PLANT SCIENCE TODAY
ISSN 2348-1900 (online)
Vol 11(2): 44–57
https://doi.org/10.14719/pst.2600

HORIZON
e-Publishing Group
HePG

RESEARCH ARTICLE

Screening and evaluation of bacterial endophytes of cowpea [*Vigna unguiculata* (L.) Walp.] for plant growth promotion and biocontrol potential

M. Siva[1], S. J. Sreeja[2], Susha S. Thara[3], G. Heera[3] & K. N. Anith[4]*

[1]Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, 641 003, Tamil Nadu, India
[2]Department of Plant Pathology, Rice Research Station, Kerala Agricultural University, Vyttila, Kochi, 682 005, Kerala, India
[3]Department of Plant Pathology, College of Agriculture, Kerala Agricultural University, Vellayani, Thiruvananthapuram 695522, Kerala, India
[4]Department of Microbiology, College of Agriculture, Kerala Agricultural University, Vellayani, Thiruvananthapuram 695522, Kerala, India

*Email: anith.kn@kau.in

OPEN ACCESS

ARTICLE HISTORY
Received: 25 April 2023
Accepted: 31 January 2024
Available online
Version 1.0 : 07 March 2024
Version 2.0 : 01 April 2024

Check for updates

Additional information

Peer review: Publisher thanks Sectional Editor and the other anonymous reviewers for their contribution to the peer review of this work.

Reprints & permissions information is available at https://horizonepublishing.com/journals/index.php/PST/open_access_policy

Publisher's Note: Horizon e-Publishing Group remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Indexing: Plant Science Today, published by Horizon e-Publishing Group, is covered by Scopus, Web of Science, BIOSIS Previews, Clarivate Analytics, NAAS, UGC Care, etc See https://horizonepublishing.com/journals/index.php/PST/indexing_abstracting

CITE THIS ARTICLE

Siva M, Sreeja S J, Thara S S, Heera G, Anith K N. Screening and evaluation of bacterial endophytes of cowpea [*Vigna unguiculata* (L.) Walp.] for plant growth promotion and biocontrol potential. Plant Science Today. 2024; 11(2): 44–57. https://doi.org/10.14719/pst.2600

Abstract

Cowpea [*Vigna unguiculata* (L.) Walp.], is a popular leguminous vegetable crop grown in the wetland fallows of Kerala, India. Web blight caused by *Rhizoctonia solani* has turned out to be a problem in cowpeas, leading to substantial yield loss. We isolated 22 endophytic bacteria from fodder cowpea var. Aiswarya and 16 from bush cowpea var. Bhagyalakshmi, and evaluated their plant growth promotion ability and potential for web blight pathogen suppression. Initially, the isolates were screened *in vitro* for their plant growth promotional traits and antagonism against *R. solani.* The potential isolates were identified as *Bacillus subtilis*, *B. amyloliquefaciens* and *B. velezensis* through 16S rDNA cataloguing. Plant growth-promoting traits like the production of IAA (Indole acetic acid), ammonia, phosphorus, and siderophore by the promising isolates were also investigated. *B. amyloliquefaciens* CBSE5 recorded the highest IAA (3.54 µg/mL) and siderophore production. *B. velezensis* CBRE5 showed the highest ammonia (177.29 µmol/mL) production and phosphate solubilization. In the *in vivo* assay, seed biopriming along with foliar application on 20 and 40 days after sowing, of a consortium of *B. amyloliquefaciens* CBSE5 and *B. velezensis* CBRE5 recorded maximum valuesfor all growth parameters *viz.* shoot length, root length, shoot fresh weight, root fresh weight, shoot dry weight, root dry weight, pod yield and number of pods per plant. The present study reports for the first time the use of endophytic *Bacillus* spp. isolated from cowpeas as plant growth-promoting bacteria, with biocontrol potential against the web blight pathogen *R. solani*.

Keywords

Antagonistic microorganisms; Bacterial consortium; Biological control; Endophytic bacteria; Plant growth promotion

Introduction

Cowpea [*Vigna unguiculata* (L.) Walp.], is an important legume crop cultivated in the tropics. It is referred to as "vegetable meat" as it has high protein content in the grains. Several pests and diseases affect the crop, and among the fungal diseases, web blight caused by *Rhizoctonia solani* Kuhn is a major problem (1). The disease is usually managed with repeated application of fungicides (2). Excess reliance on chemical fungicides leads to the accumulation of chemical residues, which affects the production of safe-to-eat veg-

etables besides environmental pollution, development of resistance to pathogens, and destruction of beneficial flora and fauna. An emerging alternative to the excessive use of agrochemicals is biological control using beneficial bacteria (3). Among the biological control agents, endophytic microorganisms with plant growth-promoting traits are preferred as they improve crop production with enhanced tolerance to various biotic stresses.

Inoculation with Plant Growth Promoting rhizobacteria (PGPR) is an easy-to-do, environment-friendly, and inexpensive technology. PGPR has multiple beneficial characteristics that improve plant growth and mitigate several stresses by regulating the physiological and enzymatic traits of plants (4, 5). They enhance the uptake of nutrients like phosphorus, zinc, calcium, and nitrogen and also suppress various phytopathogens (6). Many endophytic microorganisms, both fungi and bacteria, act as plant growth promoters in several crops. Most plant species ubiquitously harbor endophytic bacteria, which reside within the plants by colonizing various tissues, locally as well as systemically (6). They improve plant growth by increasing the availability and enhancing the transportation of essential nutrients or by modulating signaling molecules, phytohormones, and genes/proteins involved in primary and secondary metabolism in plants (7). Endophytic bacterial application is an eco-friendly input in sustainable agricultural practices for the management of plant pathogens (8). Endophytic bacteria have been isolated from cowpea plants earlier. Three endo-phytic bacteria were identified from the roots of cowpea *viz. Staphylococcus intermedius, S. caprae,* and *S. saprophyticus* in a study conducted in Bangladesh (9). Though endophytes have been isolated from many leguminous plants, reports on the use of endophytic bacteria from cowpea as plant growth promoters and biocontrol agents are very limited (10, 11). Antagonistic endophytic bacteria have been isolated from other crops like black pepper against *Phytophthora capsici* (12), from tomato against *Ralstonia solanacearum* (13), and from amaranthus against *R. solani* (14) from the location of the present study. Within the bacterial endophytic community, the Gram-positive, spore-forming, rod-shaped *Bacillus* is the most dominant group (15, 16). Endospore formation allows better survival when nutrient depletion occurs, population density thresholds exceed the limits or other stress conditions are encountered (17). The objectives of the present investigation were the isolation and characte-rization of endophytic bacteria from cowpea plants, with biocontrol potential against the web blight pathogen of the same host plant, and evaluation of the selected isolates for plant growth promotion in the bush-cowpea variety Bhagyalakshmi.

Materials and Methods

Isolation of endophytic bacteria from cowpea plants

Endophytic bacteria were isolated from plant tissues of healthy fodder cowpea variety Aiswarya and bush cowpea variety Bhagyalakshmi following standard protocols (18, 19). The samples (5 to 10 cm in length) were washed in running tap water and cut into pieces, followed by four pre-sanitization washes with sterile distilled water. The samples were soaked in 4 percent sodium hypochlorite solution for three minutes followed by treatment with 70 percent alcohol for one minute for proper surface sterilization. The bits were again washed four times with sterile distilled water. Sterility checks were done to assess the efficacy of the disinfestation process. The final wash (0.1 ml each) was plated on Nutrient Agar (NA) medium, and also transferred to 9.9 ml of Nutrient Broth (NB) and incubated at 28 ± 2°C. If no bacterial growth occurred in the sterility check after 48 hr of incubation, it was assumed that the surface sterilization was properly done and the bacteria recovered during the ensuing isolation processes were endophytes. For the isolation of endophytic bacteria, the surface-sterilized plant tissue was triturated in 1 mL of phosphate-buffer saline solution (PBS; pH 7.4) using a sterile pestle and mortar under aseptic conditions. 0.1 mL of each of the macerated tissue was spread plated on NA, King's B (KB), and Tryptic Soy Agar (TSA) medium. Further 0.1 mL of the macerate was mixed with 0.9 mL of sterile water and vortexed to obtain 10^{-1}dilution. Plating of the diluted suspension was done on NA, KB, and TSA medium and the plates were incubated at 28 ± 2°C. Following isolation, each bacterial isolate was sub-cultured and evaluated for purity. For short-term storage, the isolates were kept in a refrigerator (4°C) on NA slants. The long-term storage was in glycerol stock at -80°C.

Isolation of pathogen associated with web blight of cowpea

Leaves of cowpea variety Bhagyalakshmi with web blight symptoms were collected from Instructional Farm, College of Agriculture, Vellayani, Kerala, India. Lesions on leaves along with some healthy tissue cut into small bits. The bits were surface sterilized using 1% sodium hypochlorite solution for one minute followed by washing with sterile distilled water thrice. The bits were placed on Potato Dextrose Agar (PDA) plates supplemented with Cephalexin (100 ppm) and incubated for 2 to 3 days at 28°C. The pathogen was further purified to obtain a pure culture, which was maintained on PDA slants under refrigerated conditions with regular sub-culturing for future use. To assess pathogenicity, detached leaves from the cowpea variety Bhagyalakshmi were inoculated with the pathogen. The mycelial disc (8 mm diameter) of the fungal isolate was placed on the upper surface of the leaf after giving a pinprick injury and was covered with a piece of moist cotton. The inoculated leaves were placed in a Petri dish to maintain humid conditions. Development of lesions on leaves was observed 1-2 days after pathogen inoculation. Re-isolation and confirmation of pathogenicity of the re-isolate was also done.

