Endospore-forming phyllosphere bacteria from *Amaranthus* spp. suppress leaf blight (*Rhizoctonia solani* Kühn) disease of *Amaranthus tricolor* L.

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Abstract

Amaranthus (*Amaranthus tricolor* L.) is the most preferred leafy vegetable of Southern India. Leaf blight incited by *Rhizoctonia solani* is a devastating disease of amaranthus, especially in the popular red leaf varieties. Many phyllosphere microorganisms have been reported for their antagonism against foliar phytopathogens. Endospore-forming phyllosphere bacteria were isolated from *Amaranthus* spp. by an efficient "two-step" enrichment process and identified them as *Bacillus* spp. based on morphological, biochemical and molecular characterization. Their *in vitro* antagonistic activity against *R. solani* was tested by dual culture plate assay and detached leaf assay. All the eight isolates showed *in vitro* antagonism in the plate assay and suppression of development of disease symptom in the leaf assay. Six isolates with better antagonism were selected for further *in vivo* studies in a greenhouse experiment. Treatments included foliar spray on leaf blight susceptible red amaranthus variety Arun with cell suspension of bacterial isolates and the recommended fungicide Mancozeb (0.2%), pathogen inoculated control and absolute control. *Bacillus* sp. AL3 obtained from the red amaranthus variety Arun exhibited 44.5 % disease suppression over the pathogen inoculated control. Foliar spray with the recommended fungicide, mancozeb (0.2%), performed poorly in suppressing the disease compared to foliar application of bacterial cell suspension except for a single strain. This forms the first report of exclusive use of endospore-forming phyllosphere bacteria as biocontrol agents against *Rhizoctonia solani*.

Keywords: *Amaranthus* spp., *Bacillus* spp., Endospore-forming bacteria, Leaf blight, Phyllosphere, *Rhizoctonia solani*.

Introduction

Amaranthus (*Amaranthus tricolor* L.) is one of the commercially important leafy vegetables cultivated and consumed in Southern India. It is commonly known as "poor man's spinach" and is one of the cheapest and most accepted leafy vegetables grown in Kerala, India. It is a short duration crop with low cost of production which is an integral part in the crop rotation practices of Kerala. Leaf blight disease incited by soil borne fungal pathogen *Rhizoctonia solani* Kühn is a major constraint of amaranthus production, as leaves, the economically important plant part, are affected (Nayar et al., 1996). Even though both red and green amaranthus varieties are used for culinary purpose, people of Kerala prefer red amaranthus to the green one. But red amaranthus is more susceptible to *Rhizoctonia* leaf blight *(*Celine et al., 2003*)*. Symptoms are initiated as light cream-coloured spots on the foliage which later cover the whole leaf very quickly (Nayar et al., 1996). The severity of the disease is high under warm and humid conditions of the rainy season. Foliar spray with mancozeb (0.2 %) at biweekly intervals is the recommended chemical control measure for managing the disease (Gokulapalan et al., 1999).

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Since the consumed plant parts are the leaves, chemical spray on the leaves is not encouraged and there is need for safer and eco-friendly alternatives for managing the disease.

Foliar spray with turmeric powder and baking soda mixture in the ratio of 1:4, @ 36 g per 10L water was found to be effective in suppressing the disease (Gokulapalan et al., 1999). When the infection is severe, the disease spread is difficult to control. Plant Growth Promoting Rhizobacteria (PGPR) have been used as biological control agents against leaf blight disease. Application of PGPR strains belonging to *Pseudomonas* and *Bacillus* along with the plant activator, Acibenzolar-S-methyl (ASM) has been found to reduce the disease in the susceptible variety of amaranthus "Arun", under greenhouse conditions (Nair et al., 2007; Nair and Anith, 2009). Disease severity was less when *Pseudomonas fluorescens* PN026 and ASM were applied together compared to the chemical treatment (0.2% copper oxychloride). Uppala et al. (2010) used endophytic bacteria of different genera (*Pseudomonas* sp. EB-22 and *Bacillus* sp. EB-43) from *Amaranthus* spp. for leaf blight management in susceptible red amaranthus variety Arun under sterile soil conditions. Two bacterial isolates from the red amaranthus variety suppressed the disease severity to 37.6 percentage over the pathogen inoculated control. In all these cases the microbial agents were applied to the root zone of the crop and exploited the principle of induced systemic resistance in disease management. If direct antagonism of bacterial biocontrol agents against the fungal pathogen on the foliage, *i.e*., at the site of infection, is exploited, better suppression of the pathogen could be expected. Phylloplane bacteria from *Amaranthus* spp. would be the most appropriate candidates for such an intervention as the disease is mainly confined to the foliage of the crop.

Phylloplane is an important environment with abundant microbial activity. Inadequate nutrient accessibility, higher temperature and moisture fluctuations are the unusual conditions faced by phylloplane colonizing bacteria (Dubey et al., 2017). However, many of them are bestowed with plant growth promoting (Dubey et al., 2017) and biocontrol traits (Andrews, 1992). Recent years have witnessed endospore-forming *Bacillus* spp. gaining attention as beneficial bacteria in agriculture. Many strains within this genus possess traits responsible for plant growth promotion and disease suppression (Kloepper et al., 2004; Kumar et al., 2011a; Chowdhury et al., 2015). The ability of *Bacillus* spp. to form endospores is of particular significance. These extremely resistant structures are dormant and can tolerate a wide range of environmental stresses for extended periods. This property helps in preparation of different formulations of such beneficial microbes that give rise to stable products with extended shelf lives. The aim of this study was to isolate and identify endospore forming bacteria with antagonistic property against *R. solani* from phyllosphere of *Amaranthus* spp. and test their disease suppressive ability against amaranthus leaf blight.

