

Influence of Endophytic Bacterium, *Cellulosimicrobium* sp. FRR2 on Plant Growth of *Amaranthus campestris* L. and Bacterial Survival at Adverse Environmental Conditions

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Abstract

The endophytic microorganisms are believed to be an important bio-resource for modern agriculture because of their beneficial effects on plant growth promotion, biocontrol, stress tolerance, and diseases resistance. This study was focused to know the beneficial effect of endophytic bacterium (FRR2) isolated from the roots of *Ficus religiosa* L. on *Amaranthus campestris* L. and their tolerance ability against salinity and heavy metals. The strain FRR2 was recognized as *Cellulosimicrobium* sp. by 16s rRNA sequencing and phylogenetic study. The bacterial isolate FRR2 showed salt (at 150 mM NaCl) and metal (at 150 μ M CuSO₄ and 100 μ M ZnSO₄) tolerance ability and significantly higher growth rate of *Amaranthus campestris* in a green leafy vegetable might be due to the nitrogen fixation, indole acetic acid production, amylase and protease activities. In addition, the endophyte FRR2 application slightly increased the antioxidants activity than their controls. The results of this study revealed that *Cellulosimicrobium* sp. strain FRR2 would be an effective endophyte to increase the growth of green leafy vegetables.

Keywords: *Amaranthus campestris*, *Cellulosimicrobium* sp., Endophytes, Heavy metal, Salinity

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INTRODUCTION

Endophytes are a great example of an endosymbiotic group of microorganisms that colonize plants and microbes¹ and protect the plants from environmental stresses. They are widespread in plant tissues as they can resist host competition. The endophyte population is dependent upon various factors including host developmental stage, inoculum density, and environmental conditions.² The presence of endophytes in various parts of plants helps to enhance the growth of plants, resistance against plant pathogens, and other adverse environmental conditions. Certain endophytes also function as diazotrophic by providing fixed nitrogen to the host plant. As they are found pervasive, can be isolated from monocots, dicots ranging from higher trees to even.³

Most all plants contain a higher number of endophytes in roots compared with other tissues.⁴ In some cases of polluted soils, endophytes produce auxins for promoting plant growth, and even in some cases gibberellins and cytokines are produced.⁵ In this study, FRR2 endophytic bacterial strain was isolated *F. religiosa* L. commonly known as Peepal which is the oldest tree mentioned in Indian Literature.⁶ *F. religiosa* L is found to have medicinal properties like antibacterial, antidiabetic, anti-amnesia, antiulcer, and antioxidant properties in the presence of chemical compounds.⁷ Nowadays many researchers are using them for novel drugs designing and production.

This study was aimed to evaluate the endophyte isolated from *Ficus* and *Cardiospermum* roots on their plant growth-promoting activity of *A. campestris* and their survival levels at different abiotic stress conditions where *Amaranthus campestris* is a leafy vegetable containing a rich amount of antioxidants, vitamins, and minerals⁸ and active constituents like alkaloids, flavonoids, glycosides, steroids, glycosides, carotenoids, and saponins.⁹

MATERIALS AND METHODS

Isolation of endophytic bacteria

The fresh and healthy roots were collected from the medicinal plant, *F. religiosa* L. and *Cardiospermum halicabum* L. The collected samples were surface sterilized and crushed in

sterile distilled water using a sterile mortar and pestle. The root extracts were collected in sterile tubes and 0.1ml of the extract was inoculated on a sterile tryptic soy agar plate and incubated for 3 days at 27±2°C. The different bacterial colonies were identified by color and morphological variations.

Screening of plant growth-promoting activity of endophytes

One hundred seeds of *Amaranthus campestris* L. were surface sterilized with 0.1 % mercuric chloride and 70% ethanol. The sterilized seeds were carefully shifted to 2 ml culture tubes containing the bacterial isolates (FRR1, FRR2, and CHR3) and were allowed to soak for 3 h. The bacterial treated seeds were taken from the culture tube by draining the medium and shifted to petri plates containing sterilized cotton and tissue paper. The petri plates were kept in a dark place at 28 ± 2°C and relative humidity 60–70 % for 3 days in the culture room and sterile water was sprayed on plates to keep moisture level periodically. The germination of endophytes treated seeds was compared with control (without endophytes treated seeds) to check their plant growth promotion activity.