The identity of the pathogen was done based on morphological, cultural, and molecular methods. Molecular identification was carried out by 18S rDNA cataloging. DNA isolation from 100 mg of mycelium was done with NucleoSpin® Plant II Kit (Macherey-Nagel Inc., PA, USA) as per the manufacturer's instructions. Universal primers for the 18S ribosomal DNA; NS1: 5′-GTAGTCATATGCTTGTCTC-

3′ as forward and NS4: 5′-CTTCCGTCAATTCCTTTAAG-3′ as reverse were used. PCR amplification was carried out in GeneAmp PCR System 9700 (Applied Biosystems, USA) with 50 µl reaction mix comprising 25-50 ng DNA, 50 mM of each primer, 0.5 U/µl Taq DNA polymerase, and 0.2 mM of each dNTP. Following amplification conditions were provided: initial denaturation at 98°C for 3 min; 30 cycles of amplification with denaturation at 98°C for 1 min, annealing at 54°C for 30 sec and extension at 72°C for 15 sec, and a final extension at 72°C for 1 min. The amplified products were sequenced using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following the manufacturer protocol. The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Nucleotide BLAST analysis was performed with the consensus sequence obtained after alignment using the BioEdit program. The identity of the organism was confirmed by analyzing the BLAST output.

In vitro antagonism of endophytic bacteria against R. solani

A dual culture plate assay was used to evaluate the direct antagonism of endophytic bacteria against *R. solani* (20). Bacterial isolates were streak purified on NA medium to obtain pure colonies. Five -day-old mycelial plugs of the fungus (8 mm diameter) were inoculated at the center of a PDA plate. Two streaks (2.5 cm) with the endophytic bacterial isolate were done on both edges of the plate. Inoculated plates were incubated at 28°C for two days and three replications maintained. Plates with the fungus alone served as control. Percent growth inhibition of fungal growth and zone of inhibition (mm) if any were recorded (21, 22).

The indirect antagonistic reaction between endophytic bacteria and the pathogen was assessed as described by Athira and Anith (13). A loopful of each endophytic bacterial isolate was transferred to NB (100 mL) in a conical flask and incubated overnight at 28°C in a shaking incubator (100 rpm). Ten mL broth culture of each isolate was spined down at 4,500 rpm for 15 min in a sterile polypropylene tube. The supernatant was filter sterilized with a nitrocellulose bacteriological filter (0.2 µ). The filtrate was collected aseptically in cryovials and stored at 4°C for further use. The antagonistic effect of the culture filtrate was assessed by the agar well diffusion method on PDA plates. Three replications were maintained for each bacterial isolate. On incubation at 28°C for 48 hr., the zone of inhibition (mm) if any, was measured.

Biopriming of cowpea seeds and seedling vigor index determination

Bacterial inoculum for biopriming of cowpea seeds (var. Bhagyalakshmi) was prepared following the protocol by Anith (23). NA plates were heavily cross-streaked with endophytic bacterial isolate and incubated for 24 hr. at 28°C. 10 mL sterile distilled water was added to the plates under aseptic conditions and bacterial cells were suspended in it with a sterile glass spreader. It was collected in a sterile glass vial. The optical density (OD) was made to 0.6 at 660 nm with sterile distilled water using a spectrophotometer (Shimadzu 1900i, Japan), resulting in a suspension containing 10^8 cfu/mL.

Biopriming of cowpea seed (var. Bhagyalakshmi) was done as described by Abdul-Baki and Anderson (24). Seeds were soaked in 1% sodium hypochlorite solution for one minute and rinsed with sterile distilled water three times. Carboxymethyl cellulose (CMC) @ 0.1 g/10mL was added to the freshly prepared suspension of endophytic bacterial isolate as an adhesive. Surface sterilized seeds (30 numbers) were immersed for 4 hrs. in the suspension of each bacterial isolate (25). After soaking, the seeds were air-dried. Hydropriming (HP) was done by soaking seeds in sterile distilled water. Control treatment involved no seed priming. The bio-primed seeds were placed equidistantly between two sheets of the germination paper. Seeds were placed in a row of 10 numbers per paper towel, rolled, tagged, and kept upright for incubation at 28°C. Moisture was maintained by watering the paper towel every day. Each treatment had three replications. After seven days growth parameters of cowpea seedlings were recorded. Seedling vigor index I and II were assessed according to the formula of Abdul-Baki and Anderson (24).

SVI–I = Germination Percentage (%) × Seedling length* (cm)

*(Seedling length = Shoot length + Root length)

SVI–II = Germination Percentage (%) × Seedling dry weight*(g)

*(Seedling dry weight = Shoot dry weight + Root dry weight)

Characterization of promising endophytic bacterial isolates

Based on the antagonistic reaction against *R. solani*, and growth promotion ability assessed by the roll towel assay, three bacterial isolates were selected for further studies. Standard procedures were employed to assess the colony characters such as elevation, color, form, margin, colony size (mm), cell arrangement, Gram's reaction, and endospore formation (26). Readymade HiMedia© identification kits (HiMedia Laboratories, Mumbai, India) were used for the biochemical characterization of the bacterial isolates. The different biochemical tests performed were Indole, Methyl red, Voges Proskauer's, Citrate utilization, Glucose, Adonitol, Arabinose, Lactose, Sorbitol, Mannitol, Rhamnose, Sucrose, Lysine utilization, Ornithine utilization, Urease, Phenylalanine utilization, Nitrate reduction, H_2S production, Xylose, Maltose, Fructose, Dextrose, Galactose, Raffinose, Trehalose, Melibiose, L-Arabinose, Mannose, Inulin, Sodium gluconate, Glycerol, Salicin, Dulcitol, Inositol, Arabitol, Erythritol, alpha-Methyl-D-glucoside, Cellobiose, Melezitose, alpha-Methyl-D-mannoside, Xylitol, ONPG, Esculin hydrolysis, D-Arabinose, Malonate utilization, Sorbose, Catalase and Arginine tests.

Molecular characterization of the promising endophytic bacterial isolates was done by 16S rRNA sequence analysis using universal primers, 16s-RS-F: forward (5′-CAGGCCTAACACATGCAAGTC-3′) and 16s-RS-R: reverse (5′-

GGGCGGWGTGTACAAGGC- 3′). The sequence reaction was performed in a PCR thermocycler (GeneAmp PCR System 9700, Applied Biosystems) with the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems - USA) according to the manufacturer's protocol. After alignment, the nucleotide BLAST analysis was performed with the contig sequence obtained using the BioEdit program. The sequence of nucleotides in 16S rRNA was matched and compared with the sequence available in the database by using the BLAST tool offered by the National Centre for Biotechnology Information. BLASTn provided by NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was carried out for homology search. The phylogenetic relationship of the promising bacterial isolates with other bacteria showing sequence similarity was analyzed using software MEGA11 (version 11.0.13) (27) and displayed using iTOL (https://itol.embl.de) web-based software (version 5.0) (28).

Compatibility assessment of the endophytic bacterial isolates

Cross streak plate assay and disc diffusion methods were used for testing the compatibility among the promising endophytic bacterial isolates. A loopful of culture from a single colony of an endophytic bacterial isolate was streaked in a straight line across a solidified NA medium. After 24 hr. of incubation at 28°C, other isolates were streaked perpendicular to the test bacterial growth and incubated for another 48 hr. Three replications were maintained. Similarly, it was repeated for the other test organisms. Inhibition zone if any, between the test organism and other bacterial isolates at the site of cross streak was observed (29).

In disc diffusion assay, NA plates were swab-inoculated with each endophytic bacterial isolate, uniformly covering the entire surface. The test isolates were inoculated in NB and incubated for 24 hr. at 28°C in an incubator shaker (110 rpm). Sterile filter paper discs of 5 mm diameter were dipped separately in the culture suspension (10 µl) of these isolates. The discs were dried inside a laminar airflow chamber before placing them on the surface of solidified NA medium already swabbed with the test organism. Three replications were kept for each isolate. Plates were incubated for 48 hr. at 28°C. The test was repeated for the other isolates. Development of inhibition zone if any, around the discs was observed (28).

Assessment of plant growth-promoting traits of promising bacterial endophytes

The production of Indole Acetic Acid (IAA) by the promising endophytic bacterial isolates was estimated following the procedure described by Gordon and Weber (30). Briefly, 15 mL of sterile NB supplemented with 0.1% L-tryptophan was inoculated with 50 µL of bacterial suspension and incubated at 30°C in a shaker for 72 hr. under dark conditions. The suspension was centrifuged, and to the supernatant (2 mL) two drops of orthophosphoric acid and a further four mL of Salkowski reagent were added, mixed well, and kept under dark for 25 min. The appearance of pink color indicated IAA synthesis after 25 min. The absorbance of the reaction mixture was read at 530 nm using a spectrophotometer (Shimadzu 990i, Shimadzu Corporation, Japan). The amount of IAA in the sample was calculated from a standard graph prepared with chemical grade indole-3-acetic acid and the result was expressed as µg/mL.

The promising endophytic bacterial isolates were evaluated for their ability to dissolve the insoluble tricalcium phosphate present in Pikovskaya's agar medium (31, 32). A loopful of bacterial culture was placed at the center of three mm thick plates having solidified Pikovskaya's agar medium (HiMedia Laboratories, Mumbai, India) and incubated at 28°C for five days. The appearance of a halo zone around the colony was taken as a positive result.

Ammonia production was determined spectrophotometrically as per the method described by Cappuccino and Sherman (26) using Nessler's reagent as the indicator. 50 µL of bacterial cell suspension was transferred to 30 mL of peptone broth (4.0%), incubated at 25°C for 72 hr., and after incubation, 1 mL of Nessler's reagent (Hi-Media Laboratories, Mumbai, India) was added. The development of yellow to dark brown color indicated a positive reaction for the test. The absorbance was measured at 450 nm and the amount of ammonia production was estimated using the standard curve of ammonium sulfate.

Hydrogen cyanide (HCN) production was assessed as per the protocol described by Lorck (33). Bacterial isolate was streaked on a modified nutrient agar medium containing 4.4 g/l glycine. Sterile filter paper discs soaked in picric acid solution (in 2% sodium carbonate) were placed in the lid of each plate and incubated at 30°C for four days. Change in the color of filter paper discs soaked in picric acid solution from yellow to brown and to red indicated the production of HCN.

The production of siderophore was assessed by the Modified Chrome Azurol Sulfonate (CAS) agar plate method described by Milagres *et al*. (34). The formation of orange halo zones from dark blue hue around the bacterial colonies indicated a positive reaction for siderophore production.