Materials and Methods

Isolation of endospore- forming phyllosphere bacteria from Amaranthus spp

Five leaf samples were collected from one month old healthy red amaranthus *(Amaranthus tricolor)* variety Arun, green amaranthus variety CO-1 and the wild amaranthus *(A. viridis)* from the Instructional Farm, College of Agriculture, Vellayani, Thiruvananthapuram. Isolation of phyllosphere bacteria was done as per the protocol by Batool et al. (2016) with modifications. A "two-step" enrichment of the samples was carried out prior to isolation. Leaf samples were rinsed quickly once with sterile distilled water and placed in sterile Petri plates for drying in a laminar air flow chamber. They were then kept at 35^{o} C for two consecutive days for partial drying. The partially dried leaf sample was placed in 50 mL sterile distilled water taken in a 250 mL capacity conical flask and kept in a shaker (200 rpm) for 1.5 h. Leaf samples were taken out of the flask and the flasks with the

suspension were heated at 80°C for 15 min in a water bath. The heated leaf wash (0.1 mL) was directly plated on Nutrient Agar (M561A-500G, HiMedia Laboratories, Mumbai, India) plates. The suspension was serially diluted to 10-fold and 100-fold and 0.1mL aliquots were plated on nutrient agar medium. The agar plates were incubated at 28°C. Bacterial colonies with same morphological features based on colony characters (colour, form, elevation and margin) were grouped together. The isolates were sub-cultured, checked for purity and preserved on NA slants under refrigerated condition as well as in sterile glycerol (25%) at -80°C.

Characterization of endospore-forming phyllosphere bacterial isolates

Bacterial isolates were streak purified on NA plates to get single well isolated colonies and colony colour and morphology were observed. The cell shape, cell arrangement, Gram's reaction and endospore formation were also observed (Cappuccino and Sherman, 1992).

Characterization of the eight endospore-forming phyllospheric bacterial isolates was done biochemically by performing various biochemical tests and carbohydrate utilization tests by using readymade HiMedia© kits (KB001 HiIMViC Biochemical Test Kit; HiMedia Laboratories, Mumbai, India). Various biochemical tests performed were Indole, Methyl red, Voges Proskauer and Citrate utilization. Carbohydrate utilization pattern for glucose, adonitol, arabinose, lactose, sorbitol, mannitol, rhamnose and sucrose were carried out. The results of biochemical tests were used to arrive at a tentative genus level identification of isolates as prescribed by Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

Molecular identification of the pure cultures of endospore-forming phyllospheric bacterial isolates were done by 16S rRNA cataloguing using universal primers, 16S-RS-F; forward 5' CAGGCCTAACACATGCAAGTC3' and 16S-RS-R; reverse 5'GGGCGGWGTGTACAAGGC 3'. Genomic DNA was isolated using NucleoSpin® Tissue Kit (Macherey-Nagel, USA) following manufacturer's instructions (Hudlow et al., 2011). The quality of the DNA isolated was checked using agarose gel electrophoresis. PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X PCR buffer (100mM Tris HCl, pH-8.3; 500mM KCl), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 2.5mM $MgCl_2$, 1 unit of AmpliTaq Gold DNA polymerase enzyme, 0.1 mg/mL BSA, 0.7 µl of 4% DMSO, 5pM of forward and reverse primers and FTA disc as template. The amplification was carried out in a PCR thermal cycler (Gene Amp PCR System 9700, Applied biosystems). The amplification conditions were as follows: initial denaturation at 95°C for 5 min; 35 cycles of amplification with denaturation at 95°C for 30 sec, annealing at 60°C for 40 sec and extension at 72°C for 60 sec and a final extension at 72°C for 7 min. Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the Big Dye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufactures protocol. The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Nucleotide BLAST analysis was done with the contig sequence made after alignment using BioEdit programme. Identity of the organism was ascertained by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) output. Phylogenetic relatedness between the endospore forming phyllosphere bacterial isolates was found out by tree construction using the software MEGA X (Version 10).

Phytopathogen and culture conditions

Rhizoctonia solani was obtained from the culture collection of Department of Agricultural Microbiology, College of Agriculture, Kerala Agricultural University, Vellayani, Kerala, India. The fungal pathogen was maintained and grown on Potato dextrose agar (PDA) medium at 28°C for seven days. Virulence and pathogenicity of the

fungus was tested by artificial inoculation on detached healthy leaves of Amaranthus var. Arun as described by Nair and Anith (2009). Development of lesion was observed 24-48 h after pathogen inoculation.

In vitro antagonism of endospore-forming phyllosphere bacteria against Rhizoctonia solani

All the eight bacterial isolates were screened for their effectiveness in inhibiting the growth of *R. solani* on PDA medium by dual culture plate assay (Dennis and Webster, 1971; Anith et al., 2021). A single mycelial plug (4 mm dia) of *R. solani* from a five-day-old PDA plate was placed at the centre of a fresh PDA plate. Two streaks of 2.5 cm length of fresh bacterial culture were made on two opposite edges of the plate. *R. solani* alone in PDA plates was maintained as control. Plates were incubated at 28°C for 2 days. Observations were recorded by measuring the zone of inhibition if any.