Screening of endophytic bacteria for the production of hydrolytic enzyme

For amylase activity, the endophytes were placed on the starch agar medium and incubated at 28 °C for 24 hours. After the incubation period, the gram's iodine was added to check the formation of clear halo zones around the bacterial colony.¹⁰ The activity of protease was determined by casein degradation on skim milk agar medium. The bacterial isolates were inoculated and incubated at 28°C for 24 hours and then the clear zone of casein hydrolysis was observed.¹¹

Identification of Endophytic bacterium

The potential plant growth-promoting bacterial isolate (FRR2) was isolated from *F. religiosa* roots and identified based on a partial 16S rRNA sequence. For PCR amplification, 27F primer 5' (AGA GTT TGA TCM TGG CTC AG) 3' and 1492R primer 5' (TAC GGY TAC CTT GTT ACG ACT T) 3' were used.¹¹ To find the nucleotide sequence homology of this bacterial isolate, an online search BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>) was used. The construction of a phylogenetic tree from closely related sequences

was helped to find out the name of the bacterial isolate FRR2

Plant growth-promoting metabolites production from endophytes

For indole acetic acid analysis, the bacterial isolate was inoculated into Luria -Bertani medium supplemented with 1mg/ml L-tryptophan and incubated at 30°C for 48 h. Bacterial culture was mixed with 2 ml of salkowaski’s reagent and kept for 20 min at 37°C under dark conditions. The production of IAA was determined by the presence of pink color in the tubes.¹² To test the ammonia production, the freshly grown bacterial isolate was inoculated into the peptone water and incubated for 72 h at 26 ± 2°C. Nessler’s reagent (0.5ml) was added to the tubes. Development of brown to yellow color was indicated as a positive test for ammonia production.⁸ The nitrogen fixation was performed qualitatively by growing the bacterial isolates on solid Norris glucose medium (N-free

medium) containing 1% Bromothymol blue as a pH indicator and incubated at 37°C for 24 h.¹³

Stress tolerance study

The bacterial isolate was inoculated on a tryptic soy agar medium containing NaCl (100, 120, and 150 mM), CuSO₄ (50, 100, and 150 µM), and ZnSO₄ (50, 100, and 150 µM). The plates were incubated at 37°C for 3 days. Following the incubation period, the diameter of the bacterial colony was measured.¹⁴

DPPH free radical scavenging assay

The dried and powdered sprouts (control and bacterial treated) were mixed with methanol. At dark conditions, this mixture of methanol extract was allowed to react with diphenyl-1 picryl hydrazyl hydrate (DPPH) for 30 min. The absorbance was measured at 517nm.¹⁵

DPPH Scavenging (%) was calculated as following DPPH Scavenged (%) = (A control-A treated)/A control x100.

Table 1. Effect of endophytes on *A. campestris* L seed germination. The experiment was repeated three times

No.	Bacterial isolates	Total No. of seeds	No. of seeds germinated	Seed germination (%)
1.	Control	100	43 ± 1	43%
2.	FRR1	100	72 ± 1	72%
3.	FRR2	100	73 ± 3	73.3%
4.	CHR3	100	55 ± 3	55.5%