Plant growth promotion under pot culture conditions

In an open field, a pot culture experiment was conducted to evaluate the effectiveness of selected endophytic bacterial isolates and their combinations in promoting the growth of the cowpea variety Bhagyalakshmi. Bacterial inoculum was prepared as described above. For the preparation of the consortium, equal amounts of the individual bacterial suspension were mixed. The experiment followed a completely randomized design (CRD) with three replications. To prepare the potting mixture; sand, garden soil, and farmyard manure were mixed in a 1:1:1 ratio and autoclaved for three consecutive days at 121°C and 15 psi pressure for one hour each. Earthen pots measuring 10 cm x 10 cm were filled with six kg of the sterilized potting mixture, and two bacterized seeds were sown in each pot. Biopriming was done as described above. After germination, one seedling was uprooted to maintain a single plant per pot. The plants were fertilized with 1% solution of NPK (19:19:19) @ 50 mL/pot as soil drenching at 20-day inter-

vals. The selected bacterial endophyte suspension (10^8 cfu/mL) was given twice as a foliar spray at 20 and 40 days after sowing (DAS). Biometric observations were recorded at 60 DAS by destructive sampling, including root length (cm), shoot length (cm), root fresh weight (g), shoot fresh weight (g), number of pods per plant, and pod yield (g). Root dry weight (g) and shoot dry weight (g) of plants were recorded after uprooting and drying samples in a hot air oven at 60°C for two days. The control group consisted of plants raised without any bacterial treatment.

Statistical analysis

The collected data underwent statistical analysis using Analysis of Variance (ANOVA). Mean values, standard deviations (SD), and Critical Differences (CD) were calculated and compared using the Kerala Agricultural University - General R-shiny based Analysis Platform Empowered by Statistics (KAU - GRAPES) software, with a significance level set at 5% ($p \leq 0.05$) (35).

Results

Isolation of endophytic bacteria from cowpea plants

Twenty-two endophytic bacterial isolates were obtained from the roots, stems, and leaves of healthy fodder cowpea (var. Aiswarya), while 16 isolates were obtained from bush cowpea (var. Bhagyalakshmi) on different bacteriological media. The isolates were designated as CFRE (Cowpea Fodder Root Endophyte), CFSE (Cowpea Fodder Stem Endophyte), CFLE (Cowpea Fodder Leaf Endophyte), CBRE (Cowpea Bush Root Endophyte), CBSE (Cowpea Bush Stem Endophyte), and CBLE (Cowpea Bush Leaf Endophyte) based on their location in either the roots, stems, or leaves of the fodder or bush cowpea. The colony characters such as elevation, color, form, margin, colony size (mm), cell arrangement, and Gram reaction of the isolates obtained are presented in Tables 1 and 2.

Characteristics of the pathogen associated with web blight of cowpea

The color of the fungal growth on PDA was initially white which later changed to light brown. The fungus produced high pigmentation on PDB six days after inoculation. The margin of fungal growth was regular. The mycelium was off-white to pale in color with a diameter ranging from 4.5

Table 1. Morphological and cultural characteristics of endophytic bacteria isolated from fodder cowpea var. Aiswarya.

Isolates	Elevation	Colour	Form	Margin	Colony diameter (mm)	Gram reaction	Cell arrangement
Source: Root							
CFRE1	Raised	Creamy white	Irregular	Undulated	1.0	G^-	Single rod
CFRE2	Flat	Creamy white	Irregular	Undulated	3.5	G^+	Single rod
CFRE3	Raised	White	Irregular	Curled	4.0	G^-	Single rod
CFRE4	Flat	White	Irregular	Undulated	5.0	G^-	Single rod
CFRE5	Raised	Off-white	Irregular	Curled	1.5	G^+	Single rod
CFRE6	Raised	Off-white	Circular	Entire	1.0	G^+	Single rod
CFRE7	Raised	Off-white	Irregular	Curled	3.0	G^+	Single rod
CFRE8	Raised	Off-white	Circular	Entire	4.0	G^-	Straight cocci
Source: Stem							
CFSE1	Flat	Off-white	Circular	Entire	2.0	G^+	Single rod
CFSE2	Flat	Creamy white	Circular	Entire	1.0	G^+	Straight rod
CFSE3	Raised	Creamy white	Irregular	Undulated	2.5	G^+	Single rod
CFSE4	Raised	White	Irregular	Undulated	2.0	G^+	Single rod
CFSE5	Raised	White	Irregular	Curled	4.0	G^-	Single rod
CFSE6	Raised	White	Irregular	Undulated	3.0	G^+	Straight rod
CFSE7	Raised	Creamy white	Irregular	Curled	2.0	G^-	Straight cocci
CFSE8	Raised	White	Irregular	Curled	3.5	G^+	Single rod
Source: Leaf							
CFLE1	Raised	Yellow	Circular	Entire	1.5	G^-	Single rod
CFLE2	Raised	Off-white	Irregular	Curled	3.5	G^-	Single rod
CFLE3	Flat	White	Irregular	Undulated	3.0	G^+	Single rod
CFLE4	Raised	White	Irregular	Curled	4.0	G^+	Single rod
CFLE5	Flat	White	Irregular	Undulated	3.0	G^+	Single rod
CFLE6	Raised	Off-white	Irregular	Undulated	4.0	G^-	Single rod

*CFRE – Cowpea Fodder Root Endophyte; CFSE – Cowpea Fodder Stem Endophyte; CFLE – Cowpea Fodder Leaf Endophyte.

Table 2. Morphological and cultural characteristics of endophytic bacteria isolated from bush cowpea var. Bhagyalakshmi.

Isolates*	Elevation	Colour	Form	Margin	Colony dia (mm)	Gram reaction	Cell arrangement
Source: Root							
CBRE1	Raised	Off-white	Irregular	Curled	4.0	G^+	Single rod
CBRE2	Flat	Off-white	Circular	Entire	4.5	G^-	Straight rod
CBRE3	Raised	Off-white	Irregular	Curled	3.0	G^+	Single rod
CBRE4	Flat	Off-white	Irregular	Undulated	6.0	G^+	Single rod
CBRE5	Raised	Off-white	Irregular	Curled	4.0	G^+	Single rod
CBRE6	Raised	Creamy white	Circular	Entire	3.5	G^+	Single rod
CBRE7	Flat	Creamy white	Irregular	Undulated	3.5	G^+	Single rod
Source: Stem							
CBSE1	Raised	Creamy white	Irregular	Undulated	4.0	G^+	Single rod
CBSE2	Flat	Off-white	Irregular	Undulated	8.0	G^+	Single rod
CBSE3	Raised	White	Irregular	Curled	5.0	G^-	Single rod
CBSE4	Raised	Off-white	Irregular	Curled	3.5	G^+	Single rod
CBSE5	Raised	Off-white	Irregular	Undulated	3.5	G^+	Single rod
Source: Leaf							
CBLE1	Raised	Off-white	Circular	Entire	4.0	G^-	Straight cocci
CBLE2	Raised	Creamy white	Irregular	Undulated	4.5	G^-	Single rod
CBLE3	Raised	White	Circular	Entire	3.0	G^+	Single cocci
CBLE4	Raised	White	Circular	Entire	2.0	G^-	Cluster cocci

*CBRE – Cowpea Bush Root Endophyte; CBSE – Cowpea Bush Stem Endophyte; CBLE – Cowpea Bush Leaf Endophyte.

to 6.2 µm and grew rapidly. The hyphal branches were inclined to the direction of growth. Young hyphal branches were observed at a right angle to the main hyphae. The cross-septum formation was noticed in the branches near the point of origin. These morphological and cultural characteristics were similar to those of *Rhizoctonia solani* Kuhn. The identity of the pathogen, *Rhizoctonia solani*, was confirmed through sequence analysis of the Internal Transcribed Spacer (ITS) region of the 18S ribosomal DNA (rDNA) with GenBank Accession No. OL819875.

Antagonism of endophytic bacterial isolates against R. solani

Fifteen out of the 22 isolates from fodder cowpea var. Aiswarya exhibited inhibition against the pathogen, while nine out of 16 isolates from bush cowpea var. Bhagyalakshmi showed inhibitory activity. The isolate CBRE5 recorded the highest zone of inhibition against *R. solani*, and the mycelial growth inhibition (%) was also maximum with this isolate (Table 3). Only two isolates each from fodder cowpea var. Aiswarya and bush cowpea var. Bhagyalakshmi exhibited inhibition against *R. solani* when tested for indirect antagonism by culture filtrate (Table 4). Based on the results of direct and indirect antagonism (34) (Fig. 1; Fig. 2), 20 endophytic bacterial isolates were chosen for further experiments.

Seedling vigor index

Seed priming with the isolates CFRE3, CFRE4, CFSE3, CFLE3, CBRE3, CBRE5, CBSE5, and CBLE2 recorded 100% germination. There was a significant influence on root length, shoot length, fresh weight, and dry weight of root and shoot of cowpea seedlings with bacterization with endophytic bacterial isolates over the control (Table 5; Fig. 3). Seedling vigor index I value ranged

Table 3. Direct antagonistic activity of bacterial endophytes against *R. solani* in dual culture assay.