Antagonistic effect of the culture filtrate of the bacterial bioagents against the leaf blight pathogen was assessed by agar well diffusion method (Balouiri et al., 2016; Athira and Anith, 2020). The bacterial bioagents were purified and a loopful of cells from a single isolated bacterial colony was transferred to 100 mL Nutrient broth prepared in 250 mL Erlenmeyer flask. Inoculated flasks were incubated overnight in an incubator shaker (100 rpm) at 28°C. 10 mL of the broth culture of each of the bacterial isolates was centrifuged at 10000 rpm for 5 min in sterile polypropylene tubes. The supernatant of each of the bacterial bioagents was collected aseptically and filter sterilized using a 0.2 µm nitrocellulose bacteriological filter and stored at 4°C for further use. PDA plates with *R. solani* mycelial plugs placed at the centre were prepared as mentioned above and were incubated at 28°C for one day. After incubation, at two opposite edges of the plate, wells (8 mm dia) were cut using a sterile cork borer. The wells were partially filled with 100 µl of 1% molten agar to avoid the loss of culture filtrate. As the agar in the well solidified, 100 µl of the culture filtrate of each bacterial bioagents was added to the wells in the individual plates. Four replicates were kept for each of the bacterial strain and plates were incubated for 48 h at 28° C. Observations were recorded and the zone of inhibition of the fungal growth around the well was measured.

Screening by detached leaf assay

Detached leaf assay was done as per the procedure described by Anith et al. (2003) with slight modifications. Bacterial isolates were heavily cross streaked on NA medium. After one day of incubation at 28°C, the plates were drenched with 10 mL sterile distilled water and scraped off to obtain bacterial suspension. The bacterial suspension was vortexed to get a uniform suspension. The OD values of the suspensions, measured in a spectrophotometer (Shimadzu 990i, Shimadzu Corporation, Japan) at 600 nm, were adjusted to 0.5 by adding sterile water as required which corresponded to an approximate cell density of 10^7 cfu mL^{-1} (Athira et al., 2021). Detached leaves of amaranthus (var. Arun) were washed in sterile water, moisture removed by drying in a laminar airflow chamber and treated with individual bacterial isolates by briefly dipping them in the bacterial cell suspension for one min. Leaves dipped in sterile water served as control. The treated leaves were kept inside a laminar air flow chamber and allowed to get rid of the moisture. They were then transferred to sterile Petri dishes. From the fully grown *R. solani* plates, mycelial discs (4 mm dia) were cut out. Challenge inoculation was carried out by placing the mycelial discs on the lower surface of the leaf. Moist cotton was placed over the mycelial disc. To avoid drying of leaves the proximal end of the leaves were covered with moist cotton. Appearance of leaf lesions was observed from the first day of inoculation up to four days. The antagonistic activity of bacterial isolates was assessed by measuring the area of lesion developed (cm^2) (Paul et al., 2021). Each treatment was maintained with four replications.

In vivo disease suppression

Based on the better antagonism activity under *in vitro* conditions, six isolates (*Bacillus* sp., AL1 *Bacillus* sp., AL3 *Bacillus* sp., GL2 *Bacillus* sp., GL3 *Bacillus subtilis* WL1 and *Bacillus amyloliquefaciens* WL2) were selected. The efficacy of selected six endospore-forming phyllosphere bacteria in controlling the foliar blight disease caused by *R. solani* in the red amaranthus var. Arun was tested in a pot culture experiment under naturally ventilated greenhouse covered with low density polyethylene sheet of 200-micron thickness and 85% light transmissibility. The treatment details are given in Table 6. Seedlings were raised in the protrays with sterilized vermiculite as planting medium during May, 2020. Vermiculite was sterilized by autoclaving at 121° C for 1 h each for three consecutive days. Protrays having 40 cells, each cell measuring a diameter of 5 cm were filled with the sterile vermiculite. Seed of amaranthus were surface sterilized in 1% sodium hypochlorite aqueous solution for 3 min under aseptic conditions. Seed were further washed thrice with sterile distilled water to get rid of the sodium hypochlorite. Two surface sterilized seed were sown per cell of protray and after germination thinning was carried out to maintain a single seedling per cell. Seedlings were irrigated with sterile water twice a day. After 15 days of seeding, at four leaf stage, seedlings were transplanted to plastic pots (15 cm dia) filled with one kg each of unsterile potting mixture (soil, coir pith and cow dung in the ratio 2:1:1) during May-June, 2020.

After 15 days of transplanting, foliar spray with six promising endospore-forming phyllosphere bacteria selected from the *in vitro* antagonism tests was carried out. The bacterial isolates used were *Bacillus* sp. AL1, *Bacillus* sp. AL3, *Bacillus* sp. GL2, *Bacillus* sp. GL3, *Bacillus subtilis* WL1 and *Bacillus amyloliquefaciens* WL2. Inoculum for spraying was prepared following the procedures described by Kollakkodan et al. (2021). Bacterial cell inoculum (10^7 cfu mL^{-1}) was prepared by adjusting the OD of the suspension to 0.5 at 600 nm using a spectrophotometer (Shimadzu 990i, Shimadzu Corporation, Japan) and sprayed on to the foliage of plants. Plants sprayed with Mancozeb (0.3 %) were kept as chemical control. Pathogen inoculated and absolute (uninoculated) controls were also maintained. The experiment was designed in CRD having four replications with five plants each. They were irrigated twice daily.

