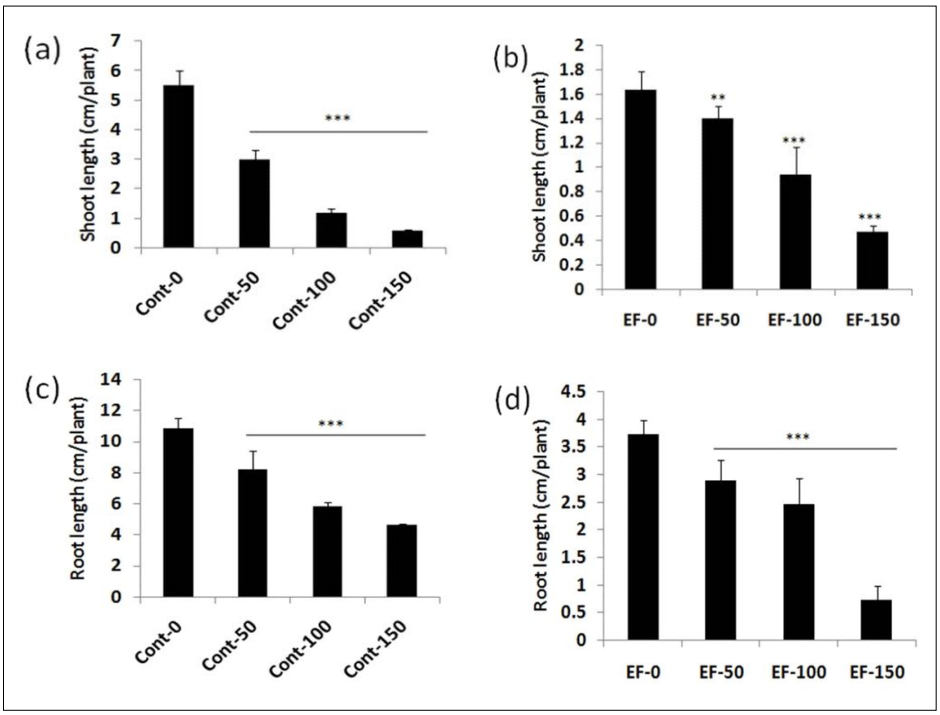


Fig 3: Effect of salt on root and shoot length (a) shoot length of control seedling at different salt concentration (b) shoot length of endophyte free seedling at different salt concentration (c) root length of control seedling at different salt concentration (d) root length of endophyte free seedling at different salt concentration

seedling length, dry weight and germination percentage was observed in the green grams inoculated with isolate KHDEB5 under salt stress varying upto a wide range of salt concentration (upto 500 mM NaCl) (Das *et al.,* 2020). From same leguminaceae family plant, four endophytes, *Bacillus cereus* NUU1, *Achromobacter xylosoxidans* NUU2, *Bacillus thuringiensis* NUU3 and *Bacillus subtilis* NUU4 were isolated from *Cicer arietinum* root, however only *B. subtilis* NUU4 significantly improved plant growth, symbiotic relation with rhizobia and yield under saline soil condition (Egamberdieva *et al.,* 2017).

Proline content in control and endophyte free seedling under salt stress

Proline is very important osmolyte to protect plant under abiotic stress condition and increasing content with increasing salt concentration upto certain extent (Kaur and Asthir, 2015). At 0mM salt concentration, the proline content in shoot was not significant difference between control and endophyte free seedlings (Fig 4a,b). Proline content in control seedling shoot at 50 and 100 mM was increased by 69.2% and 115.4% respectively compared with 0mM salt treatment (Fig 4a). Proline content was not increasing with increasing salt, because at 150mM proline content in control seedling was increased by 54.7% as compared with 0mM salt (Fig 4a). In endophyte free seedling shoot, proline content was maximum at 50 mM salt then followed by decreasing with increasing salt (Fig 4b). At 50, 100 and 150mM salt concentration, proline content was increased by 85.9, 35.1 and 29.6% as compared with 0mM salt of endophyte free seedling shoot (Fig 4b).

In saline soil condition (100 mM), *Cicer arietinum* treated with *Mesorhizobium ciceri* strain IC53 alone or along with endophyte *B. subtilis* NUU4, have been in increased the proline content in root by 11% and 29% respectively (Egamberdieva *et al.,* 2017). In this study, proline content was maximum at 100 mM in control seedling shoot, while in case of endophyte free seedling, proline content was reached maximum early at 50 mM. This result revealed that, endophyte free seedlings were not adaptive in salt condition as compared with control seedlings.

