Mysore J. Agric. Sci., 57 (2) : 120-128 (2023)

Seed-inhabiting Endophytic Bacteria of Finger Millet [*Eleusine coracana* (L.) Gaertn] Enhance Early Seedling Growth and Development

M. Hemapriya, K. N. Nataraja and R. Uma Shaanker
Department of Crop Physiology, College of Agriculture, UAS, GKVK, Bengaluru - 560 065
e-Mail : umashaanker@gmail.com

Authors Contribution

M. Hemapriya : Conceptualization, formal analysis, investigation and original draft writing ;
K. N. Nataraja : Reviewing and editing the manuscript;
R. Uma Shaanker : Reviewing and editing

Corresponding Author :
R. Uma Shaanker
Department of Crop Physiology, College of Agriculture, UAS, GKVK, Bengaluru

Received : September 2022
Accepted : March 2023

Abstract

Seed microbiome has a strategic importance as it connects the maternal microbiome with next generation, seedling microbiome. Seed microbiome (bacteria & fungi) can be viewed as the transfer of microbial memory of life history adaptations to the next generation. Against this background, present study was taken up to study the role of seed inhabiting bacterial endophytes on finger millet seedling growth and development. Two bacterial endophytes were isolated from surface-sterilized seeds of finger millet and molecularly identified by 16S rRNA sequencing as *Paenibacillus quercus* (EC1) and *Bacillus cereus* (EC2). Enrichment of seed bacterial isolates enhanced seedling growth while removal of the endophytic bacteria from finger millet seeds compromised seedling growth and development. In summary our studies indicated that seed borne endophytic bacteria are crucial for early seedling establishment.

Keywords: Seed, Endophytes, Finger millet, Seedling establishment

In recent years, increasing attention has been paid to endophytes and the role they play in modulating plant growth and development. Both, endophytic fungi and bacteria seem to enjoy a unique relationship with the plant that range from mutualism to symbiosis. In fact, their interactions with the plant are nested so deeply and intricately, that often the endophytes and other plant associated microbiome is also referred to as 'second genome' of the plant (Berendsen *et al.,* 2012). Their colonization does not cause any apparent symptoms and neither are they rejected by the plants.

Endophytes have been reported to promote plant fitness, either by directly enhancing nutrient availability, production of phytohormones, organic acids or indirectly by preventing the growth or activity of phytopathogen (Manasa *et al.,* 2015). The microbiome spans the entire plant, ranging from the rhizosphere to the phyllosphere, including the seed. While abundant literature exists to document the nature of microbiome in rhizosphere and phyllosphere, relatively a little is known about the seed microbiome and how these might help in seedling establishment.

Seed borne endophytes have been reported in different crops such as rice, wheat, maize and millets (Verma *et al.,* 2018; Johnston-Monje & Manish Raizada, 2011; Herrera *et al.,* 2016 and Kumar *et al.,* 2021). It is estimated that over 9000 diverse microbial species inhabit the seeds (Shade *et al.,* 2017 and Adam *et al.,* 2018). Principally, seeds acquire their microbiota through multiple paths, including through the xylem or nonvascular tissue of the mother plant, the floral pathway *via* the stigma of the mother or simply by contact in the soil (Barret *et al.,* 2016). While the former two involve mainly vertical transmission from seed to seed, the latter clearly is a case of horizontal transfer. Seed inhabiting endophytes are also known to stimulate the expression of various genes in developing seedling of crop plants related to root

architecture development and defense against biotic and abiotic stresses (Gogna *et al.,* 2015 and Tasmiya *et al.,* 2021).

Against this background, in this study an attempt has been made to explore the possible role of seed inhabited bacterial endophytes in the establishment of finger millet seedlings. We hypothesized that finger millet seeds may carry crucial bacteria that improve seedling growth and development. Our finding suggests that seeds of finger millet harbour important bacterial endophytes, *Paenibacillus quercus* (EC 1) and *Bacillus cereus* (EC 2); enrichment of these bacteria enhanced seedling growth while removal of these bacteria from seeds hampered seedling growth and development.