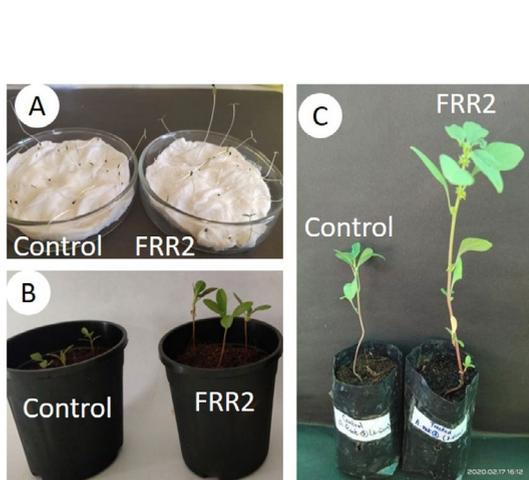


Fig. 1. Effect of endophyte (FRR2) on *A. campestris* L seed germination (A) in tissue paper placed on petri plates, seedling (B) growth at pots, and plant growth (C) in plastic bags.

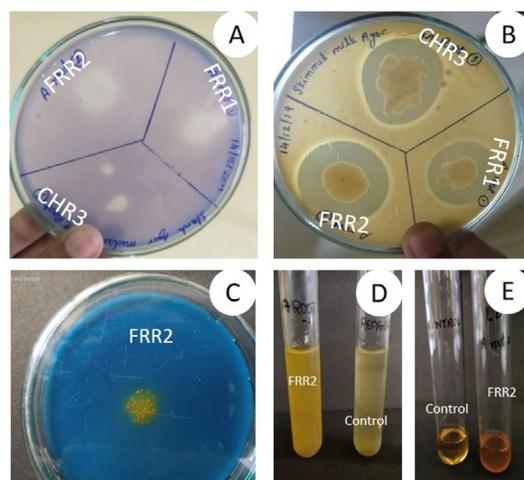


Fig. 2. Production of amylase (A), protease (B) by endophytes (FRR1, FRR2, and CHR3) and nitrogen fixation (C), ammonia (D), and Indole acetic acid (E) of FRR2.

(A control -the absorbance of the control reaction and A Treated- the absorbance in the presence of the sample of the extracts).

A. *campestris* L plant growth at shed net condition

The bacterial isolate (FRR2) was mixed to the mixture of soil and coir pith (1:1). The seeds of *A. campestris* L were surface sterilized and sowed in pots containing bacteria treated and non-treated (control) soil at shed net conditions (under natural light; 60-70 % relative humidity; ~18° C to 28° C temperature) with periodic irrigation. The height of the plant was compared between control and bacterial treatment.

Statistical analysis

The salt tolerance study, DPPH free radical scavenging assay, and plant growth experiments were repeated three times and standard error was calculated from the obtained values by using Sigma Pot software (10).

RESULTS

Characters of endophytes favor planting growth

Plant growth-promoting activity of three different endophytes (FRR1, FRR2, and CHR3) was tested on *Amaranthus campestris* L seeds. After 10 days, the plates were examined for germination of seeds. The endophytic bacterial isolate FRR2 treatment showed a higher percentage of seed germination in comparison with control and other endophytes (Table 1 and Fig. 1A).

The metabolites including amylase, protease, IAA, ammonia, and nitrogen fixation were analyzed in the endophytic cultures. FRR1 and CHR3 produced only amylase and protease. Among the three endophytes, the production of amylase, protease, indole acetic acid, ammonia, and nitrogen fixation was higher in FRR2 than in other cultures (Table 2 and Fig. 2).

Identification of endophytic bacterial isolate FRR2

We isolated two endophytic bacteria (FRR1 and FRR2) from *Ficus religiosa* L and an endophytic bacteria (CHR3) from *Cardiospermum halicabum* L. FRR2 showed potential plant growth-promoting activity than other endophytes so it was subjected to taxonomic identification by 16S RNA sequencing. The bacterial isolate, FRR2 was identified as *Cellulosimicrobium* sp. by phylogenetic analysis based on the endophytic bacterial sequence of 16s rRNA. The accession

no. MZ497513 was given for the bacterial strain of FRR2 from the NCBI database.