Inoculation with virulent strain of *Rhizoctonia solani* was carried out to top three fully unfolded intact healthy leaves of each plant of all treatments except the absolute control. Mycelial plugs from five-days-old fully grown PDA plates of *R. solani* were made using 4 mm cork borer. Inoculation of *R. solani* was done on lower leaf surface of intact leaves after 7 days of bacterial suspension spray. The mycelial plugs were covered with thin layer of moist cotton to provide humid condition. Observations on disease incidence and disease severity were recorded on 3rd and 5th day after inoculation (DAI) of the pathogen. Disease severity was calculated for each plant inoculated with pathogen. Severity of disease was graded by using the 0-9 scale (Nair and Anith, 2009).

Grade	Description
0	No infection
1	1 – 10 percent of leaf area infected
3	11 – 25 percent of leaf area infected
5	26 – 50 percent of leaf area infected
7	51 – 75 percent of leaf area infected
9	76 – 100 percent of leaf area infected

Percent Disease index (PDI) was calculated using the formula:

$$PDI = \frac{\text{Sum of individual rating}}{\text{No. of leaves assessed x Maximum grade used}} \times 100$$

Statistical analysis

Statistical analysis was done using R based analysis platform of Kerala Agricultural University, GRAPES (General Rshiny Based Analysis Platform Empowered by Statistics; https://www.kaugrapes.com/home) by one way Analysis

of Variance (ANOVA) and the treatment means were compared using Duncan's Multiple Range (DMRT) at a probability of 0.05 %.

Results and Discussion

Characterization of endospore-forming phyllosphere bacterial isolates

Three isolates each were obtained from the red amaranthus variety Arun (AL1, AL2, AL3) and the green amaranthus variety CO-1 (GL1, GL2, GL3) respectively on NA medium. From the wild type amaranthus two isolates (WL1, WL2) were obtained. As per Bergey's Manual of Determinative Bacteriology all the isolates were tentatively identified as *Bacillus* spp. (Holt et al., 1994). All of the isolates were Gram positive and showed presence of endospores in the staining process. Morphological, biochemical and 16SrRNA gene sequence analysis confirmed the position of the

Table 1. Characteristics of endospore-forming phyllosphere bacterial isolates from *Amaranthus* spp.

Isolates*	Cell shape and arrangement	Colony characters				Gram's reaction	Endospore formation
		Colour	Form	Elevation	Margin		
AL1	Single rod	Creamy white	Irregular	Raised	Undulate	G^+	+
AL2	Single rod	Creamy white	Irregular	Raised	Undulate	G^+	+
AL3	Single rod	Creamy white	Irregular	Raised	Undulate	G^+	+
GL1	Single rod	Creamy white	Irregular	Raised	Undulate	G^+	+
GL2	Single rod	Brownish creamy white	Irregular	Flat	Undulate	G^+	+
GL3	Single rod	Creamy white	Irregular	Raised	Undulate	G^+	+
WL1	Single rod	Creamy white	Irregular	Raised	Undulate	G^+	+
WL2	Single rod	Creamy white	Irregular	Raised	Undulate	G^+	+

*AL – Isolates from leaves of variety Arun; GL – Isolates from leaves of green variety CO-1; WL – Isolates from leaves of wild amaranthus

Table 2. Biochemical characters of endospore-forming phyllosphere bacterial isolates from *Amaranthus* spp.

Biochemical test	Isolates							
	AL1	AL2	AL3	GL1	GL2	GL3	WL1	WL2
Indole	-	-	-	-	-	-	-	-
Methyl red	+	+	-	-	+	+	-	+
Voges Proskauer's	+	+	+	+	+	+	+	+
Citrate utilization	-	-	-	-	+	-	-	-
Glucose utilization	-	-	-	+	-	-	-	-
Adonitol utilization	-	-	-	-	-	-	-	-
Arabinose utilization	-	-	-	-	+	-	-	-
Lactose utilization	-	-	-	-	-	-	-	-
Sorbitol utilization	-	-	-	-	+	+	-	-
Mannitol utilization	-	-	-	-	-	-	-	-
Rhamnose utilization	-	-	-	-	-	-	-	-
Sucrose utilization	-	-	-	-	-	-	-	-

*AL – Isolates from leaves of variety Arun; GL – Isolates from leaves of green variety CO-1; WL – Isolates from leaves of wild amaranthus

Table 3. BLAST search details of 16S rRNA gene sequences producing most significant alignment of contig sequences of endospore-forming phyllosphere bacterial isolates from *Amaranthus* spp

Isolates*	Max score	Total score	Query cover (%)	Best match in Genbank data base	Accession no.
AL1	2239	2239	98%	*Bacillus* sp.	JX155393.1
AL2	2206	2206	98%	*Bacillus subtilis*	MN960406.1
AL3	2239	2239	99%	*Bacillus* sp.	KJ669214.1
GL1	2248	2248	99%	*Bacillus amyloliquefaciens*	AB547229.1
GL2	2268	2268	98%	*Bacillus* sp.	MF765317.1
GL3	2161	2161	98%	*Bacillus* sp.	KJ669214.1
WL1	2268	2268	98%	*Bacillus subtilis*	HE98518.1
WL2	2261	2261	98%	*Bacillus amyloliquefaciens*	AB547229.1

*AL – Isolates from leaves of variety Arun; GL – Isolates from leaves of green variety CO-1; WL – Isolates from leaves of wild amaranthus