Isolates	Inhibition zone (mm)*	Mycelial growth inhibition (%)
CFRE1	2.75 (1.80) ± 0.27^{ij}	37.40 (37.68) ± 3.90^{cdefg}
CFRE2	6.33 (2.61) ± 0.5^{f}	39.63 (39.01) ± 1.69^{bcde}
CFRE3	3.00 (1.87) ± 0.00^{i}	31.48 (34.11) ± 3.57^{hij}
CFRE4	5.58 (2.46) ± 0.49^{g}	38.15 (38.14) ± 2.31^{bcdefg}
CFRE5	1.58 (1.44) ± 0.20^{lm}	31.48 (34.11) ± 3.57^{hij}
CFRE7	4.75 (2.29) ± 0.27^{h}	35.55 (36.59) ± 2.2^{efgh}
CFSE3	2.33 (1.68) ± 0.40^{jk}	37.41 (37.70) ± 2.22^{cdefg}
CFSE4	2.66 (1.77) ± 0.25^{ij}	34.07 (35.71) ± 0.64^{ghij}
CFSE5	1.75 (1.49) ± 0.27^{kl}	30.00 (33.20) ± 1.11^{j}
CFSE6	4.16 (2.15) ± 0.40^{h}	36.30 (37.04) ± 0.64^{defg}
CFSE7	1.08 (1.25) ± 0.20^{m}	30.37 (33.43) ± 1.69^{ij}
CFSE8	7.50 (2.82) ± 0.44^{de}	40.37 (39.43) ± 3.39^{bcd}
CFLE2	2.66 (1.77) ± 0.25^{ij}	41.48 (40.09) ± 0.64^{bc}
CELE3	8.16 (2.94) ± 0.75^{c}	41.11 (39.87) ± 1.11^{bc}
CFLE5	5.50 (2.44) ± 0.54^{g}	34.44 (35.93) ± 0.00^{fghi}
CBRE1	8.83 (3.05) ± 1.16^{ab}	42.22 (40.52) ± 2.22^{b}
CBRE3	7.33 (2.78) ± 1.50^{e}	39.99 (39.20) ± 4.84^{bcd}
CBRE5	9.33 (3.13) ± 0.51^{a}	48.52 (44.14) ± 1.28^{a}
CBRE7	7.83 (2.88) ± 0.40^{cde}	38.15 (38.13) ± 2.79^{bcdefg}
CBSE1	4.66 (2.27) ± 0.51^{h}	36.29 (37.04) ± 1.70^{defg}
CBSE3	1.00 (1.22) ± 0.00^{m}	23.70 (29.13) ± 0.64^{k}
CBSE4	8.00 (2.91) ± 0.63^{cd}	40.00 (39.22) ± 2.22^{bcd}
CBSE5	8.33 (2.97) ± 0.81^{bc}	41.85 (40.29) ± 5.59^{b}
CBLE2	2.05 (1.58) ± 0.62^{kl}	38.51 (38.34) ± 3.57^{bcdef}
Control	0.00 (0.71) ± 0.00^{n}	0.00 (0.29) ± 00^{l}
SEm±	0.24	1.46
CD (0.05)	0.67	4.16

* Mean (± SD) of six replications. Values followed by similar superscripts are not significantly different at the 5% level ($p \leq 0.05$). Values in parenthesis are square root transformed.

Table 4. Indirect antagonistic activity of bacterial endophytes against *R. solani* in culture filtrate assay.

Isolates	Inhibition zone (mm)*
CFSE3	1.30 (1.34) ± 0.10[b]
CFLE3	2.20 (1.64) ± 0.10[a]
CBRE5	2.33 (1.68) ± 0.25[a]
CBLE2	1.40 (1.37) ± 0.17[b]
Control	0.00 (0.71) ± 0.0[c]
SEm±	0.09
CD (0.05)	0.32

* Mean (± SD) of six replications. Values followed by similar superscripts are not significantly different at the 5% level ($p \leq 0.05$). Values in parenthesis are square root transformed values.

Fig. 1. Direct antagonistic activity of bacterial endophytes against *R. solani* in dual culture assay. The zone of inhibition was calculated by measuring the distance between the leading edge of the fungus and the outer edge of the bacterial growth in the interacting area after two days of incubation.

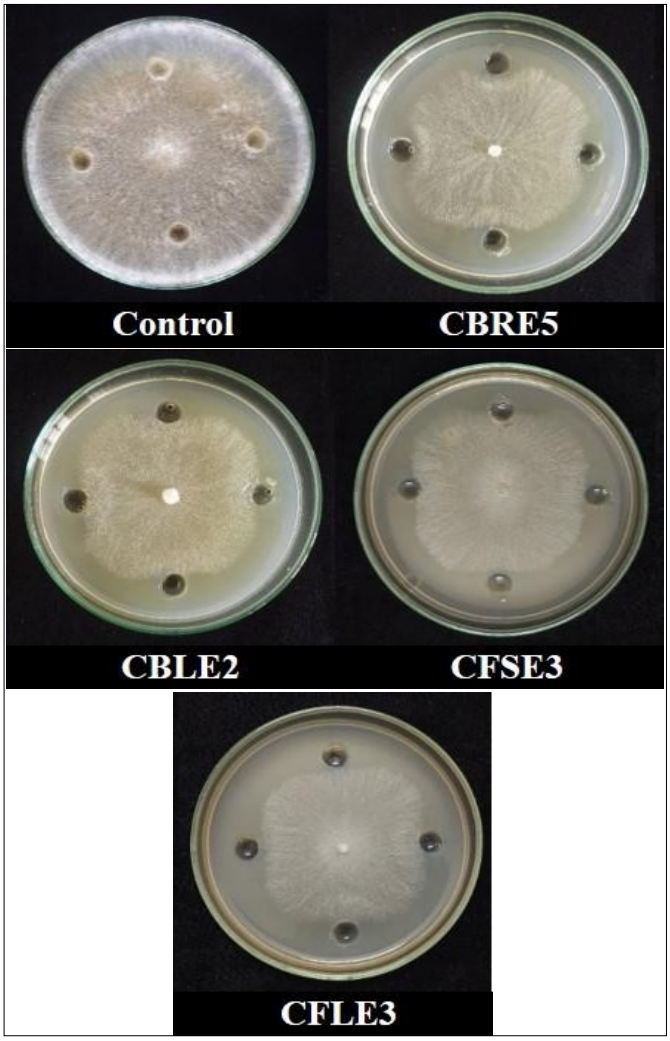

Fig. 2. Agar well diffusion assay with culture filtrate of bacterial endophytes against *R. solani* in PDA medium. The wells in the control plate were added with sterile distilled water.

from 4087.33 (CFLE3) to 2905.46 (CFRE1) while seedling vigour index II values ranged from 7.66 (CFLE3) to 3.96 (CFSE6) (Table 5). The isolates CFLE3, CBRE5, and CBSE5 were selected as the best three bacterial endophytes for further studies.

Fig. 3. Representative plants showing growth of cowpea seeds bioprimed with different endophytic *Bacillus* spp. in the roll towel assay. Observations were taken on seven days of growth. **A**: CFRE1, **B**: CFRE2, **C**: CFRE3, **D**: CFRE4, **E**: CFRE7, **F**: CFSE3, **G**: CFSE4, **H**: CFSE6, **I**: CFSE8, **J**: CFLE2, **K**: CFLE3, **L**: CFLE5, **M**: CBRE1, **N**: CBRE3, **O**: CBRE5, **P**: CBRE7, **Q**: CBSE1, **R**: CBSE4, **S**: CBSE5, **T**: CBLE2, **U**: Hydropriming .

Table 5. Effect of biopriming with endophytic bacteria on cowpea seedling vigour index (var. Bhagyalakshmi).

Treatments	Seedling vigor index – I *	Seedling vigor index – II*
CFRE1	2,905.46 ± 314.92^{cdef}	5.30 ± 1.00^{cde}
CFRE2	2,518.93 ± 32.57^{f}	4.78 ± 0.77^{cdef}
CFRE3	2,808.66 ± 349.34^{def}	5.26 ± 0.59^{cde}
CFRE4	3,187.66 ± 281.64^{bcde}	5.64 ± 1.02^{bcde}
CFRE7	2,983.80 ± 210.54^{cdef}	4.70 ± 0.65^{def}
CFSE3	3,351.33 ± 67.11^{bcd}	5.79 ± 0.71^{bcd}
CFSE4	2,812.50 ± 374.17^{def}	5.17 ± 0.26^{cdef}
CFSE6	2,679.90 ± 282.17^{ef}	3.96 ± 0.40^{f}
CFSE8	2,868.53 ± 609.58^{def}	4.52 ± 0.88^{ef}
CFLE2	2,933.73 ± 219.12^{cdef}	5.76 ± 1.70^{bcd}
CFLE3	4,087.33 ± 1054.58^{a}	7.66 ± 0.23^{a}
CFLE5	2,803.33 ± 188.22^{def}	5.36 ± 0.42^{cde}
CBRE1	2,897.63 ± 396.11^{cdef}	5.47 ± 0.86^{cde}
CBRE3	3,079.00 ± 106.41^{bcdef}	5.42 ± 0.85^{cde}
CBRE5	3,452.33 ± 28.43^{bc}	6.73 ± 0.64^{ab}
CBRE7	2,916.66 ± 301.10^{cdef}	5.29 ± 0.53^{cde}
CBSE1	3,128.66 ± 185.44^{bcde}	5.55 ± 0.97^{bcde}
CBSE4	2,885.00 ± 199.09^{cdef}	5.15 ± 0.46^{cdef}
CBSE5	3,598.00 ± 136.63^{ab}	6.70 ± 0.573^{ab}
CBLE2	3,090.66 ± 213.14^{bcdef}	5.96 ± 0.23^{bc}
Control	2,618.06 ± 324.08^{ef}	5.37 ± 0.25^{cde}
Hydropriming	2,728.80 ± 347.50^{ef}	4.83 ± 0.24^{cdef}
SEm±	204.52	0.42
CD (0.05)	584.89	1.22

* Mean (± SD) of three replications. Values followed by similar superscripts are not significantly different at a 5% level ($p \leq 0.05$).

Characterization of the promising endophytic bacterial isolates

Based on colony characteristics, Gram's reaction, endospore staining, and biochemical tests, it was determined that the three promising endophytic bacterial isolates belonged to the genus *Bacillus*. Further confirmation of the species of these isolates was done through 16S rRNA analysis, which identified them as *Bacillus subtilis* CFLE3 (GenBank Accession No. OL719066), *B. amyloliquefaciens* CBRE5 (GenBank Accession No. OL719067) and *B. velezensis* CBSE5 (GenBank Accession No. OL719068).

The compatibility between the three endophytic bacterial isolates following the disc diffusion method and cross streak plate assay indicated that they were compatible as no growth inhibition was observed.