Stress tolerance study

The salt and heavy metal tolerance assay were performed by inoculating the endophytic bacterial strain FRR2 in NaCl (100, 120, and 200 mM), CuSO₄ (50, 100, and 150 μM), and ZnSO₄ (50, 100, and 150 μM). After incubation, the colony diameter was measured in order to check its ability for salt tolerance and the values were plotted in a graph (Fig. 3). Except for the high concentration of ZnSO₄ (150 mM), FRR2 endophyte was survived and grown as normal (control).

Effect of FRR2 endophytes on *A. campestris* L growth

The endophyte (FRR2) treated and non-treated *A. campestris* L seeds were shown in the ten pots separately and observed the changes (Fig. 1B and C). A higher rate of seedling and

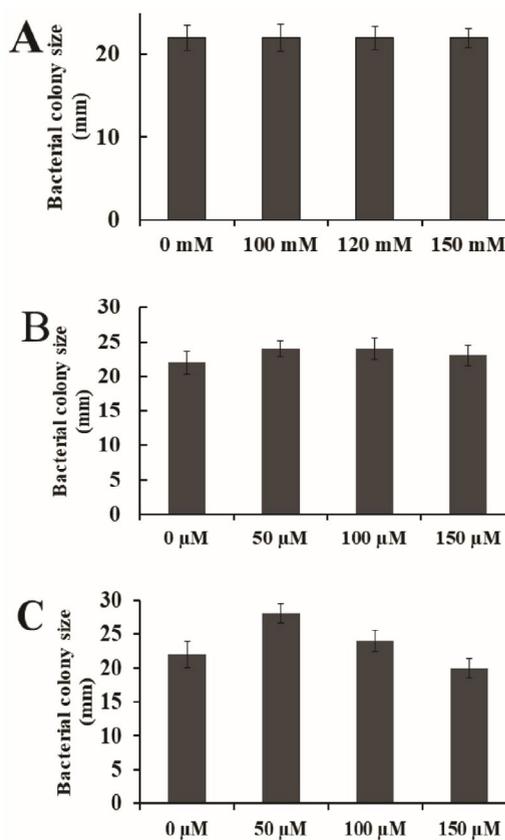


Fig. 3. Effect of NaCl (A), CuSO₄ (B), and ZnSO₄ (C) on the growth of FRR2 endophyte at 2 days old culture.

plant growth was noticed in seeds treated with endophyte (FRR2).

DPPH free radical scavenging assay

The methanolic extract of endophytic bacterial strain FRR2 treated plants showed high antioxidant activity when compared with their control (bacteria un-treated plants) (Fig. 4).

DISCUSSION

Several bacteria resident the plant parts and give support to their host plant growth and survival. In the present study, an endophytic bacterium *Cellulosimicrobium* sp. was identified as a potential beneficial agent to increase *A. campestris* L. growth. It is a gram-positive and aerobic bacterium that can improve plant nutrients due to the exudation of metabolites for plant growth. The green leafy vegetable (*A. campestris* L.) is more important for human and animal nutrients. Radhakrishnan and Lee¹¹ reported that the application of the endophytic bacterium

Bacillus methylotrophicus KE2 promoted the growth of lettuce and improved the nutritional values. Similarly, in the current study, endophytic bacterial treatment exhibited a greater amount of seed germination, seedling, and plant growth than their controls due to nitrogen fixation, production of IAA, and ammonia. IAA is produced by some microbes and involves the development of roots and shoots. Endophytic bacteria can fix the atmospheric nitrogen and make it available to the plants. Ammonia is a vital source of nitrogen to plants.