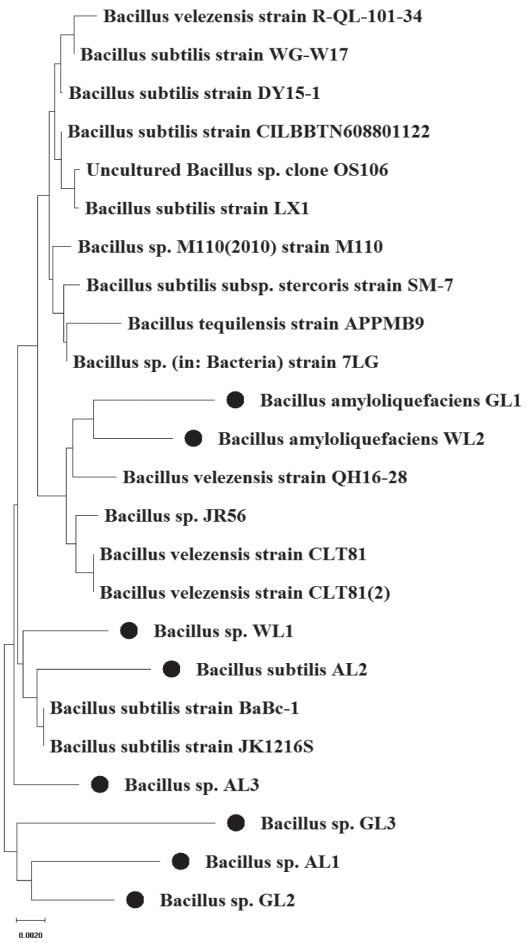

Figure 1. Phylogenetic relatedness of endospore-forming phyllosphere bacterial isolates from *Amaranthus* spp. Black bullets represent the endospore-forming isolates from *Amaranthus* spp.

isolates within the genus *Bacillus* (Table 1-3). Phylogenetic analysis based on alignment of nucleotide sequences of 16S rRNA gene of endospore forming phylosphere bacterial isolates from *Amaranthus* spp. showed relatedness with other spore forming bacilli (Fig 1).

In vitro antagonism of endospore-forming phyllosphere bacteria against Rhizoctonia solani

Presence of zone of inhibition in dual culture plate was considered as a sign of direct antagonism of endospore forming phyllosphere bacterial isolates against *R. solani.* All isolates inhibited growth of

Table 4. In vitro antagonism of endospore - forming phyllosphere bacterial isolates against *Rhizoctonia solani*

Isolates	Zone of inhibition (mm) in dual culture plate assay*	Zone of inhibition (mm) in culture filtrate assay*
AL1	3.17 ± 0.75^{c}	1.37 ± 0.52^{cd}
AL2	2.00 ± 0.63^{d}	1.12 ± 0.64^{cde}
AL3	4.83 ± 0.75^{b}	2.12 ± 0.64^{b}
GL1	1.00 ± 0.63^{e}	0.50 ± 0.53^{e}
GL2	3.00 ± 0.63^{c}	1.75 ± 0.71^{bc}
GL3	8.17 ± 0.75^{a}	2.88 ± 0.64^{a}
WL1	4.50 ± 1.05^{b}	2.25 ± 0.71^{ab}
WL2	7.67 ± 1.03^{a}	0.87 ± 0.64^{de}

*Mean (+ SD) of 4 replications (n = 8). Values in a column followed by same letter do not differ significantly according to Duncan's Multiple Range Test ($p \leq 0.05$)

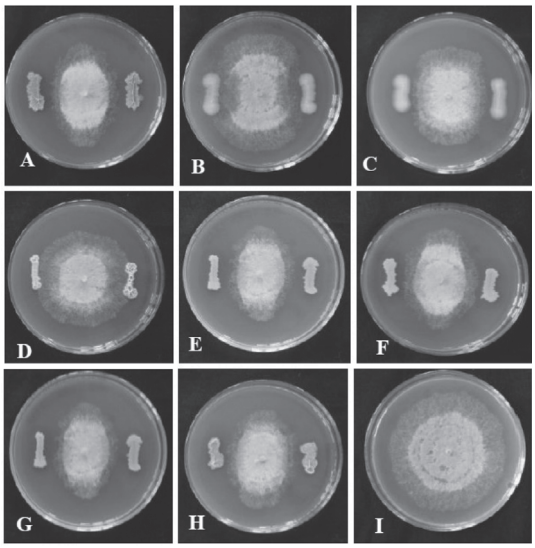

Figure 2. Antagonism of endospore-forming phyllosphere bacterial isolates against *Rhizoctonia solani* in dual culture plate assay
A-*Bacillus* sp. AL1, **B-** *Bacillus subtilis* AL2, **C-** *Bacillus* sp. AL3, **D-** *Bacillus amyloliquefaciens* GL1, **E-** *Bacillus* sp. GL2, **F-***Bacillus* sp. GL3, **G-** *Bacillus subtilis* WL1, **H-** *Bacillus amyloliquefaciens* WL2, **I-** Control

the fungal pathogen. Culture filtrate of all bacterial isolates also showed inhibition of growth of *R. solani* (Table 4; Fig 2-3).

R. solani on artificial inoculation on detached healthy leaves of red amaranthus variety Arun under *in vitro* produced typical blight symptoms.

