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

S. Sandhya¹, Ramalingam Radhakrishnan¹,²*, Ramaraj Sathasivam³, Muthukrishnan Arun⁴, Gurusaravanan Packiaraj⁵ and Sang Un Park³,⁶*

¹Department of Microbiology, Karpagam Academy of Higher Education, Coimbatore - 641 021, Tamil Nadu, India.
²Department of Botany, Jamal Mohamed College (Autonomous), Affiliated to Bharathidasan University, Tiruchirappalli - 620 020, Tamil Nadu, India.
³Department of Crop Science, Chungnam National University, 99 Daehak-ro, Yuseong-gu, Daejeon 34134, Republic of Korea.
⁴Department of Biotechnology, Bharathiar University, Coimbatore - 641 046, Tamil Nadu, India.
⁵Department of Botany, Bharathiar University, Coimbatore - 641 046, Tamil Nadu, India.
⁶Department of Smart Agriculture Systems, Chungnam National University, 99 Daehak-ro, Yuseong-gu, Daejeon 34134, Republic of Korea.

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 µM CuSO₄ and 100 µM 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

*Correspondence: rkbot@jmc.edu; supark@cnu.ac.kr  (Received: July 08, 2021; accepted: October 04, 2021)
INTRODUCTION

Endophytes are a great example of an endosymbiotic group of microorganisms that colonize plants and microbes 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, inoculums density, and environmental conditions. 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.

Most all plants contain a higher number of endophytes in roots compared with other tissues. In some cases of polluted soils, endophytes produce auxins for promoting plant growth, and even in some cases gibberellins and cytokines are produced. In this study, FRR2 endophytic bacterial strain was isolated from Ficus and Cardiospermum roots.

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. 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.

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. 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 1 mg/ml L-tryptophan and incubated at 30°C for 48 h. Bacterial culture was mixed with 2 ml of salkowski’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. 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 (0.5 ml) was added to the tubes. Development of brown to yellow color was indicated as a positive test for ammonia production. 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.

**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.

**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 517 nm. DPPH Scavenging (%) was calculated as following DPPH Scavenged (%) = (A control - A treated)/A control x 100.

### 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
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 Lee\(^\text{11}\) 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.\(^\text{16,17}\) A previous study revealed that *Acinetobacter calcoaceticus* SE370 produced malic, citric, succinic, and gibberellic acids and

| 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 = ++++

**Table 2. Endophytic bacterial secretions for plant growth promotion**

![Fig. 4. Effect of FRR2 endophyte on DPPH activity of *A. campestris* L plant. Control – Endophytes untreated plants; Treated – Endophytes treated plants.](image-url)
promoted the Chinese cabbage plant growth. 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. Similarly, Liu et al. observed the protease and cellulose activity of endophytes were improved the phosphate solubilization in *F. songorica*. 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. 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.

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. 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.

**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.

**ACKNOWLEDGMENTS**

The authors thank Jamal Mohamed College (Autonomous), Tiruchirappalli, India, and Karpagam Academy of Higher Education, Coimbatore, India for encouraging research through seed money projects. The authors thank the Department of Biotechnology (DBT), Department of Science and Technology (DST) under the Government of India for providing facilities to carry out the research.

**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.

**REFERENCES**

1. Gouda S, Das G, Sen SK, Shin HS, Patra JK. Endophytes: A treasure house of bioactive compounds of medicinal importance. *Front Microbiol.* 2016;7:1538. doi: 10.3389/fmicb.2016.01538
2. Dudeja SS, Giri R. Beneficial properties, colonization, establishment and molecular diversity of endophytic bacteria in legumes and non legumes. *African J Microbiol Res.* 2014;8(15):1562-1572. doi: 10.5897/AJMR2013.6541
3. Luo S-L, Chen L, Chen J-L, et al. Analysis and characterization of cultivable heavy metal-resistant bacterial endophytes isolated from Cd-hyperaccumulator *Solanum nigrum* L. and their potential use for phytoremediation. *Chemosphere.* 2011;85(7):1130-1138. doi: 10.1016/j.chemosphere.2011.07.053
4. Anjum N, Chandra R. Endophytic bacteria: Optimizaton
of isolation procedure from various medicinal plants and their preliminary characterization. Asian J Pharm Clin Res. 2015;8(4):233-238.

5. Li HY, Wei DQ, Shen M, Zhou ZP. Endophytes and their role in phytoremediation. Fungal Diversity. 2012;54(1):11-18. doi: 10.1007/s13225-012-0165-x

6. Singh S, Ali A, Jeyabalang G, Singh R, Singh K. Standardization of bark of Ficus religiosa Linn. by developing different quality control parameters. Int J Adv Sci Technol. 2015;2(1):13-20. doi: 10.30750/jrst.213

7. Gautam S, Meshram A, Bhagyawant SS, Srivastava N. Ficus religiosa-potential role in pharmaceuticals. Int J Pharm Sci Res. 2014;5(5):1616-1623.