Material and Methods

Seed Material

Eleusine coracana (L.) Gaertn, an allotetraploid (2n = 4X = 36), is an annual robust grass belonging to family Poaceae, widely grown as a millet in the semi-arid tropics and subtropics of the world under rainfed conditions. *Eleusine coracana* (L.) Gaertn was domesticated around 5000 years ago in western Uganda and the Ethiopian highlands. Later, finger millet was introduced into Western Ghats of India around 3000 BC. Thus, India became the secondary centre of diversity for finger millet. For this study, seeds of *Eleusine coracana,* GPU28 accession was procured from the Department of Crop Physiology, University of Agricultural Sciences, Bangalore, GKVK, Bengaluru - 65, Karnataka and India.

Isolation of Endophytes from Seeds of *Eleusine coracana*

Seeds of finger millet species, GPU28 were soaked in autoclaved water for 24h. Seeds were thoroughly washed with distilled water, surface sterilized with 70 per cent (v/v) ethanol for 3 min followed by 1 per cent (v/v) sodium hypochlorite for 3 min and 70 per cent (v/v) ethanol for 3min and then washed with sterile double distilled water thrice to remove sterilization solution (Arnold *et al*., 2000). Surface sterilized seeds were cut into two halves and were placed onto Potato Dextrose Agar (PDA), Czapek Dok, Malt extract agar amended with streptomycin for fungal colonies emergence while Nutrient Agar (NA) media, LB agar, Trypticase soy agar for bacterial colonies emergence, respectively (Taylor *et al.,* 1999 and Parthasarathy & Sathiyabama (2014). To check for the effectiveness of sterilization, surface impressions of the seed segments were made on media and incubated at 28 ± 2 °C. The plates were monitored for endophytes growth.

Morphological and Molecular Characterization of Bacterial Endophytes

Purified bacterial isolates were classified into operational taxonomic units (OTUs) based on shape / form, texture, margin, elevation, pigmentation. Voucher numbers were assigned to all the bacterial OTUs and deposited in the library collection at the School of Ecology and Conservation laboratory, University of Agricultural Sciences, GKVK, Bangalore.

For molecular characterization, the genomic DNA of the bacterial endophytes were extracted by alkaline lysis method (Sambrook *et al.,* 1989). The bacteria isolated were cultured individually in NB broth for 24 h at 30 °C. The cells were pelleted by centrifuging at 12,000 rpm for 2 min. The pellet was re-suspended in 650 µl of extraction buffer (100 mM Tris-HCl, 100 mM EDTA, 250 mM NaCl and pH 8.2) and incubated in water bath at 65 °C for 30 min. To the extract, 100 µl of 5 M potassium acetate solution was added and placed on ice for 10 min for precipitation of protein and carbohydrates. The supernatant was collected by centrifuging at 12,000 rpm for 8 min. The DNA was precipitated by adding equal volume of chilled isopropanol for 2 h at -20 °C and then was centrifuged at 12,000 rpm for 5 min. The pellet obtained was washed with 70 per cent chilled ethanol, air dried and dissolved in 20 µl of sterile distilled water. The RNA present in the samples were removed by treating the sample with 3 µl of RNAase at 37 °C for 1h. The DNA was fractioned on 1 per cent (w/v) agarose and visualized following staining with ethidium bromide and quantified using Nano Drop V3.2.1.

The Mysore Journal of Agricultural Sciences

Further, the DNA was amplified using 16S rRNA primers (16SF: 5' GTTAGATCTTGGC TCAGGACGAACGC 3') and (16SR: 5' GATCCAGCCGCACCTTCCGATACG 3'). The PCR amplification was carried out by using Eppendorf Master cycler; the total volume of PCR reaction mixtures was 20 µl containing 2.0 µl of 1X PCR Taq. Buffer with $MgCl_2$ (1.5 mM), 2.0 µl of 10 mM dNTP's mix, 1.0 µl of 16S rRNA primers (forward and reverse, 0.5 µl each), 0.3 µl of Taq DNA Polymerase (1U Genei Bengaluru), 1.0 µl of Template DNA (~50 ng/ µl) and 13.7 µl of sterile distilled water. The PCR conditions used for amplifications were initial denaturation at 94 ºC for 5 min followed by 35 cycles of 94 ºC for 1 min, 58 ºC for 30s, 72 ºC for 1 min, and a final extension at 72 ºC for 10 min. After PCR amplification, PCR products were visualized on 1 per cent agarose gel with 1X TAE buffer. Purification and sequencing of amplified PCR products were done by Sanger sequencing (AgriGenome Labs Pvt. Ltd., Cochin, Kerala). The sequences were queried in the Basic Local Alignment Search Tool (BLAST) in the NCBI GenBank database (www.ncbi.nlm.nih.gov). The sequence with the highest homology and with a maximum query coverage and maximum score was used to ascertain the identity of the endophytic bacteria. A phylogenetic tree was constructed for each of the isolates following MEGA version X.