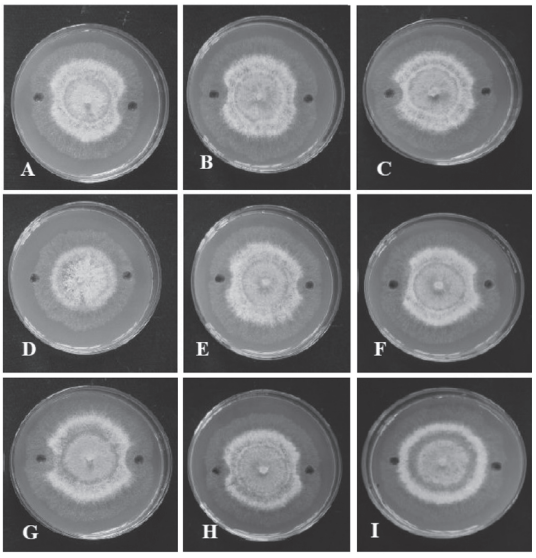

Figure 3. Indirect antagonism by culture filtrate of endospore-forming phyllosphere bacterial isolates against *Rhizoctonia solani*. **A-***Bacillus* sp. AL1, **B-** *Bacillus subtilis* AL2, **C-** *Bacillus* sp. AL3, **D-** *Bacillus amyloliquefaciens* GL1, **E-** *Bacillus* sp. GL2, **F-** *Bacillus* sp. GL3, **G-** *Bacillus subtilis* WL1, **H-** *Bacillus amyloliquefaciens* WL2, **I-** Control

Table 5. Lesion development in detached leaf assay

Treatments	Lesion Area (cm^2)*	
	3 DAI	4 DAI
AL1	0.96 ± 0.32^{ab}	3.27 ± 2.54^{ab}
AL2	2.62 ± 2.06^{bc}	8.18 ± 2.83^{bc}
AL3	1.12 ± 0.68^{ab}	7.16 ± 3.66^{abc}
GL1	2.77 ± 0.70^{bc}	9.59 ± 3.59^{cd}
GL2	2.52 ± 1.73^{bc}	6.70 ± 2.61^{abc}
GL3	1.13 ± 0.77^{ab}	6.99 ± 4.32^{abc}
WL1	0.94 ± 1.28^{ab}	3.47 ± 2.38^{ab}
WL2	$0.42 \pm 0.40a$	3.11 ± 1.41^{a}
Pathogen Control	3.49 ± 1.59^{c}	12.23 ± 3.17^{d}

*DAI- Days after inoculation. Mean (+ SD) of 4 replications (n = 12). Values in a column followed by same letter do not differ significantly according to Duncan's Multiple Range Test ($p \leq 0.05$)

Symptoms initiated as translucent irregular green patches on the inoculated areas of leaves after two days of inoculation. Gradually the patches enlarged and covered the entire leaf lamina. Later, the leaves turned creamy and dried up. In the detached leaf assay for assessing *in vitro* suppression of the pathogen, there was significant difference between lesion development on leaves treated with bacterial isolates and those treated with water as control (Table 5; Fig. 4).

Figure 4. Screening of endospore-forming phyllosphere bacterial isolates against *Rhizoctonia solani* by detached leaf assay. **A-** *Bacillus* sp. AL1, **B-** *Bacillus subtilis* AL2, **C-** *Bacillus* sp. AL3, **D-** *Bacillus amyloliquefaciens* GL1, **E-** *Bacillus* sp. GL2, **F-***Bacillus* sp. GL3, **G-** *Bacillus subtilis* WL1, **H-** *Bacillus amyloliquefaciens* WL2, **I-** Absolute control, **J-** Pathogen inoculated control.

In vivo disease suppression

Treatments had significant effect on the PDI calculated by scoring the lesion size on inoculated leaves at 3 DAI and 5 DAI. All the pathogen inoculated plants developed symptoms. Plants treated with *Bacillus* sp. AL3 showed the least percentage disease index (PDI) (18.75) and had the maximum disease suppression (44.51 %) over pathogen control when scoring was done 5 DAI (Table 6; Fig 5). Disease suppression was better in plants treated with bacterial isolates than that in the chemical spray except those treated with *Bacillus* sp. AL1.

Table 6. Effect of endospore forming phyllosphere bacterial treatments on the per cent disease index at 3 DAI and 5 DAI

Treatments	Per cent disease indexon 3 DAI*	Disease suppression over the pathogen control on 3 DAI (%)	Per cent disease index on 5 DAI*	Disease suppression over the pathogen control on 5DAI (%)
T1 *Bacillus* sp. AL1	$14.81 \pm 2.93^{bc}_{b}$	15.80	$28.70 \pm 11.64^{bc}_{b}$	15.06
T2 *Bacillus* sp. AL3	11.81 ± 3.41	32.86	18.75 ± 2.76	44.51
T3 *Bacillus* sp. GL2	$13.66 \pm 3.58^{bc}_{b}$	22.34	$20.83 \pm 7.80^{bc}_{bc}$	38.35
T4 *Bacillus* sp. GL3	11.80 ± 2.31	32.92	25.51 ± 10.48	24.50
T5 *Bacillus subtilis* WL1	15.51 ± 3.57^{bc}	11.82	25.92 ± 1.07^{bc}	23.29
T6 *Bacillus amyloliquefaciens* WL2	$12.26 \pm 2.05^{bc}_{c}$	30.30	$20.60 \pm 5.47^{bc}_{c}$	39.03
T7 Pathogen control	17.59 ± 4.60	-	33.79 ± 6.30	-
T8 Chemical control(0.3% Mancozeb)	14.25 ± 5.41^{bc}	18.99	26.85 ± 14.13^{bc}	20.54
T9 Absolute control	0.00 ± 0.00^{a}	100	0.00 ± 0.00^{a}	100

DAI - Days after inoculation. * Mean (+ SD) of 4 replications (n = 20). Values in a column followed by same letter do not differ significantly according to Duncan's Multiple Range Test ($p \leq 0.05$)