Proline content in control and endophyte free seedling root was increasing with increasing salt concentration (Fig 4c, d). At 0 mM salt concentration, proline content was 0.0078±0.00043 and 0.0064±0.00017 (µmol/mg) in control and endophyte free seedling root respectively (Fig 4c,d).In control seedling root, the proline content at 50, 100 and 150mM salt concentration was increased by 43.2, 114.6 and 166.7% than 0 mM salt concentration (Fig 4c). However, in endophyte free seedling root, the proline content at 50, 100 and 150 mM salt concentration was increased by 42.3, 88.8 and 149% than 0mM salt concentration (Fig 4d). Plant synthesized proline content in root and shoot tissue at low level for development and growth, albeit shoot synthesize more proline than root in normal condition (KaviKishor *et al.,* 2015). During seed formation proline accumulated more in seed, however in

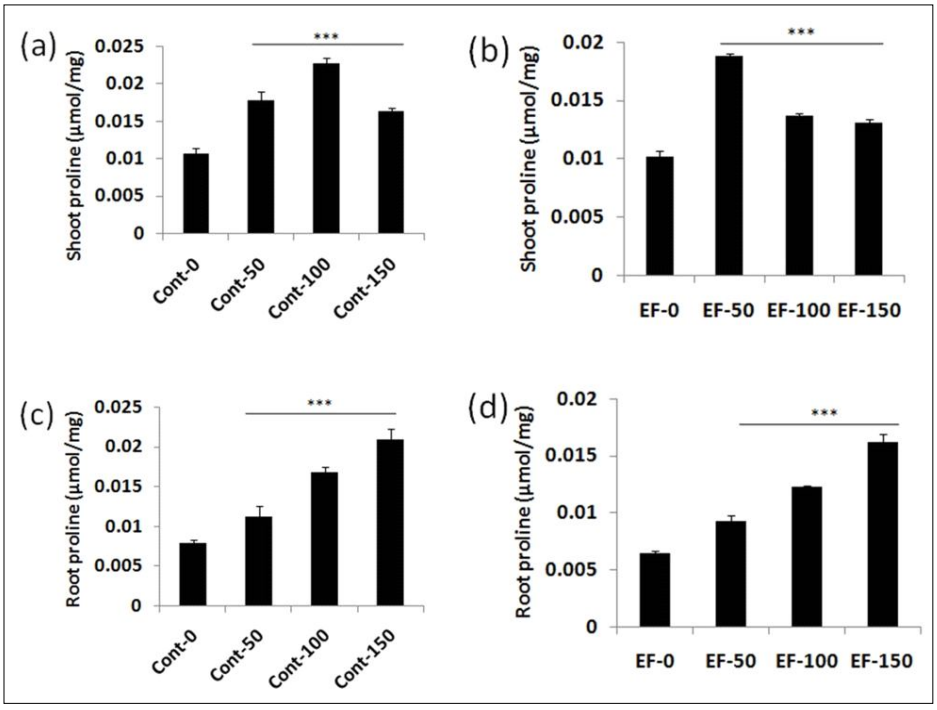


Fig 4: Effect of salt on proline content in root and shoot tissue (a) proline content in shoot of control seedling at different salt concentration (b) proline content in shoot of endophyte free seedling at different salt concentration (c) proline content in root of control seedling at different salt concentration (d) proline content in root of endophyte free seedling at different salt concentration.