Enrichment of Pre-germinated Seeds with Bacterial Endophytes

Surface sterilized seeds were allowed to pre-germinate (protruding of shoot and root) and uniformly pre-germinated seeds were taken, and treated with 5 ml (10^6 - 10^8 cells/ml) of *Paenibacillus quercus* (EC1) and *Bacillus cereus* (EC2) and consortium of two bacterial endophytes for 3 h. Another set of uniformly pre-germinated seeds were treated with sterile water which served as a control. Both, endophyte enriched and non-enriched pre-germinated seeds were transferred to germination paper. A total of 30 seedlings were used in each treatment, with three replications (each with 10 seedlings). Seedling growth rate was assessed every other day of treatment till 7th day.

Standardization of Antibiotic Concentration

An antibiotic, streptomycin sulphate was used to assess the role of bacterial endophytes in seedling growth and development. Surface sterilized seeds were treated with different concentration of streptomycin sulphate (10, 25 and 50 µg/ml) for 24 h with constant shaking at 120 rpm. Seeds soaked in autoclaved water served as a control. To check the complete disinfection / removal of native bacterial endophytes, streptomycin treated seeds were plotted on nutrient agar plate and observed for 5 days. No bacterial growth around seeds confirmed the complete disinfection.

After 24 h of treatment, seeds were washed thrice with sterile distilled water to remove the traces of streptomycin sulphate. Treated and control seeds were placed on plastic pots (4 inch) containing coir peat and allowed for germination and growth at room temperature for 10 days. After 10 days of treatment seedling height (shoot and root length) was measured. The experiments were conducted in triplicates with 25 seedlings for each replication.

Statistical Analysis

All experiments were conducted with a minimum of three replications. One way analysis of variance (ANOVA) was performed to statistically validate the results obtained. The means of various treatments were compared using a Duncan's new multiple range test (MRT) test at 95 per cent confidence interval. All statistical analysis was done in MS-Excel and SPSS software.

Results and Discussion

Isolation and Identification of Endophytes from Seeds of Finger Millet

Surface sterilized finger millet seeds were cut into two halves and inoculated onto six different media (Potato Dextrose Agar, Czapek Dok, Malt extract agar amended with streptomycin sulphate for fungal and Nutrient Agar media, LB agar, Trypticase soy agar for bacterial colonies emergence, respectively). Surprisingly, finger millet seeds did not yield any

fungal colonies. These results are intriguing and are indeed supported by an independent study by Mousa *et al.* (2015) on finger millet. The exact reasons for the absence of culturable-fungal endophytes in the seed are not clear and at best can only be conjectured. Among them are a) some structural constraints offered by the seed coat for entry of fungal mycelia into intercellular spaces of seed coat tissue. Indeed, McDonough and Rooney (1986) reviewed the structure of seeds of major millets. He suggested that finger (*Eleusine coracana* (L.) Gaertn.), proso [*Panicum miliaceum* (L.)] and foxtail (*Setaria italica* (L.) P. Beauv.) millets are utricles. In an utricle, the seed is covered by the membranous pericarp, which is not fused with seed coat. The entire utricle is further enclosed by chaff-like bracts called lemma and palea and b) inhibition of fungal entry and growth by endophytic bacteria resident in the seed coat. Further studies would be required to unravel these possibilities.