Nitrogen is essential for plant growth including photosynthesis. A current study found that the endophytic bacteria yield ammonia and it may help plants. Several studies revealed that secretion of IAA, ammonia, and nitrogen fixation from the bacteria was significantly promoted plant growth.^{16,17} A previous study revealed that *Acinetobacter calcoaceticus* SE370 produced malic, citric, succinic, and gibberellic acids and

Table 2. Endophytic bacterial secretions for plant growth promotion

No.	Bacterial isolate	Amylase production	Protease production	Indole acetic acid production	Ammonia production	Nitrogen fixation
1.	FRR1	++	++	-	-	-
2.	FRR2	+++	++++	+	+	++
3.	CHR3	+	+++	-	-	-

X > 10 mm = + ; 10 mm to 14 mm = ++ ; 15 mm to 20 mm = +++ ; < 20 mm = ++++

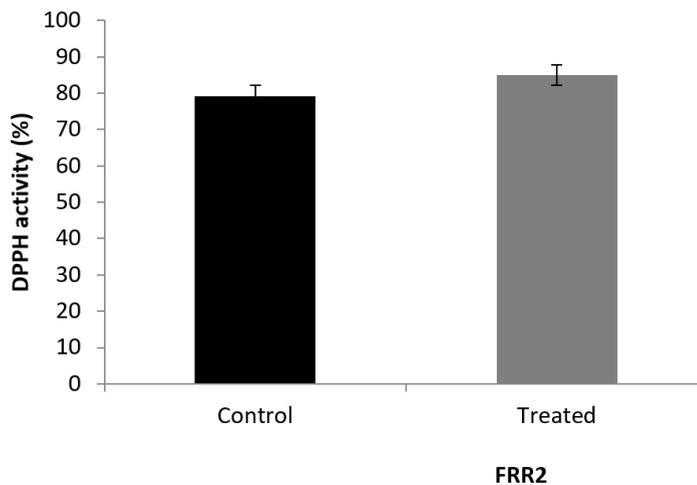


Fig. 4. Effect of FRR2 endophyte on DPPH activity of *A. campestris* L plant. Control – Endophytes untreated plants; Treated – Endophytes treated plants.

promoted the Chinese cabbage plant growth.¹⁸ The secretion of amylase and protease activities of endophytes also support the growth of plants. Plant tissues store starch as a food source. In bacteria, amylase is an enzyme responsible for converting starch into sugar and easily it makes available to plants. Protease enzymes can degrade the proteins into peptide bonds and then into amino acids and it makes them available to plants. Endophytic bacterium *B. subtilis* EGY16 produced cellulose, chitinolytic, and protease and enhanced plant growth.¹⁹ Similarly, Liu et al.²⁰ observed the protease and cellulose activity of endophytes were improved the phosphate solubilization in *F. songorica* L. Antioxidants are important in preventing various diseases in human beings. The food containing more amount of antioxidants is high priced in the market. The antioxidants studies were conducted in several Indian green leafy vegetables to identify the important plant species for human health.²¹ In addition, the higher level of antioxidants (DPPH activity) in *Cellulosimicrobium* sp. FRR2 associated plants is evidence of endophytic treatment will increase the antioxidants in plants. In our previous study showed that *Bacillus amyloliquefaciens* subsp. *plantarum* GR53 increased the antioxidants in Chinese cabbage and reduced the Rhizoctonia disease effects.²²

Plant growth-promoting bacteria survive in the different salinity and heavy metal accumulated soil which is supporting plant growth. In the current study, *Cellulosimicrobium* sp. FRR2 increased the plant growth of *A. campestris* L and survived in high salinity (150 mM NaCl), heavy metals (100 µM of Cu and Zn). Several bacteria present in the rhizosphere of plant tissues have the capacity to survive against salinity and mitigate the salinity effects in plants.²³ A previous study revealed that *Enterobacter asburiae* KE17 tolerates the Cu and Zn stress up to 150 µM and their association mitigated the soybean plant growth against heavy metal stresses.¹⁴

CONCLUSION

This study revealed that the treatment of endophytic bacteria *Cellulosimicrobium* sp. FRR2 on spinach (*Amaranthus campestris* L.) would be helpful to promote plant growth and to sustain adverse environmental conditions. Further study

will be focused on field application and production of biofertilizers.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

SS, RR, and SP designed and proceeded with the experiment. RS, MA, and GP reviewed the manuscript.

FUNDING

None.

DATA AVAILABILITY

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

Not applicable.

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