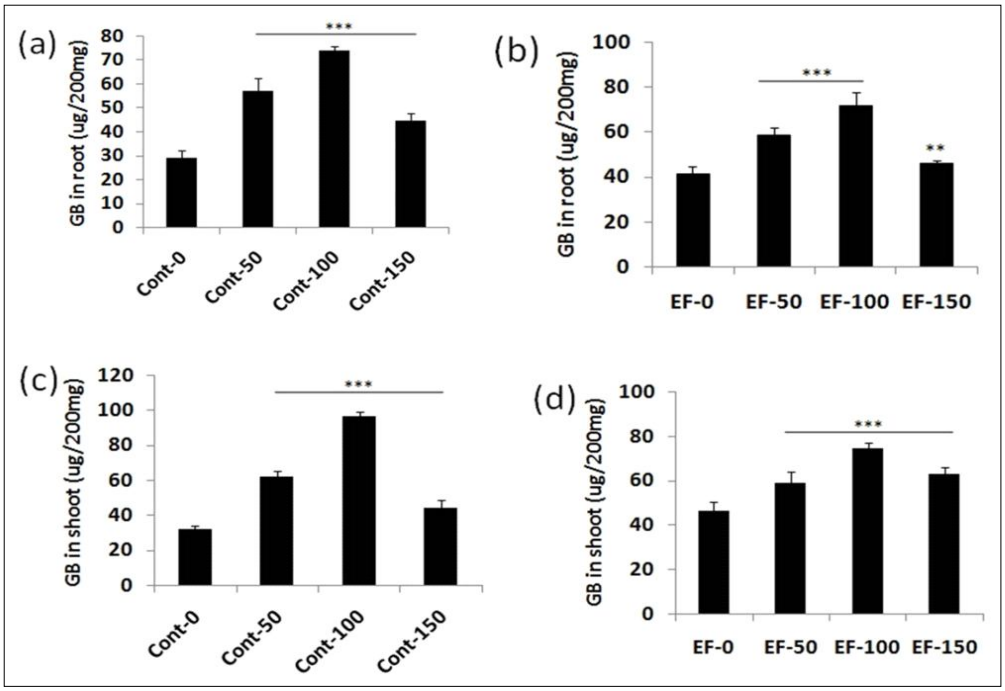


Fig 5: Effect of salt on glycine betaine (GB) content in root and shoot tissue (a) GB content in root of control seedling at different salt concentration (b) GB content in root of endophyte free seedling at different salt concentration (c) GB content in shoot of control seedling at different salt concentration (d) GB content in shoot of endophyte free seedling at different salt concentration.

salt stress condition induced more in root and shoot to tolerate salt stress (Mattioli *et al.,* 2009).

Glycine betaine content in control and endophyte free seedling under salt stress

Glycine betaine (GB) is an amphoteric quaternary ammonium compound and is ubiquitously reported in microorganism, animal and from plant. GB is non-toxic, soluble in water and remain selectrical neutral at wide range of pH (Gupta *et al.,* 2014). GB content in root and shoot tissue was increased upto 100 mM, afterward decreased at 150mM salt concentration (Fig 5). Either in shoot or root, the GB content was higher in control seedling than endophyte free seedling plant (Fig 5). In control seedling, the GB was 91.5 and 198.4% higher in shoot at 50 and 100 mM as compared with 0 mM salt (Fig 5a). But at 150 mM GB in shoot decreased and it was only 37.6% higher than 0 mM salt concentration (Fig 5a). GB content in endophyte free seedling shoot was also following same pattern. At 50 and 100 mM, GB content was 27.5 and 61% higher in endophyte free seedling shoot respectively as compared with 0 mM salt, while at 150 mM GB content was 36.9% higher than 0 mM salt concentration (Fig 5b).

In abiotic stress condition, spatio-temporal accumulation of GB provides osmotic adjustment, stabilizes proteins and protects the photosynthetic apparatus (Annunziata *et al.* 2019). Salt stress damage to photosynthetic pigment and apparatus, accumulation of GB during salt stress and foliar spray of GB led to pigment stabilization and increase in photosynthetic rate and growth of plant (Cha-Um and Kirdmanee, 2010). Transgenic wheat (betaine aldehyde dehydrogenase, *BADH*) was accumulated upto 42 mmol L^{-1}, showed higher net photosynthetic rate and stomatal conductance over control at 200 mM salt concentration (Umar *et al.* 2018; Tian *et al.,* 2016).

In root, GB content was induced maximum at 100 mM salt concentration in both control and endophyte free seedling. In control seedling root, GB content was 95.3 and 153.2% higher at 50 and 100 mM salt concentration respectively than 0 mM salt, while at 150 mM it was 52.1% higher than 0 mM salt (Fig 5c). In case of endophyte free seedlings, GB content was 41.3 and 72.2% higher at 50 and 100 mM salt concentration respectively than 0 mM salt, however at 150 mM, it was only 10.6% higher than 0 mM salt (Fig 5d). In present study, GB content in root and shoot was increasing with increasing salt upto 100 mM. But GB was higher in shoot as compared with root at each salt concentration as support the previous finding spatio-temporal accumulation of GB (Annunziata *et al.,* 2019). Abide to GB and proline, the external spay of polyamines (putrescine, spermidine and spermine) help to salt tolerance by enhanced the contents of glutathione and ascorbate, increased activities of antioxidant enzymes (dehydroas corbatereductase, glutathione reductase, catalase and glutathione peroxidase) and glyoxalase enzyme (glyoxalase II) in moong seedling (Nahar *et al.,* 2016).

CONCLUSION

In present finding elucidated the importance of endophytes associated with seed. Endophyte seedling were retaining less proline and GB content in root and shoot as compared with control seedling *i.e* without bacteriocide or fungicide treated seedling. Total eight endophytic bacteria were present interior of seed which opened a new window to find out the potential endophytic bacteria which will be responsible for seedling germination and growth under ionic stress situation.

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Conflict of interest

Author declare that they have no conflict of interest.

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