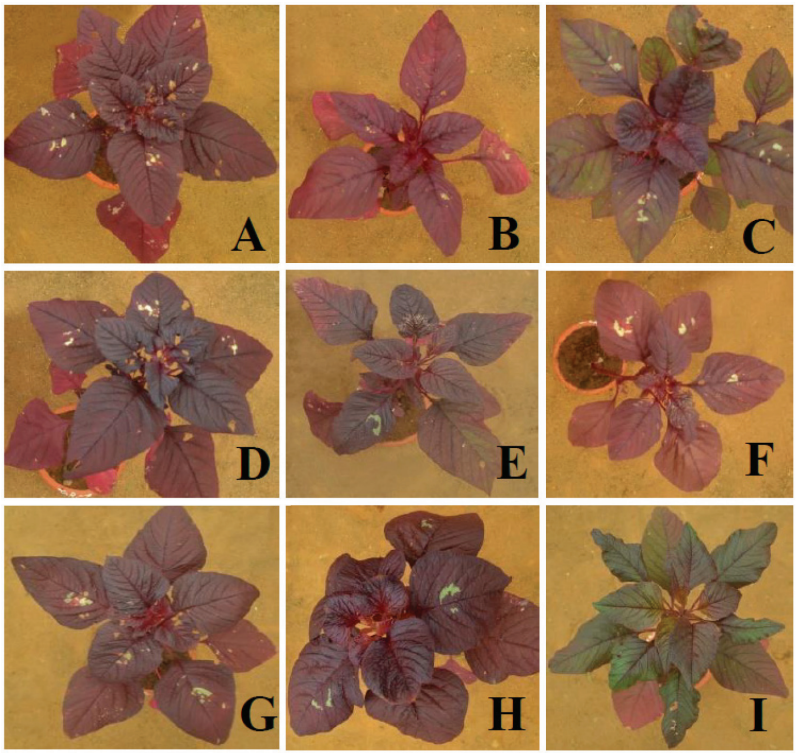

Figure 5. Leaf blight disease suppression in amaranthus var. Arun on inoculation with endospore-forming phyllosphere bacteria. **A-** *Bacillus* sp. AL1, **B-** *Bacillus* sp. AL3, **C-** *Bacillus* sp. GL2, **D-** *Bacillus* sp. GL3, **E-***Bacillus subtilis* WL1, **F-***Bacillus amyloliquefaciens* WL2, **G-** Pathogen inoculated control, **H-**Chemical control (0.3% Mancozeb), **I-** Absolute control

Strong experimental data are available on the potential role of phyllosphere bacteria in disease suppression and plant growth promotion in many crops (Lindow and Brandl, 2003; Madhaiyan et al., 2006; Nysanth et al., 2019). Isolation of epiphytic endospore forming bacteria is an easy procedure which is done by heat treatment of the target source at 80°C for 10 minutes in water and then plating the suspension on bacteriological media (Priest and Grigorova, 1990). In the present study isolation of endospore-forming bacteria from red amaranthus variety Arun, green Amaranthus variety CO-1 and wild relative (*Amaranthus viridis*) was performed through a novel "two step" enrichment method. By partially drying the leaf samples at a higher temperature that is not harmful to the life of the bacteria, but would enhance the chances of endospore formation, a better recovery of endospore-formers compared to the conventional method was realized (Yashaswini et al., 2021). Molecular characterization of the isolates by 16S rRNA gene cataloguing also confirmed their position in the genus *Bacillus*. On isolation of endospore-forming phyllosphere bacteria on nutrient agar medium, too many colonies could be obtained. Therefore, those with same colony morphology were clustered together, and representative selection was made from such discrete groups. Thus, the number of isolates to be screened was made to a minimum. However, with the genus *Bacillus*, an interesting observation was that different species may have same colony morphology and some of the same species may have different colony morphology, even on a single kind agar medium. This is one of the problems associated with such preliminary grouping. However, neighborhood joining phylogenetic tree based on the sequence of the 16S rRNA gene of endospore-forming phyllosphere bacterial isolates obtained from *Amaranthus* spp. showed a wide diversity.

Even though a number of reports are available on the exploitation of plant associated bacteria for the control of *Rhizoctonia* leaf blight in amaranthus, till date no studies have been done on the use of endospore-forming phyllosphere bacteria for the same. Initially the inhibitory effect of the endospore-forming phyllosphere bacterial isolates on *R. solani* was carried out on PDA medium using the conventional dual culture plating technique which is relatively a simple and rapid method suitable for assessing direct antifungal activity. We found strong antifungal activity of the endospore-forming phyllosphere bacterial isolates against the pathogen *R. solani* under *in vitro* conditions. Inhibition of mycelial growth of fungal pathogen by bacterial antagonists may be due to the production and secretion of antimicrobial compounds (Leyns et al., 1990) or due to the disorganization of fungal hyphae by chitin degradation, mycelial deformation with terminal and intercalary swellings (Manjula et al., 2002). The ability to produce antibiotics, cell wall degrading enzymes and antifungal volatiles, of strains of *Bacillus* spp. are considered as the excellent biological control mechanisms against a wide range of plant pathogens (Fira et al., 2018). Our results also showed that culture filtrate of the isolates had strong *in vitro* inhibition activity against *R. solani.* Cell free culture filtrate may contain media diffusible compounds that induced hyphal deformation and enlargement of cytoplasmic vacuoles in *R. solani* (Huang et al., 2013). Production of antifungal volatiles by *Bacillus subtilis* has been implicated in hyphal deformation of *R. solani* (Fiddaman and Rossall, 1993).