However, bacterial colonies were obtained from most of the seeds. A total of two bacterial isolates were morphologically grouped as EC 1 and EC 2. Further these bacterial isolates were molecularly identified using 16S rRNA sequencing as *Paenibacillus quercus* (EC 1) and *Bacillus cereus* (EC 2) (Table 1). Phylogenetic relationship of the sequences to closest matches in public database, based on 16S rRNA gene sequences was constructed by using neighbour-joining method in MEGA version X environment. The reliability of the relationships of lineages on the inferred trees was tested by bootstrap analysis for 1000 replicates (Fig. 1). Genera *Paenibacillus* and *Bacillus* belongs to the phylum, firmicutes. Recent past studies showed that *Paenibacillus* and *Bacillus* are the most common

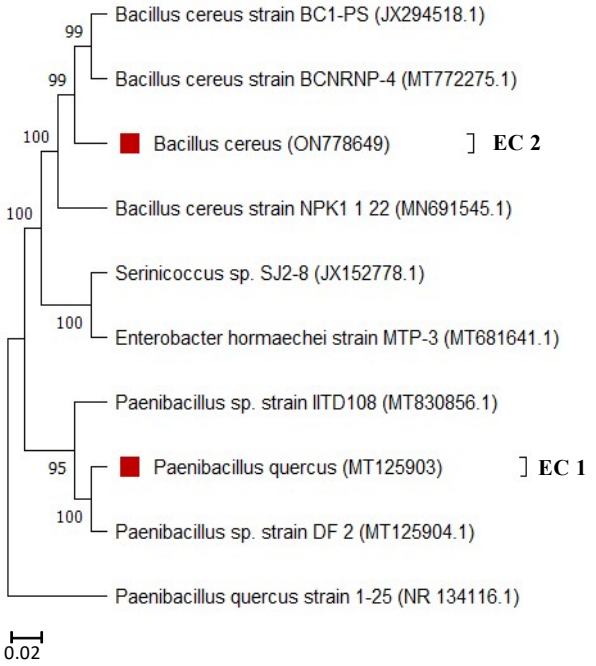

Fig. 1: Neighbor-joining phylogenetic tree showing the relationships of the bacterial isolates from seeds of finger millets. Support bootstrap values are shown in the branches. Scale bar indicates 0.02 substitutions per nucleotide position

seed associated endophytes (Prasannakumar *et al.,* 2020 and Kumar *et al.,* 2020).

Effect of Seed Bacterial Endophytes on Finger Millet early Seedling Growth and Development

To check the growth promotion ability of seed bacterial endophytes, uniformly pre-germinated finger millet seeds were treated with 5ml of endophytic bacterial suspension and on the other hand, seeds treated with water served as a control. Endophytes enriched seeds showed relatively higher shoot and root length compared to the control at every stage of development (Fig. 2). However, seeds enriched with

TABLE 1

Molecular identification of seed bacterial endophytes of finger millet using 16S rRNA

Plant species	Bacterial isolate	Sequence Length (bp)	Best Blast search	Query cover (%)	Identity (%)	Gen Bank accession number
E. coracana (GPU28)	EC 1	879	*Paenibacillus quercus*	100	95.80	MT125903
	EC 2	944	*Bacillus cereus*	100	100	ON778649

The Mysore Journal of Agricultural Sciences

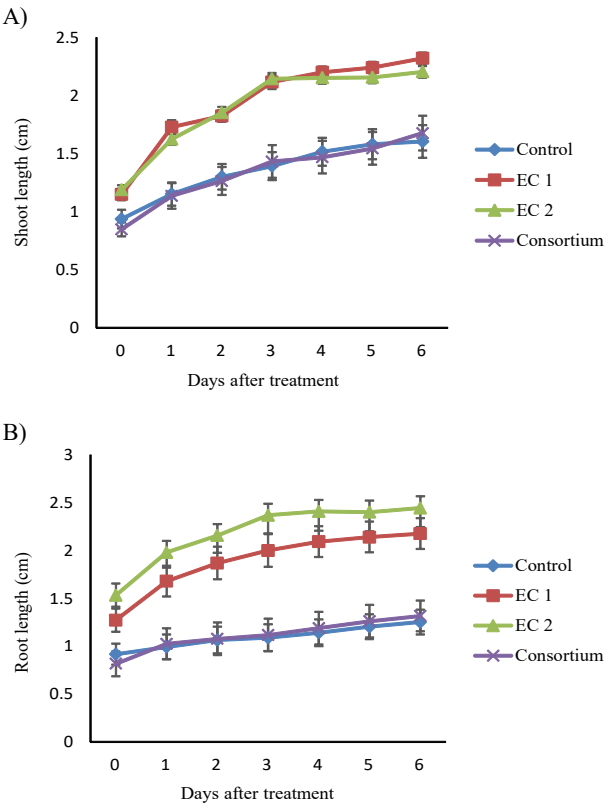

Fig. 2: Effect of bacterial endophytes enrichment in the early seedling growth and development of finger millet. A) shoot and B) root length of finger millet seedling days after treatment. Values represents mean ± SE. (EC1 - *Paenibacillus quercus*, EC2 - *Bacillus cereus* and consortium -EC1 + EC2)