Plant growth-promoting traits of the promising endophytic bacterial isolates

The IAA and ammonia were quantitatively estimated (Fig. 4). Production of IAA by the endophytic bacterial isolates ranged from 1.51 to 3.91 µg/mL (Fig. 5A). The highest ammonia production was recorded by *B. velezensis* CBRE5 (177.29 µmol/mL) (Fig. 5B). *B. velezensis* CBRE5 and *B. subtilis* CFLE3 produced a clear zone around their growth on

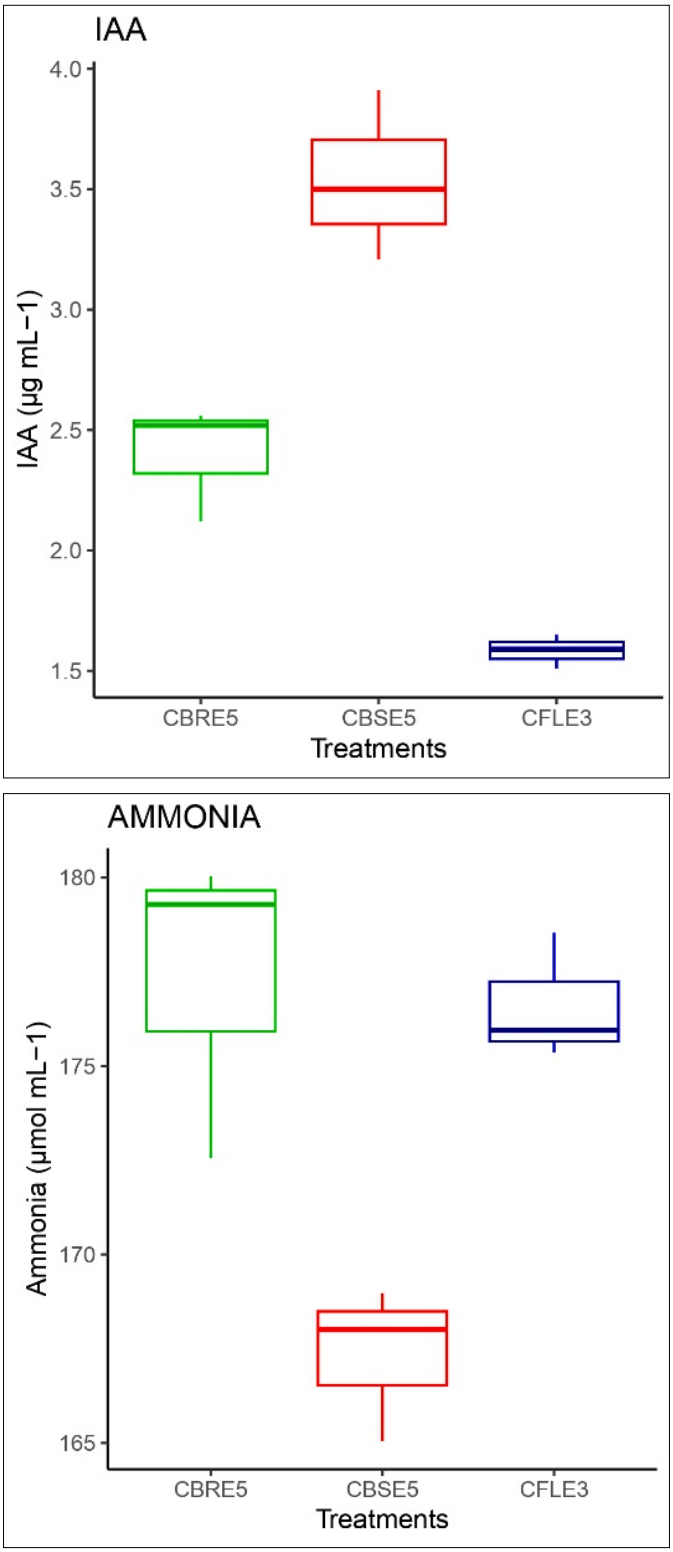

Fig. 4. Production of IAA (µg/mL) and ammonia (µmol/mL) by bacterial endophytes.

Pikovskaya's medium which indicated their ability to solubilize tricalcium phosphate (Fig. 5C). The zone of orange coloration confirmed siderophore produ-ction in all three isolates. The maximum zone of coloration was shown by *B. amyloliquefaciens* CBSE5 (6.00 mm) (Fig. 5D). None of the isolates were found to be cyanogenic (Table 6).

Plant growth promotion under pot culture

There was a significant increase in all the growth parameters of cowpea plants treated with endophytic bacterial isolates over the absolute control. However, seed biopriming for 4 hr. along with the foliar application (20 and 40

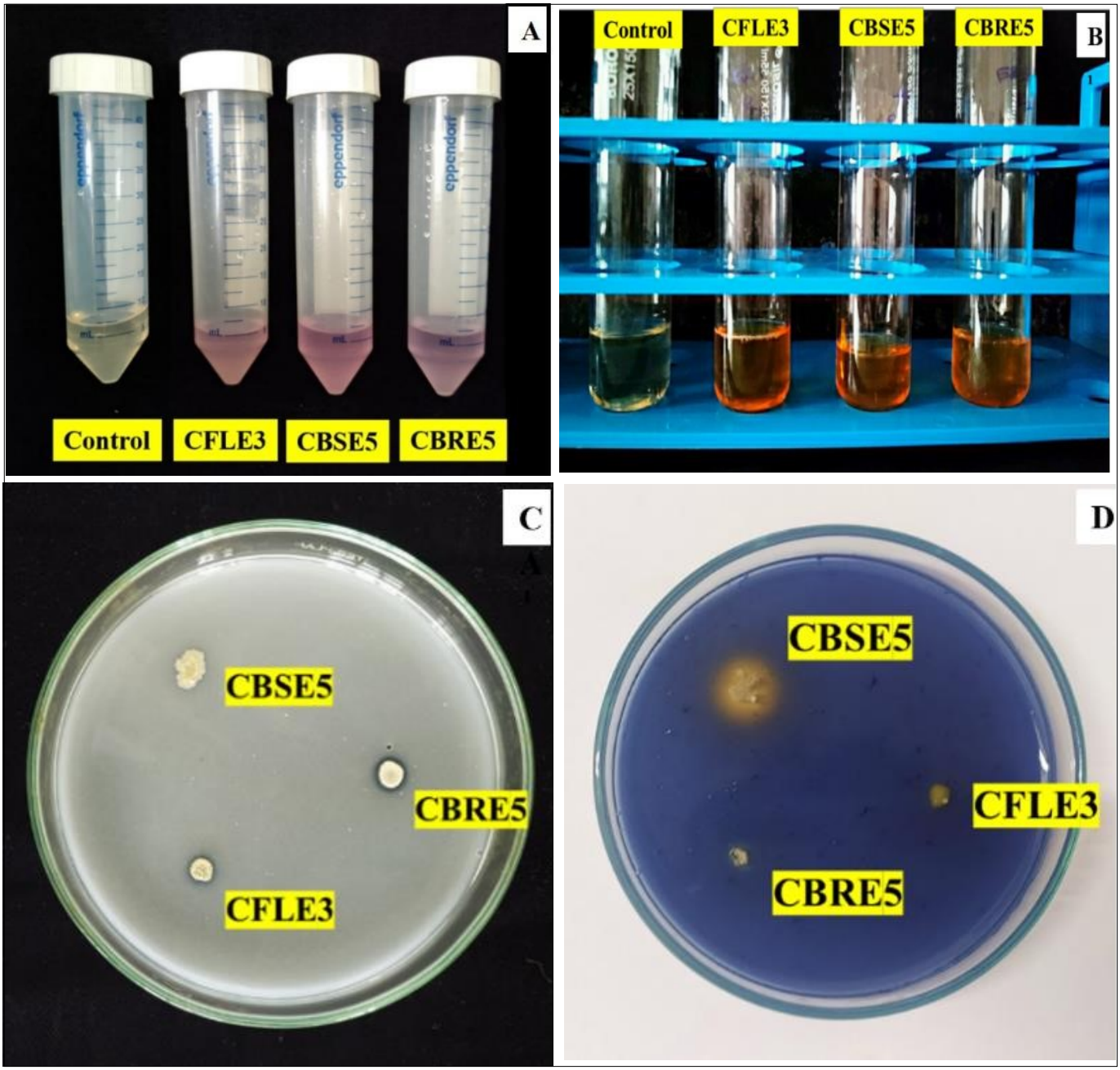

Fig. 5. Qualitative screening of (**A**) Indole acetic acid (IAA) and (**B**) Ammonia production (**C**) Phosphate solubilization and (**D**) Siderophore production by endophytic bacterial isolates.

Table 6. Plant growth-promoting traits of promising endophytic bacterial isolates under *in vitro.*

Isolates	IAA (µg/mL) *	Phosphate solubilization	Ammonia (µmol/mL) *	Siderophore production
B. subtilis CFLE3	1.58 ± 0.07^{c}	+	176.61 ± 1.69^{a}	+
B. amyloliquefaciens CBSE5	3.54 ± 0.35^{a}	-	167.34 ± 2.04^{b}	++
B. velezensis CBRE5	2.40 ± 0.24^{b}	++	177.29 ± 4.12^{a}	+
SEm±	0.14	-	1.634	-
CD (0.05)	0.51	-	5.654	-

* Mean (± SD) of three replications. Values followed by similar superscripts in a column are not significantly different at a 5% level ($p \leq 0.05$).

DAS) of a consortium of *B. amyloliquefaciens* CBSE5 and *B. velezensis* CBRE5 recorded maximum valuesfor all growth parameters except the number of days taken for flowering (Table 7). Bacterial inoculation also had a profound influence on the rooting pattern of the plants (Fig. 6).

Discussion

The objective of this study was to isolate endophytic bacteria from healthy cowpea plants, investigate their potential in promoting plant growth, and evaluate their ability to suppress the pathogen responsible for web blight disease, *R. solani*. Many potential endophytic bacteria were isolated by trituration of surface disinfected plant tissues of other crops such as black pepper (12), tomato (13), and amaranthus (14) from the same location of the present study, but no attempts have been made to isolate bacterial endophytes from cowpea. However, Marzan *et al.* (9) have iso-

Table 7. Biometric characters of cowpea plants (var. Bhagyalakshmi) on inoculation with endophytic bacteria.