One of the shortfalls of dual culture plate assay is lack of involvement of the host. Any assay involving interaction of the host plant, pathogen and the antagonist would resemble the field conditions in a better way and is expected to generate a more realistic basis for antagonism (Anith et al., 2003). In a detached leaf assay, the occurrence of lesion on the leaves treated with phyllosphere bacterial isolates was less than that observed in the control. The reduction in the lesion size is supposed to be due to direct antagonism. Ability to adversely affect the early steps in the establishment of the disease at the site of infection is thought to be one of the factors involved in success of biocontrol by plant associated

antagonists (Baker et al., 1983; Yoshida et al., 2001). Similarly, in a detached leaf assay, *Bacillus cereus* BT8 suppressed *P. capsici* in cacao (Melnick et al., 2008). *Bacillus subtilis* MBI 600, *B. subtilis* sub sp *subtilis* AP 209 and AP 52, and *B. amyloliquefaciens* AP 219 significantly reduced the sheath blight lesions on detached rice leaves (Kumar et al., 2011b).

The antagonistic activity in dual culture plate assay of certain isolates did not have co-relation with that of the detached leaf assay. For instance, the isolates *Bacillus* sp. GL3 from the green amaranthus CO-1 produced maximum zone of inhibition in the dual culture plate assay but did not perform well in detached leaf assay. This could be due to the inability of the antagonist to produce antifungal metabolites on the surface of leaves. As the conditions in dual culture and detached leaf assays vary greatly, such contradictory results may occur (Kollakkodan et al., 2021). In contrast, the isolate *Bacillus amyloliquefaciens* WL2 produced larger zone of inhibition in dual culture assay and minimum lesion size in detached leaf assay. Similar results of dual plate assay having correlation with detached leaf assay were observed earlier (Huang et al. 2005; Rajkumar et al., 2005).

In the greenhouse experiment application of *Bacillus* sp. GL3 had better suppression of the pathogen when disease scoring was done on the third day of inoculation. However, at 5 DAI *Bacillus* sp. AL3 showed remarkable disease suppression compared to *Bacillus* sp. GL3. This could be due to the better survival ability of the AL3 strain on the phyllosphere of the red amaranthus variety Arun, as it has been isolated from the same variety itself. The variety CO-1 and the wild species are resistant to the infection by *R. solani*. We suggest that when antagonists against a foliar pathogen are to be isolated, the best source could be susceptible but healthy host plant itself. There was 44.5 % disease suppression on inoculation with *Bacillus* sp. AL3 over the pathogen inoculated control whereas spraying with the recommend chemical (Mancozeb 0.2%) resulted in only 20.5 % disease suppression. Except the isolate AL1, all other phylloplane bacterial strains had better disease suppressive ability than the chemical control. Mancozeb was sprayed to the plants in a prophylactic manner as recommended (Gokulapalan et al., 1999) and was carried out five days before the artificial inoculation of the fungus to the plants. Since mancozeb is a contact fungicide, direct interaction between the chemical and the pathogen must be required to effect disease suppression. Decreased disease suppression by mancozeb in the pot culture experiment could be attributed to low persistence of it in the foliage of amaranthus. However, when microbials with competitive survival ability are applied to the crop plants, the chance of their persistence in the site of application may last long as they may multiply using the nutrients secured from the plants. Thus, sustained protection to the inoculated plants be ensured.

Present study reports the possibility of using endospore forming phyllosphere bacteria in suppressing the *Rhizoctonia* leaf blight for the first time. Reports on biocontrol activity of *Bacillus* spp. against *R. solani* in different crops such as tomato (Szczech and Shoda, 2004), cucumber (Huang et al., 2012) and lettuce (Chowdhury et al., 2013) strengthen the results of our investigation. Recently Yashaswini et al. (2021) have reported that root endophytic endospore-forming *Bacillus* spp. isolated from *Amaranthus* spp. could suppress leaf blight disease in the red amaranthus variety Arun. *Bacillus* species are ubiquitous plant epiphytes with ability to produce many beneficial plant-growth promoting and anti-fungal metabolites (Hollensteiner et al., 2017). Results of the present study show that spore-forming phylloplane bacteria obtained from a healthy leaf blight susceptible amaranthus varieties could impart suppression of the disease in a vulnerable variety. Stability and survival of antagonists in a user-friendly bio-formulation is important for field application and successful commercialization. Selection and development of endospore-forming bacteria as

biocontrol agents thus assumes importance. Poor nutrient conditions and exposure to sunlight, heat or cold and other environmental adversities makes phylloplane a peculiar environmental niche. Production of endospores by *Bacillus* spp. ensures their survival under adverse environmental conditions and makes them successful biocontrol agents at the phyllosphere.

Isolation of endospore-forming phyllosphere bacteria from *Amaranthus* spp. was carried out by a novel two-step enrichment process with high efficiency. Greenhouse studies revealed that spray application of cell suspensions of phyllosphere bacteria was capable of suppression of *R. solani* induced leaf blight disease in the susceptible variety Arun. Isolate *Bacillus* sp AL3, obtained from the same variety, significantly suppressed leaf blight to a tune of 44.5 % compared to the pathogen inoculated control. Prophylactic spraying with the recommended chemical, mancozeb (0.2%) to the leaves five days before artificial inoculation with the pathogen was less efficient in suppressing the disease than foliar application of spore forming bacteria except in a single case. The present study demonstrates significance of endospore-forming phyllosphere bacterial isolates obtained from *Amaranthus* spp. in suppression of leaf blight disease in the susceptible, popular red amaranthus variety Arun.

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