consortium of EC 1 and EC 2 showed shoot and root length on par with control (Fig. 2 and Fig. 3). *Paenibacillus* sp. (EC 1) and *Bacillus* sp. (EC 2) are the most common cultivable bacterial genus and have been frequently recorded as seed associated microbes and they also help in the seedling establishment (Kumar *et al.,* 2020)

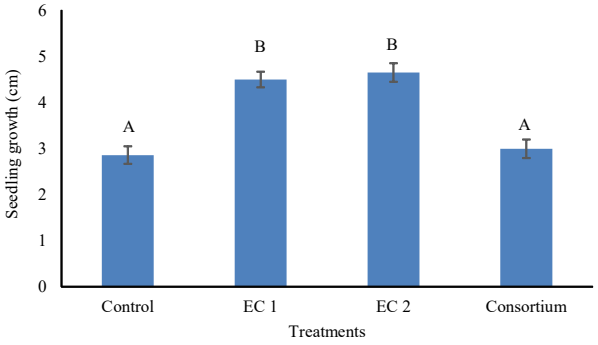

Fig. 3: Growth rate of finger millet seedling after seven days of treatment. Values represents mean ± SE and different letters indicate significant differences (P < 0.05) based on Duncan's test. (EC1 - *Paenibacillus quercus*, EC2 - *Bacillus cereus* and consortium -EC1 + EC2)

Standardization of Antibiotic Concentration

One of the challenges in analysing the role of microbiome in seedling growth is to obtain seeds that are free of microbiome. Unfortunately, most methods of culture, save aseptic culture of plants, do not preclude microbiome contaminations from the ambient environment. Under these circumstances, use of an antibiotics could help cleanse the tissue of the microbiome. In this study, an antibiotic, streptomycin sulphate was used to cleanse the seed tissues of their bacterial endophytes. Streptomycin sulphate (an amino glycoside antibiotic), a protein synthesis inhibitor, binds to 30S subunit of the bacterial ribosome and interferes with the binding of formyl-methionyl-tRNA to the 30S subunit (Zhu *et al.,* 2001).

To comply to this requirement, an experiment was conducted to evaluate and arrive at an appropriate concentration of streptomycin that could be used to treat the finger millet seeds to cleanse them of the bacterial endophytes. Surface sterilized seeds of finger millet were incubated at different concentrations of streptomycin sulphate (10, 25 and

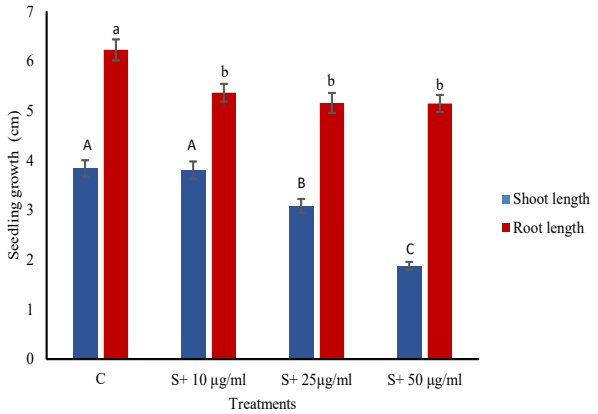

Fig. 4: Shoot & root length of ragi seedling after treating seeds with different concentration of antibiotics for an 24hrs. Values represents mean ± SE and different letters indicate significant differences (P < 0.05) based on Duncan's test. (S^+ indicate streptomycin sulphate).

50 µg/ml) for 24 h. The seeds were allowed to grow and their root and shoot lengths (cm) were measured after ten days.