8. Prasad MP, Dagar S. Identification and characterization of endophytic bacteria from fruits like avacado and black grapes. Int J Curr Microbiol Appl Sci. 2015;3(8):937-947.

9. Peter K, Gandhi P. Rediscovering the therapeutic potential of Amaranthus species: A review. Egyptian J Basic Appl Sci. 2017;4(3):196-205. doi: 10.1016/j.ejab.2017.05.001

10. Karthik C, Arulselvi PI. Biotic effect of chromium (VI) on plant growth-promoting traits of novel Cellulosimicrobium funkei strain ARB isolated from Phaseolus vulgaris rhizosphere. Geomicrobiol J. 2016;34(5):434-442. doi: 10.1080/01490451.2016.1219429

11. Radhakrishnan R, Lee I-J. Gibberellins producing Bacillus methyloptrophicus KE2 supports plant growth and enhances nutritional metabolites and food values of lettuce. Plant Physiol Biochem. 2016;109:181-189. doi: 10.1016/j.plaphy.2016.09.018

12. Govarthanan M, Mythili R, Selvankumar T, Kamalakan S, Rajasekar A, Chang YC. Bioremediation of heavy metals using an endophytic bacterium Paenibacillus sp. RM isolated from the roots of Tridax procumbens. 3 Biotech. 2016;6(2):242. doi: 10.1007/s13205-016-0560-1

13. Jain A, Jain A, Mudaliyar V, Durve-Gupta A. Isolation, characterization and application of endophytic bacteria isolated from medicinal plants. Int Res J Nat Appl Sci. 2017;4(8):4-20.

14. Kang SM, Radhakrishnan R, You YH, et al. Enterobacter asburiae KE17 association regulates physiological changes and mitigates the toxic effects of heavy metals in soybean. Plant Biol. 2015a;17(5):1013-1022. doi: 10.1111/plb.12341

15. Blois MS. Antioxidant determinations by the use of a stable free radical. Nature. 1958;181:1199-1200. doi: 10.1038/1811199a0

16. Goswami D, Vaghela H, Parmar S, Dhandhukia P, Thakker JN. Plant growth promoting potentials of Pseudomonas spp. strain OG isolated from marine water. J Plant Interact. 2013;8(4):281-290. doi: 10.1080/17429145.2013.768360

17. Lee KE, Radhakrishnan R, Kang SM, et al. Enterococcus faecium LKE12 cell-free-extract accelerates host plant growth via gibberellins and indole-3-acetic acid secretion. J Microbiol Biotechnol. 2015;25(9):1467-1475. doi: 10.4014/jmb.1502.02011

18. Kang SM, Radhakrishnan R, You YH, et al. Mustard and Chinese cabbage plant growth promotion by optimal-medium-cultured Acinetobacter calcoaceticus SE370. J Pure Appl Microbiol. 2016;10(3):1693-1699.

19. Abdelshafy-Mohamad OA, Ma JB, Liu YH, et al. Beneficial endophytic bacterial populations associated with medicinal plant Thymus vulgaris alleviate salt stress and confer resistance to Fusarium oxysporum. Front Plant Sci. 2020;11:47. doi: 10.3389/fpls.2020.00047

20. Liu YH, Guo JW, Salam N, et al. Culturable endophytic bacteria associated with medicinal plant Ferula songorica: molecular phylogeny, distribution and screening for industrially important traits. 3 Biotech. 2016;6(2):209. doi: 10.1007/s13205-016-0522-7

21. Gupta S, Prakash J. Studies on Indian green leafy vegetables for their antioxidant activity. Plant Foods Hum Nutr. 2009;64(1):39-45. doi: 10.1007/s11130-008-0096-6

22. Kang SM, Radhakrishnan R, Lee IJ. Bacillus amyloliquefaciens subsp. plantarum GR53, a potent biocontrol agent resists Rhizoctonia disease on Chinese cabbage through hormonal and antioxidants regulation. World J Microbiol Biotechnol. 2015b;31(10):1517-1527. doi: 10.1007/s11274-015-1896-0

23. Radhakrishnan R, Baek KH. Physiological and biochemical perspectives of non-salt tolerant plants during bacterial interaction against soil salinity. Plant Physiol Biochem. 2017;116:116-126. doi: 10.1016/j.plaphy.2017.05.009