Treatments	Root length (cm)/ plant *	Shoot length (cm)/ plant *	Root fresh weight (g) /plant *	Shoot fresh weight(g)/ plant *	Root dry weight (g)/ plant *	Shoot dry weight (g) / plant *	No. of pods/ Plant *	Pod yield (g)/ plant *
T1	27.00 ± 5.29^{b}	39.13 ± 4.49^{bc}	31.83 ± 3.17^{bcd}	106.50 ± 10.33^{cd}	5.13 ± 0.98^{a}	18.00 ± 4.35^{bc}	27.33 ± 5.50^{bcd}	192.16 ± 12.57^{c}
T2	33.33 ± 5.68^{b}	45.16 ± 2.15^{ab}	29.16 ± 5.79^{cd}	119.33 ± 5.50^{bc}	4.83 ± 1.04^{a}	21.16 ± 4.19^{abc}	31.33 ± 2.51^{abc}	224.66 ± 6.53^{ab}
T3	30.33 ± 3.78^{b}	44.73 ± 3.95^{ab}	37.33 ± 4.50^{bc}	111.66 ± 3.61^{bcd}	5.40 ± 0.52^{a}	19.16 ± 7.23^{bc}	25.66 ± 3.78^{cd}	199.33 ± 1.52^{c}
T4	27.33 ± 7.02^{b}	43.53 ± 3.93^{ab}	35.00 ± 5.67^{bcd}	123.16 ± 5.53^{b}	5.23 ± 1.12^{a}	23.16 ± 2.46^{ab}	30.66 ± 4.16^{abc}	220.73 ± 6.21^{b}
T5	31.66 ± 2.30^{b}	45.60 ± 3.07^{ab}	40.33 ± 8.08^{ab}	122.33 ± 5.50^{b}	5.50 ± 0.50^{a}	21.83 ± 3.25^{abc}	33.33 ± 0.57^{ab}	216.66 ± 5.26^{b}
T6	40.93 ± 13.02^{a}	47.40 ± 4.61^{a}	49.33 ± 4.93^{a}	139.16 ± 9.43^{a}	6.33 ± 1.04^{a}	27.83 ± 3.01^{a}	36.00 ± 6.55^{a}	236.66 ± 14.95^{a}
T7	25.93 ± 5.25^{b}	36.70 ± 3.84^{c}	26.06 ± 7.92^{d}	99.83 ± 9.87^{d}	3.16 ± 0.57^{b}	15.33 ± 1.04^{c}	21.66 ± 3.05^{d}	155.33 ± 8.22^{d}
SEm±	3.94	2.19	3.45	4.35	0.50	2.35	2.40	4.54
CD (0.05)	11.97	6.67	10.43	13.20	1.52	7.13	7.28	13.77

T1: *B. subtilis* CFLE3, **T2**: *B. amyloliquefaciens* CBSE5, **T3**: *B. velezensis* CBRE5, **T4**: CFLE3 + CBSE5, **T5**: CFLE3 + CBRE5, **T6**: CBSE5 + CBRE5, **T7**: Absolute control. * Mean (± SD) of three replications. Values followed by similar superscripts in a column are not significantly different at a 5% level ($p \leq 0.05$)

Fig. 6. Representative root samples showing rooting pattern of cowpea plants treated with different endophytic *Bacillus* spp. and their combinations in pot culture. Roots excavated 60 days after sowing. **T1**: *B. subtilis* CFLE3, **T2**: *B. amyloliquefaciens* CBSE5, **T3**: *B. velezensis* CBRE5, **T4**: *B. subtilis* CFLE3 + *B. amyloliquefaciens* CBSE5 , **T5**: *B. subtilis* CFLE3 + *B. velezensis* CBRE5, **T6**: *B. amyloliquefaciens* CBSE5 + *B. velezensis* CBRE5.

lated endophytic bacteria from the roots of cowpea plants in Chittagong, Bangladesh. We isolated a total of 38 endophytic bacteria from the healthy root, stem, and leaves of fodder cowpeavar. Aiswarya (22 isolates) and bush cowpea var. Bhagyalakshmi (16 isolates).

Host plant age, genotype, geographical location, and soil type are some of the factors affecting the endophytic bacterial diversity of a plant (36). We used three different bacteriological media *viz.,* Nutrient agar (NA), King's B (KB), and Tryptic Soy Agar (TSA) medium for the isolation of endophytic bacteria from root, stem and leaves of healthy cowpea plants. It was found that the population and diversity of the isolates obtained were highly influenced by the growth media and plant part used for isolation in the study. In our study, a greater number of endophytic bacterial isolates were obtained from the roots of cowpeas followed by stems and leaves. Concerning the media used for isolation, more bacterial endophytes were obtained on the TSA medium followed by KB and NA. The selection of growth medium affects the number and diversity of endophytes that can be isolated from specific plant tissue, as no medium can meet the nutritional and growth requirements of all the bacteria. The isolates obtained showed diversity in colony color, elevation, form, margin, size, and the arrangement of cells.

The pathogen responsible for web blight disease was isolated from infected leaves of cowpea var. Bhagyalakshmi and identified as *Rhizoctonia solani* (GenBank Accession No. OL819875) through sequence analysis of ITS rDNA. Previous studies have reported the isolation of *R. solani* from the study location causing diseases in amaranth (14), cowpea, and rice (37). However, the extent of the pathogen's host range in different plant species remains unknown.

Endophytic bacteria may employ several mechanisms to exert biocontrol, such as the production of antifungal or antibacterial agents, siderophore production, nutrient competition, niche exclusion, and induction of systemic acquired host resistance or immunity (38). The initial screening for antagonistic activity of bacterial endophytes against the fungal pathogen was done using the dual culture plate technique, which is an easy-to-perform assay (39, 40, 21, 22). The presence of an inhibition zone in dual culture assay was considered as a sign of antagonism of endophytic bacteria against the pathogen. The current study is the first report on the antagonistic activity of bacterial endophytes from cowpea against *R. solani* inciting web blight in cowpea. However, antagonism against *R. solani in vitro* by endophytic *Bacillus* has been reported by Uppala *et al*. (41). Nagendran *et al*. (42) also reported inhi-

bition of the fungus by endophytic *Bacillus amyloliquefaciens in vitro*. Also, Yashaswini *et al*. (14) noted antagonistic activity by endospore-forming endophytic bacterial isolates from *Amaranthus* spp. in dual culture plate technique against *R. solani*.

Two isolates each from fodder cowpea and bush cowpea had inhibition against the pathogen with their secondary metabolites obtained by separating cells from the culture. Fungal cell collapse by lysis, necrosis, and hyphal disintegration have been reported by the hydrolytic action of amylase, pectinase, and cellulase present in the culture filtrate of bacterial isolates (43). Antibiotics and secondary metabolites produced by endophytic *Bacillus* inhibit the growth of *R. solani in vitro* as reported by Nagendran *et al*. (42). The cell-free extracts of endophytic bacteria may contain diffusible compounds that can induce hyphal deformation in *R. solani* (44). Consequently, based on the findings of direct and indirect antagonism, 20 promising endophytic bacterial isolates were chosen for further investigations.

Seed biopriming improves the viability and vigor of seeds before sowing. In addition, biopriming with antagonistic microorganisms can act as a protective barrier against seed deterioration due to phytopathogens (45). Seed germination and seedling vigour index are two main constituents that need to be assessed to get an understanding of the performance of antagonists in biopriming. A previous report suggested that four hours of biopriming of chili seed with endophytic *B. subtilis* and VLY 62 and *B. amyloliquefaciens* VLY 24 recorded maximum seedling vigor index (25). We observed that bacterial ino-culation significantly improved the seedling vigor index in cowpeas. Seed biopriming with endophytic *Bacillus* spp. has been shown to bring positive change in the growth and yield of other crop plants also (14, 25). Cowpea seedlings treated with isolate CFLE3 recorded a maximum vigor index. A significant influence on seedling vigour index (SVI) (90.99 to 223.66) by various endospore-forming endo-rhizosphere bacterial isolates from *Amaranthus viridis* was reported by Yashaswini *et al*. (14). Based on their *in vitro* antagonism and seedling vigour index, we identified CFLE3, CBRE5, and CBSE5 as the top three endophytic bacterial isolates for further investigation.

The promising endophytic bacterial isolates were tentatively identified following standard keys as per Bergey's Manual of Determinative Bacteriology as belonging to the genus *Bacillus.* The identity of the species of the isolates was further confirmed through 16S rRNA analyses and they were identified as *Bacillus subtilis*(CFLE3), *B. amyloliquefaciens* (CBRE5), and *B. velezensis* (CBSE5). The distribution of the isolates in the phylogenetic tree revealed diversity among the *B. amyloliquefaciens* CBSE5 and *B. velezensis* CBRE5 (Fig. 7). *Bacillus* sp. promotes plant growth directly or indirectly through the production of indole acetic acid (IAA), hydrogen cyanide (HCN), siderophores, phosphate solubilization, and antifungal metabolites (46). There is evidence that *B. subtilis* triggers the production of defense-related enzymes (phenylalanine ammonia lyase, peroxidase, and polyphenol oxidase), plant growth-promoting traits (IAA, siderophore, HCN, phosphate, zinc, and potas-