There was a significant difference in the shoot and root length of 50 µg/ml streptomycin sulphate treated seedlings compared to the control (Fig. 4). We found that seed treatment with 50 µg/ml concentration of streptomycin sulphate for 24 h is enough to remove bacterial endophytes, since no bacterial colonies were emerged on Nutrient agar media (Fig. 5). We also observed that seedlings developed from antibiotic treated seedlings were found weak compared to the control as visible in (Fig. 6). Further, the frequency distribution of shoot and root length of seeds treated with S^+ 50 µg/ml was skewed to shorter length compared to the control (Fig. 7). A recent study by Verma and White (2018) also showed that elimination of bacteria from seeds compromises seedling growth and development.

The present study demonstrates that finger millet seeds harbour functionally important endophytic bacteria, which are crucial for early seedling establishment. This study suggests that the microbiome associated with seeds can be used as a bio-inoculant for sustainable agriculture, but it has to be tested in field conditions. Further research has to

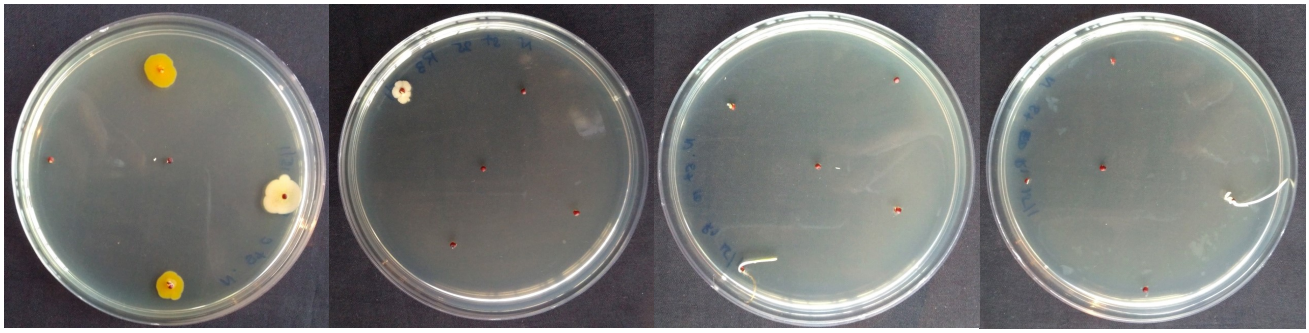

Fig. 5: Absence of bacterial endophytes in the seeds treated with S^+ 50µg/ml. (S^+ indicate streptomycin sulphate).

Fig. 6: Effect of antibiotic (S^+ indicate streptomycin sulphate) treatment on seedling growth at 10 days

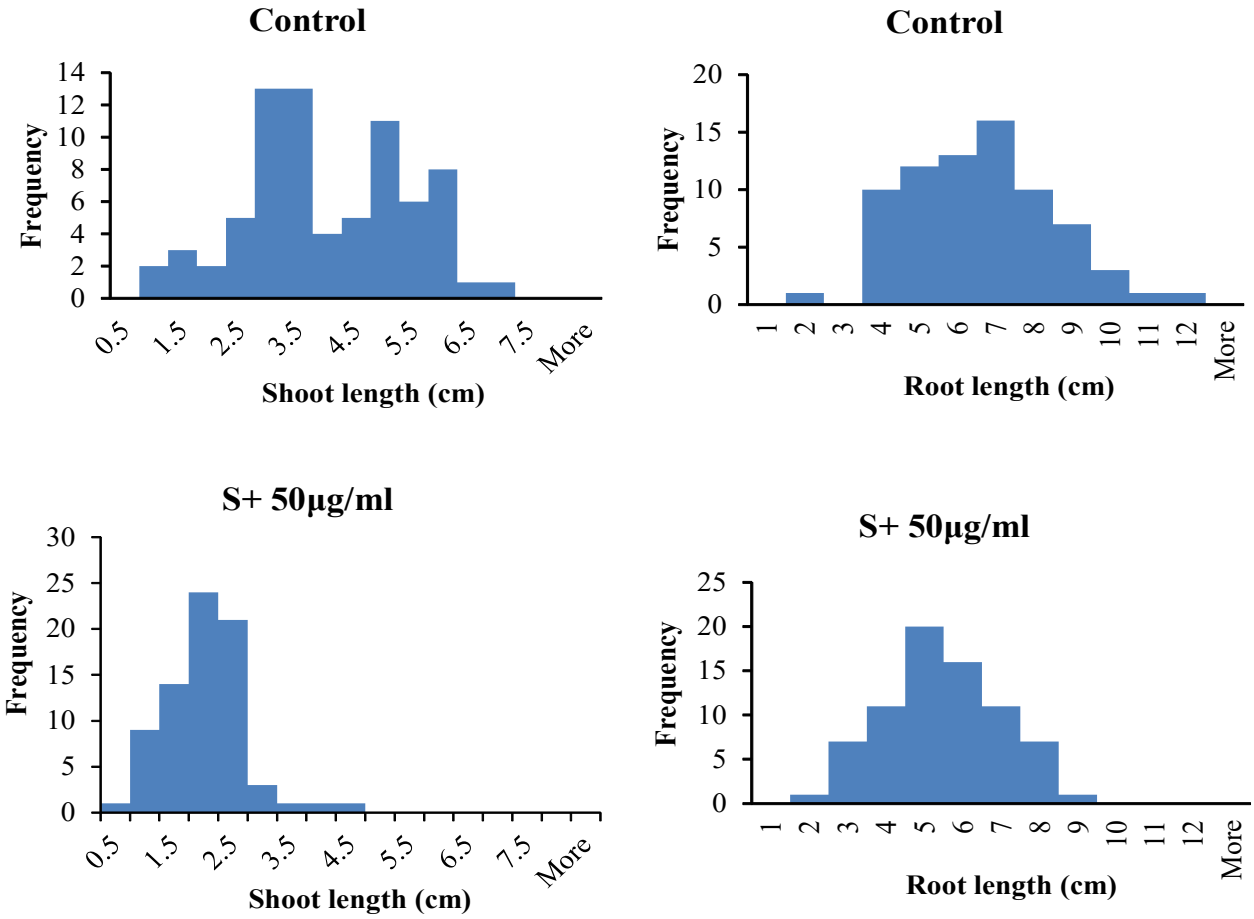

Fig. 7: Frequency distribution of shoot and root length of 10 days old seedlings raised from control and streptomycin sulphate (50µg/ml) treated seeds

be done to find out the possible mechanisms by which endophytes help seedling growth and development.

Acknowledgments : The authors thank the Indian Council of Agricultural Research (ICAR) - Centre for Advanced Agricultural Science and Technology (CAAST), Activity1c - 'Next generation technologies for microbiome enabled seed priming' (ICAR_ NAHEP; F. No./NAHEP/CAAST/2018–19; AB/ AC7703) for support. M.H. would like to thank the Council of Scientific and Industrial Research (CSIR), Government of India for providing research fellowship.

References

Adam, E., Bernhart, M., Muller, H., Winkler, J. and Berg, G., 2018, The Cucurbita pepo seed microbiome: genotype-specific composition and implications for breeding. *Plant and Soil*, https://doi.org/10.1007/s11104-016- 3113-9.

Arnold, A. E., Maynard, Z., Gilbert, G. S., Coley, P. D. and Kursar, T. A., 2000, Are tropical fungal endophytes hyper diverse? *Ecological Letters*, **3** (4) : 267 - 274.

Barret, M., Guimbaud, J. F., Darrasse, A. and Jacques, M. A., 2016, Plant microbiota affects seed transmission of phytopathogenic microorganisms. *Molecular Plant Pathology*, **17** (6) : 791 - 795.

Berendsen, R. L., Pieterse, C. M. and Bakker, P. A., 2012, The rhizosphere microbiome and plant health. *Trends in Plant Science*, **17** (8) : 478 - 486.

GOGNA, R., SHEE, K. AND MORENO, E., 2015, Cell competition during growth and regeneration. *Annual Review of Genetics*, **49** : 697 - 718.

HERRERA, S. D., GROSSI, C., ZAWOZNIK, M. AND GROPPA, M. D., 2016, Wheat seeds harbour bacterial endophytes with potential as plant growth promoters and biocontrol agents of Fusarium graminearum. *Microbiological Research*, **186** : 37 - 43.