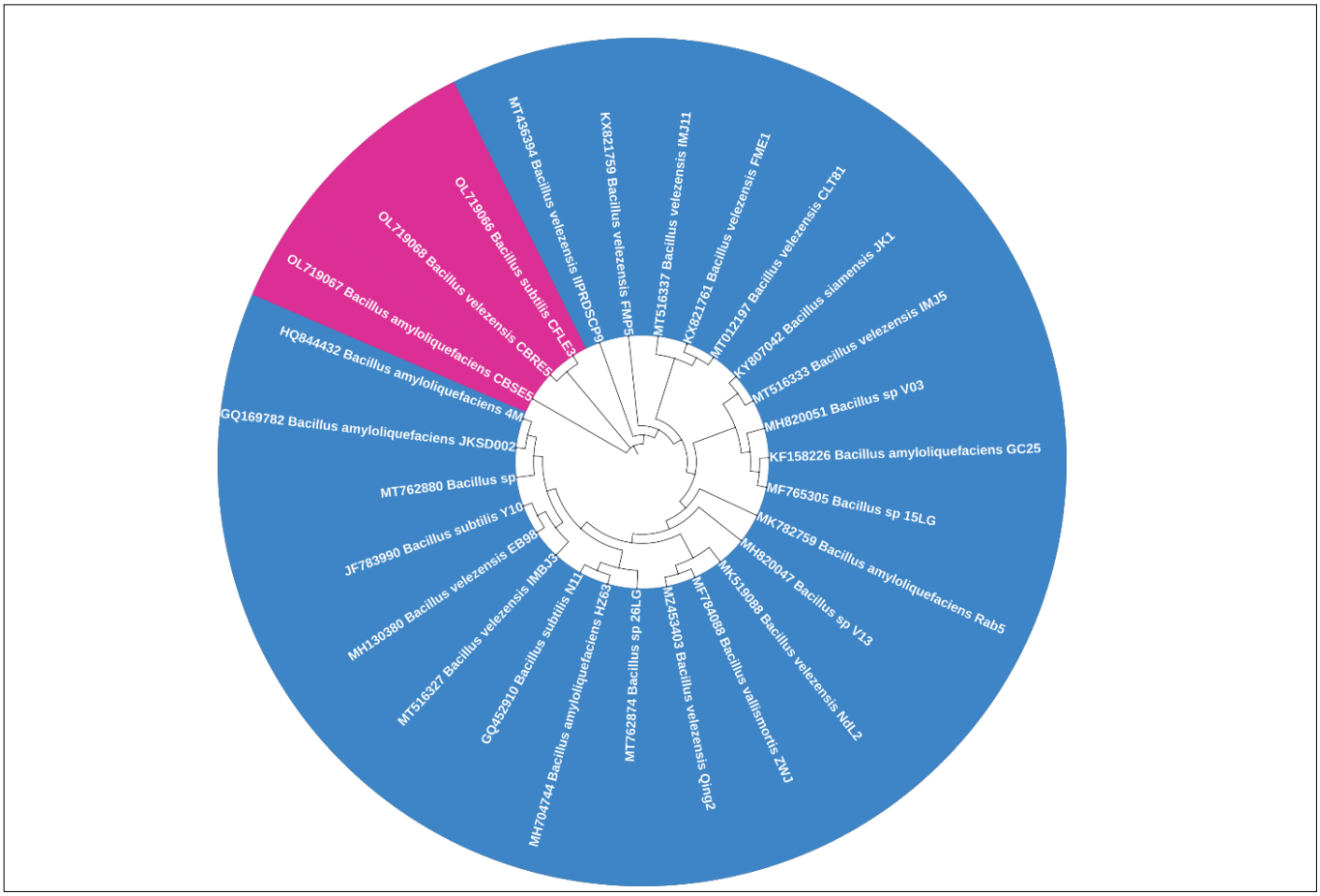

Fig. 7. Phylogenetic tree generated from 16S rRNA sequence analysis using MEGA11 (version 11.0.13) and displayed using iTOL (version 5.0). Cowpea endophytes forms a separate group and are highlighted in red shade.

sium solubilization) and antibiotics (bacylisin, surfactin, and fengycin) which are responsible for the ubiquitous nature, excellent colonization and antagonistic activity against *R. solani* in rice (47). Among the strains used in our study, *B. amyloliquefaciens* CBSE5 recorded the highest IAA production followed by *B. velezensis* and *B. subtilis* CFLE3. Indole-3-acetic acid (IAA) plays a crucial role in various plant growth and developmental processes such as cell division, nodule formation, seed germination, root and shoot development, and enhances photosynthe-tic rate. Additionally, IAA helps in providing resistance to plants against both biotic and abiotic stress (48). IAA production positively influences root growth and development, thereby enhancing nutrient uptake. Endophytic bacteria can solubilize the insoluble phosphorus in soil and make it available to plants thereby enhancing plant growth (49). Here, *B. velezensis* CBRE5 and *B. subtilis* CFLE3 indicated their ability for phosphate solubilization while no solubilization zone was shown by *B. amyloliquefaciens* CBSE5. The production of ammonia was recorded maximum by *B. velezensis* CBRE5 followed by *B. subtilis* CFLE3 and *B. amyloliquefaciens* CBSE5 in the present study. Siderophore production by endophytic bacteria suppresses the growth of deleterious microbes. Here, we noticed the zone of orange coloration in the specific medium confirming production of siderophore for all three endophytic bacterial isolates. Under iron stress conditions, these strains have been shown to produce siderophores that chelate the available iron, reducing the availability of iron for *R. solani*. This can limit the proliferation and root colonization of the pathogen, thereby potentially mitigating its impact on the plant.

The present study utilized seed biopriming for four hours and foliar application (at 20 and 40 DAS) as the mode of application for endophytic bacteria onto host plants. The best three bacterial endophytes and their combinations were evaluated for plant growth promotion. Several reports emphasize the growth promotion abilities of endophytic *B. subtilis, B. amyloliquefaciens,* and *B. velezensis* isolated from crop plants such as amaranth, chili, and pepper grown in similar locations (25, 13, 50). Now, it is evident that endophytic *Bacillus* spp. is effective in plant growth promotion in cowpeas also. *Bacillus* spp. have been reported to have additive and synergistic effects when used in combination, possibly due to the combination of different modes of action and plant growth-promoting traits (38). In the present case, the combined seed biopriming for 4 h along with the foliar application of *B. amyloliquefaciens* CBSE5 and *B. velezensis* CBRE5 (20 and 40 DAS) was the most effective treatment for plant growth promotion. The additive or synergistic effects behind the improved growth promotion in the combined application may be due to the production of IAA and solubilization of phosphorous by the combination of *B. amyloliquefaciens* CBSE5 and *B. velezensis* CBRE5.

Conclusion

Seed biopriming is a sustainable practice that ensures better seed health which ultimately results in improved crop production. Based on our results, it can be concluded that the combined application of the two endophytic bacterial isolates, *B. amyloliquefaciens* CBSE5 and *B. velezensis* CBRE5 through seed biopriming for 4 hr. followed by foliar application at 20 and 40 DAS was effective for promoting plant growth in bush cowpea. This treatment resulted in a 52% increase in pod yield compared to the control group *in vivo*. The application of plant growth-promoting endophytic bacteria with biocontrol potential is a novel approach to plant health management. A consortium of endophytic bacteria with multifarious mechanisms could simultaneously promote plant growth and suppress plant diseases effectively. Cowpea being a component crop that provides balanced nutrition to tropical populations, further validation of our findings under field conditions would pave the path for eco-friendly and sustainable safe–to–eat crop production.

Acknowledgements

The authors are grateful to the Kerala Agricultural University for providing facilities for carrying out the research.

Authors contributions

SM carried out the experiments, collected and analyzed the data, and drafted the original manuscript. SSJ supervised the work and drafted and reviewed the manuscript. SST provided the resources. HG provided the resources. KNA conceptualized and supervised the research, and drafted, reviewed, and edited the manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

References

1. Priyanka, Meena AK, Mathur AC, Bagri RK, Sharma RS. Current status and prospect of web blight of cowpea: A review. Legume Res. 2022;45:529-35. https://doi.org/10.18805/LR-4316
2. Basandrai AK, Basandrai D, Sharma BK. Fungicidal management of web blight of urd bean caused by *Rhizoctonia solani*. Legume Res. 2016;39:1038-42. https://doi.org/10.18805/lr.v39i6.6646
3. Ab Rahman SFS, Singh E, Pieterse CMJ, Schenk PM. Emerging microbial biocontrol strategies for plant pathogens. Plant Sci. 2018;267:102-11. https://doi.org/10.1016/j.plantsci.2017.11.012
4. Khan N, Bano A, Rahman MA, Guo J, Kang Z, Babar MA. Comparative physiological and metabolic analysis reveals a complex mechanism involved in drought tolerance in chickpea (*Cicer arietinum* L.) induced by PGPR and PGRs. Sci Rep. 2019;9:2097.| https://doi.org/10.1038/s41598-019-38702-8
5. Xia Y, Farooq MA, Javed MT, Kamran MA, Mukhtar T, Ali J *et al*. Multi-stress tolerant PGPR *Bacillus xiamenensis* PM14 activating sugarcane (*Saccharum officinarum* L.) red rot disease resistance. Plant Physiol Biochem. 2020;151:640-49. https://doi.org/10.1016/j.plaphy.2020.04.016
6. Zia R, Nawaz MS, Yousaf S, Amin I, Hakim S, Mirza MS, Imran A. Seed inoculation of desert PGPR induce biochemical alterations

and develop resistance against water stress in wheat. Physiologia Plantarum. 2021;172(2):990-1006. https://doi.org/10.1111/ppl.13362

7. Reinhold-Hurek B, Hurek T. Living inside plants: Bacterial endophytes. Curr Opin Plant Biol. 2011;14:435-43. https://doi.org/10.1016/j.pbi.2011.04.004

8. Dutta D, Puzari KC, Gogoi R, Dutta P. Endophytes: Exploitation as a tool in plant protection. Braz Arch Biol Technol. 2014;57:621-29. https://doi.org/10.1590/S1516-8913201402043

9. Marzan LW, Alam R, Hossain MA. Characterization identification and antibiogram studies of endophytic bacteria from cowpea (*Vigna unguiculata* (L.) Walp). Bangladesh J Agric Res. 2018;43:175-86. https://doi.org/10.3329/bjar.v43i2.37312

10. Siva M, Sreeja SJ, Thara SS, Heera G, Anith KN. Endophytic *Bacillus* spp. suppress *Rhizoctonia solani* web blight of bush cowpea. Rhizosphere. 2023;25:100682. https://doi.org/10.1016/j.rhisph.2023.100682

11. Bhutani N, Maheshwari R, Kumar P, Suneja P. Bioprospecting of endophytic bacteria from nodules and roots of *Vigna radiata*, *Vigna unguiculata* and *Cajanus cajan* for their potential use as bioinoculants. Plant Gene. 2021;28:100326. https://doi.org/10.1016/j.plgene.2021.100326

12. Kollakkodan N, Anith KN, Radhakrishnan NV. Diversity of endophytic bacteria from *Piper* spp. with antagonistic property against *Phytophthora capsici* causing foot rot disease in black pepper (*Piper nigrum* L.). J Trop Agric. 2017;55:63-70.