JOHNSTON-MONJE, D. AND RAIZADA, M.N., 2011, Conservation and Diversity of Seed Associated Endophytes in *Zea* across Boundaries of Evolution, Ethnography and Ecology. *Plos one*, **6** (6) : e20396.

KUMAR, K., PAL, G., VERMA, A. AND VERMA, S. K., 2020, Seed inhabiting bacterial endophytes of finger millet (*Eleusine coracana* L.) promote seedling growth and development and protect from fungal disease. *South African journal of botany*, **134** : 91 - 98.

KUMAR, K., VERMA, A., PAL, G., WHITE, J. F. AND VERMA, S. K., 2021. Seed Endophytic Bacteria of Pearl Millet (*Pennisetum glaucum* L.) Promote Seedling Development and Defend Against a Fungal Phytopathogen. *Frontiers in microbiology*, **12** : 774293.

MANASA, K. M., RAVIKANTH, G., NATARAJA, K. N. AND UMASHAANKER, R., 2015, Isolation and characterization of endophytic fungi from saline habitat adapted plants. *Mysore J. Agric. Sci.*, **49** (2) : 299 - 301.

MCDONOUGH, C. M., ROONEY, L. W. AND EARP, C. F., 1986, Structural characteristics of *Eleusine corocana* (finger millet) using scanning electron and fluorescence microscopy. *Food Structure*, **5** (2) : 9.

MOUSA, W. K., SHEARER, C.R., LIMAY-RIOS, V., ZHOU, T. AND RAIZADA, M. N., 2015, Bacterial endophytes from wild maize suppress Fusarium graminearum in modern maize and inhibit mycotoxin accumulation. *Frontiers in Plant Science*, **6** : 805.

PARTHASARATHY, R. AND SATHIYABAMA, M., 2014, Gymnemagenin-producing endophytic fungus isolated from a medicinal plant Gymnema sylvestre R.Br. *Appl Biochem Biotechnol*, **172** : 3141 - 3152.

PRASANNAKUMAR, M. K., MAHESH, H. B., DESAI, R. U., KUNDURU, B., NARAYAN, K. S., TELI, K., PUNEETH, M. E., RAJADURAI, R. C., PARIVALLAL, B. AND BABU, G. V., 2020, Metagenome sequencing of finger millet-associated microbial consortia provides insights into structural and functional diversity of endophytes. *3 Biotech*, **10** (1) : 1 - 17.

SAMBROOK, J., FRITSCH, F. F. AND MANIATIS, T., 1989, Molecular cloning: A laboratory manual, Cold Spring Harbor Laboratory Press, New York, pp. : 931 - 957.

SHADE, A., JACQUES, M. A. AND BARRET, M., 2017, Ecological patterns of seed microbiome diversity, transmission and assembly. *Current Opinion in Microbiology*, **37** : 15 - 22.

TASMIYA IMTIYAZ AND EARANNA, N., 2021, Characterization of bacterial endophyte imparting drought tolerance in Rice (*Oryza sativa* L.). *Mysore J. Agric. Sci.*, **55** (4) : 364 - 370.

TAYLOR, J. E., HYDE, K. D. AND JONES, E. B. G., 1999, Endophytic fungi associated with the temperate palm, Trachycarpus fortunei, within and outside its natural geographic range. *New Phytol*, **142** : 335 - 346.

VERMA, S. K. AND WHITE, J. F., 2018, Indigenous endophytic seed bacteria promote seedling development and defend against fungal disease in browntop millet (*Urochloa ramosa* L.). *Journal of applied microbiology*, **124** (3) : 764 - 778.

VERMA, S. K., KINGSLEY, K., BERGEN, M., ENGLISH, C., ELMORE, M., KHARWAR, R. N. AND WHITE, J. F., 2018. Bacterial endophytes from rice cut grass (*Leersia oryzoides* L.) increase growth, promote root gravitropic response, stimulate root hair formation and protect rice seedlings from disease. *Plant and Soil*, **422** (1) : 223 - 238.

ZHU, M., BURMAN, W. J., JARESKO, G. S., BERNING, S. E., JELLIFFE, R. W. AND PELOQUIN, C. A., 2001, Population pharmacokinetics of intravenous and intramuscular streptomycin in patients with tuberculosis. *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy*, **21** (9) : 1037 - 1045.