13. Athira S, Anith KN. Plant growth promotion and suppression of bacterial wilt incidence in tomato by rhizobacteria bacterial endophytes and the root endophytic fungus *Piriformospora indica*. Indian Phytopathol. 2020;73:629-42. https://doi.org/10.1007/s42360-020-00283-2

14. Yashaswini MS, Nysanth NS, Anith KN. Endospore-forming bacterial endophytes from *Amaranthus* spp. improve plant growth and suppress leaf blight (*Rhizoctonia solani* Kuhn) disease of *Amaranthus tricolor* L. Rhizosphere. 2021;19:100387. https://doi.org/10.1016/j.rhisph.2021.100387

15. Araujo WL, Maccheroni Jr W, Aguilar-Vildoso CI, Barroso PA, Saridakis HO, Azevedo JL. Variability and interactions between endophytic bacteria and fungi isolated from leaf tissues of citrus rootstocks. Can J Microbiol. 2001;47:229-36. https://doi.org/10.1139/w00-146

16. Kobayashi DY, Palumbo JD. Bacterial endophytes and their effects on plants and uses in agriculture. In: Bacon CW, White JF Jr. (eds) Microbial Endophytes. CRC Press. New York Marcel Dekker. 2000;199-233. https://doi.org/10.1201/9781482277302

17. Stragier P, Losick R. Molecular genetics of sporulation in *Bacillus subtilis*. Annu Rev Genet. 1996;30:297-341. https://doi.org/10.1146/annurev.genet.30.1.297

18. Hallmann J, Quadt-Hallmann A, Mahaffee WF, Kloepper JW. Bacterial endophytes in agricultural crops. Can J Microbiol. 1997;43:895-914. https://doi.org/10.1139/m97-131

19. Vyshakhi AS, Anith KN. Co-inoculation with the root endophytic fungus *Piriformospora indica* and endophytic bacteria improves growth of solanaceous vegetable seedlings. Int J Veg Sci. 2021;27:536-51. https://doi.org/10.1080/19315260.2021.1885555

20. Nair CB, Anith KN. Efficacy of acibenzolar-S-methyl and rhizobacteria for the management of foliar blight disease of amaranth. J Trop Agric. 2009;47:43-47.

21. Anith KN, Nysanth NS, Natarajan C. Novel and rapid agar plate methods for *in vitro* assessment of bacterial biocontrol isolates antagonism against multiple fungal phytopathogens. Lett Appl Microbiol. 2021;73(2):229-36. https://doi.org/10.1111/lam.13495

22. Nysanth NS, Sivapriya SL, Natarajan C, Anith KN. Novel *in vitro* methods for simultaneous screening of two antagonistic bacteria against multiple fungal phytopathogens in a single agar plate. 3 Biotech. 2022;12(6):140. https://doi.org/10.1007/s13205-022-03205-3

23. Anith KN. Mature coconut as a bio-fermentor for multiplication of plant growth promoting rhizobacteria. Curr Sci. 2009;1647-53. https://doi.org/10.2307/24107306

24. Abdul-Baki AA, Anderson JD. Vigour determination of soybean seed by multiple criteria. Crop Sci. 1973;13:630-33. https://doi.org/10.2135/cropsci1973.0011183X001300060013x

25. Athira PV, Radhakrishnan NV, Anith KN. Seed biopriming and spraying at fruit set with microbial agents suppress anthracnose disease and improve growth and yield in chilli. J Trop Agric. 2021;59(2):273-85.

26. Cappuccino JG, Sherman N. Microbiology-a laboratory manual. Harlow, England. 1999.

27. Tamura K, Stecher G, Kumar S. MEGA11: Molecular evolutionary genetics analysis version 11. Mol Biol Evol. 2021;38(7):3022-27. https://doi.org/10.1093/molbev/msab120

28. Letunic I, Bork P. Interactive tree of life (iTOL) v5: An online tool for phylogenetic tree display and annotation. Nucleic Acids Res. 2021;49(1):293-96. https://doi.org/10.1093/nar/gkab301

29. Balouiri M, Sadiki M, Ibnsouda SK. Methods for *in vitro* evaluating antimicrobial activity: A review. J Pharma Anal. 2016;6:71-9. https://doi.org/10.1016/j.jpha.2015.11.005

30. Gordon SA, Weber RP. Colorimetric estimation of indoleacetic acid. Plant Physiol. 1951;26:192. https://doi.org/110.1104/pp.26.1.192

31. Pikovskaya RI. Mobilization of phosphorus in soil in connection with vital activity of some microbial species. Microbiologiya 1948;17:362-70.

32. Gupta R, Singal R, Shankar A, Kuhad RC, Saxena RK. A modified plate assay for screening phosphate solubilizing microorganisms. J Gen Appl Microbiol. 1994;40:255-60. https://doi.org/10.2323/jgam.40.255

33. Lorck H. Production of hydrocyanic acid by bacteria. Physiol Plant. 1948;1:142-46. https://doi.org/10.1111/j.1399-3054.1948.tb07118.x

34. Milagres AM, Machuca A, Napoleao D. Detection of siderophore production from several fungi and bacteria by a modification of Chrome Azurol S (CAS) agar plate assay. J Microbiol Methods. 1999;37:1-6. https://doi.org/10.1016/S0167-7012(99)00028-7

35. Gopinath PP, Parsad R, Joseph B, Adarsh VS. GrapesAgri 1: Collection of shiny apps for data analysis in agriculture. J Open Source Softw. 2021;6:3437. https://doi.org/10.21105/joss.03437

36. Afzal I, Shinwari ZK, Sikandar S, Shahzad S. Plant beneficial endophytic bacteria: Mechanisms diversity host range and genetic determinants. Microbiol Res. 2019;221:36-49. https://doi.org/10.1016/j.micres.2019.02.001

37. Kumar HJ. Studies on the variability of *Rhizoctonia solani* Kuhn. infecting rice cowpea and amaranth. M.Sc. (Ag) thesis, Kerala Agricultural University, Thrissur. 2020.

38. Latha P, Karthikeyan M, Rajeswari E. Endophytic bacteria: Prospects and applications for the plant disease management. In: Ansari R. Mahmood I. (eds) Plant Health Under Biotic Stress. Springer Singapore. 2019;1-50. https://doi.org/10.1007/978-981-13-6040-4_1

39. Dennis C, Webster J. Antagonistic properties of species-groups of *Trichoderma*: I. Production of non-volatile antibiotics. Trans Br Mycol Soc. 1971;57:25-39. https://doi.org/10.1016/S0007-1536(71)80077-3

40. Anith KN, Radhakrishnan NV, Manomohandas TP. Screening of antagonistic bacteria for biological control of nursery wilt of black pepper (*Piper nigrum*). Microbiol Res. 2003;158: 91-97. https://doi.org/10.1078/0944-5013-00179

41. Uppala S, Beena S, Chapala M, Bowen KL. Amaranth endophytes and their role in plant growth promotion. In: Plant growth promotion by Rhizobacteria for sustainable agriculture. Scientific Publishers Jodhpur. 2010;531-37. https://doi.org/10.13140/RG.2.1.3291.3441

42. Nagendran K, Karthikeyan G, Mohammed Faisal P, Kalaiselvi P, Raveendran M, Prabakar K, Raguchander T. Exploiting endophytic bacteria for the management of sheath blight disease in rice. Biol Agric Hort. 2014;30;8-23. https://doi.org/10.1080/01448765.2013.841099

43. Kohl J, Kolnaar R, Ravensberg WJ. Mode of action of microbial biological control agents against plant diseases: Relevance beyond efficacy. Front Plant Sci. 2019;10:845. https://doi.org/10.3389/fpls.2019.00845

44. Huang X, Yong X, Zhang R, Shen Q, Yang X. The supernatant of *Bacillus pumilus* SQR-N43 has antifungal activity towards *Rhizoctonia solani.* J Basic Microbiol. 2013;53:657-63**.** https://doi.org/10.1002/jobm.201200291

45. El-Mohamedy RSR. Biopriming of okra seed to control damping off and root rot diseases. Annals Agric Sci. 2004;49(1):339-56.

46. Jamali H, Sharma A, Srivastava AK. Biocontrol potential of *Bacillus subtilis* RH5 against sheath blight of rice caused by *Rhizoctonia solani*. J Basic Microbiol. 2020;60:268-80. https://doi.org/10.1002/jobm.201900347

47. Ji SH, Gururani MA, Chun, SC. Isolation and characterization of plant growth promoting endophytic diazotrophic bacteria from Korean rice cultivars. Microbiol Res. 2014;169(1):83-98. https://doi.org/10.1016/j.micres.2013.06.003

48. Duca D, Lorv J, Patten CL, Rose D, Glick BR. Indole-3-acetic acid in plant–microbe interactions. Antonie Van Leeuwenhoek. 2014;106(1):85-125. https://doi.org/10.1007/s10482-013-0095-y

49. Alori ET, Glick BR, Babalola OO. Microbial phosphorus solubilization and its potential for use in sustainable agriculture. Front Microbiol. 2017;8:971. https://doi.org/10.3389/fmicb.2017.00971

50. Yashaswini MS, Nysanth NS, Gopinath PP, Anith KN. Endospore-forming phyllosphere bacteria from *Amaranthus* spp. suppress leaf blight (*Rhizoctonia solani* Kühn) disease of *Amaranthus tricolor* L. J Trop Agric. 2022;60:94-106.