PLANT ASSOCIATED ENDOSPORE FORMING BACTERIA FROM AMARANTHUS AS GROWTH PROMOTERS AND BIOCONTROL AGENTS AGAINST RHIZOCTONIA LEAF BLIGHT

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by

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(2018-11-131)

THESIS

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2020

DECLARATION

I, hereby declare that this thesis entitled **"PLANT ASSOCIATED ENDOSPORE FORMING BACTERIA FROM AMARANTHUS AS GROWTH PROMOTERS AND BIOCONTROL AGENTS AGAINST RHIZOCTONIA LEAF BLIGHT"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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Certified that this thesis entitled **"PLANT ASSOCIATED ENDOSPORE FORMING BACTERIA FROM AMARANTHUS AS GROWTH PROMOTERS AND BIOCONTROL AGENTS AGAINST RHIZOCTONIA LEAF BLIGHT"** is a record of research work done independently by Miss Yashaswini M. S. (2018-11-131) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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Yashaswini M. S.

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LIST OF ABBREVIATIONS

SDW	Sterile Distilled Water
min	Minutes
et al.	And other co-workers
cm	Centimetre
cfu	Colony forming units
CRD	Completely Randomized Design
CD	Critical difference
DAT	Days after transplanting
^{0}C	Degree celsius
dia	Diametre
g	Gram
hrs	Hours
IAA	Indole-3- Acetic Acid
µg	Microgram
µl	Microlitre
mg	Milligram
mL	Millilitre
mm	Millimetre
rpm	Revolution per minute
viz.	Namely
N	Nitrogen
No.	Number
NA	Nutrient Agar
OD	Optical density
IAA	Indole Acetic Acid
%	Per cent
P	phosphorous

K	Potassium
PDA	Potato Dextrose Agar
Sl.	Serial
SVI	Seedling vigour index
sp.	Species
SD	Standard Deviation
i.e.	That is
var.	Variety
AR	Arun Root
AL	Arun Leaves
GR	Green Root
GL	Green Leaves
WR	Wild Root
WL	Wild Leaves
DAI	Days after Inoculation
PBS	Phosphate Buffer Saline

Introduction

1. INTRODUCTION

Amaranth (*Amaranthus tricolor* L.) is one of the major tropical leafy vegetables grown in Southern India. Amaranthus is the largest consumed leafy vegetable in Kerala and is well known for its high nutritional value. It is widely known as "poor man's spinach" as it is cheaply available. Amaranthus is easy to cultivate and it exhibits rapid growth. As it is a short duration crop, it fits well in the crop rotation practices of Kerala. Priya *et al*. (2007) reported that the crop possesses a high content of essential micronutrients such as beta-carotene, iron, calcium, vitamin C and folic acid. It belongs to the C4 group of plants, which possess higher water-use efficiency and photosynthesis under high temperature. Leaf blight disease caused by soil borne pathogen *Rhizoctonia solani* is a major threat for amaranthus production. The severity of the disease is increased under warm and humid conditions of the rainy season and the symptoms include the appearance of light cream coloured spots on the foliage (Nayar *et al*., 1996). In grave outbreaks more than 90 percent of the plants in the field will be infected, which causes considerable economic loss owing to the reduction in the marketability of the produce. Foliar spray of mancozeb at fortnightly intervals is recommended as a chemical control measure for managing the disease (Gokulapalan *et al*., 1999). However, extensive use of agrochemicals posed environmental pollution and increased human health hazards.

Microbial biopesticides are potential sustainable substitutes, offering "environmentally friendly" alternative approaches to pest management which can be followed in organic farming practices and can also be incorporated into integrated pest management (IPM) (Jacobsen *et al*., 2004; Chandler *et al*., 2011). Besides, they are specific, less toxic to non-target organisms, pose minimum or no environmental risks and are cost effective in development, production and application (Glass and Lindemann, 1992).

Beneficial plant–microbe interactions may promote plant growth, health and development. Endophytic bacteria are those colonize the internal tissues of their host

plants and their relationships with their host can be symbiotic, mutualistic, commensalistic and trophobiotic. Through rhizosphere or phyllosphere, most of the endophytes appear to originate, but some may be transmitted through the seed. Many endophytic bacteria help in plant growth promotion and can act as biocontrol agents (Ryan *et al.*, 2008).

Phylloplane is an important matrix for microbial life. Bacteria that colonize the phylloplane are exposed to limited nutrient availability, higher temperature and moisture fluctuations. In response to climate change and environmental pollution, they act as the key players for plant's growth (Dubey *et al*., 2017) and are also identified as biocontrol agents (Andrews, 1992).

During recent years increased interest has emerged towards endospore forming *Bacillus* spp. Many strains within this genus possess several traits responsible for plant growth promotion and disease suppression (Kloepper *et al.*, 2004b; Kumar *et al*., 2011; 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 the preparation of different formulations of microbes that give rise to stable products with extended shelf lives. *Bacillus* species are also common endophytes in many plants (Kobayashi and Palumbo, 2000; Araujo *et al*., 2001). Many species of *Bacillus* are well known as plant growth-promoters and as biocontrol agents (Kumar *et al*., 2011).

Recognizing the importance of endospore forming endophytic bacteria in plant growth promotion and role of phylloplane endospore forming bacteria in the management of *Rhizoctonia solani* causing leaf blight of amaranthus, the present study was taken up with the following objectives.

1. Isolation and characterization of endospore forming bacteria from endorhizosphere and phyllosphere of Amaranthus.

2. *In vitro* evaluation of endospore forming endorhizospheric bacteria for plant growth promotion.

3. *In vitro* evaluation of endospore forming phylloplane bacteria as biocontrol agent against *Rhizoctonia solani*.

4. To study the effect of endospore forming endorhizospheric bacteria on growth promotion in pot culture experiment.

5. To study disease suppressive ability of selected endospore forming phylloplane bacteria against *Rhizoctonia solani.*

Review of literature

2. REVIEW OF LITERATURE

Amaranthus (*Amaranthus tricolor* L.) is one of the commercially important leafy vegetables consumed and cultivated 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. It belongs to the family Amaranthaceae. The genus Amaranthus includes 50–60 species cultivated for leaf (greens) and grains. The crop has excellent nutritional value because of its high content of essential micronutrients such as beta-carotene, iron, calcium, vitamin C and folic acid. A few species have been reported to possess medicinal properties also. Every parts of this plant can be used for culinary purposes. As it is a short duration crop it fits well with the crop rotation practices of Kerala. It responds well to organic manures, and has a low cost of production (Priya *et al*., 2007).

Among the different fungal diseases of amaranth, leaf blight disease caused by *Rhizoctonia solani* Kuhn, is the most destructive one. Even though chemical control of the disease with fungicides can lessen the severity of this aerial blight disease (Gokulapalan *et al*., 1999), application of chemicals on a regular basis causes serious health problems. To avoid pesticide residues in agricultural products, alternative methods for the management of pests and diseases using non-hazardous, eco-friendly agents have to be explored.

Roy (1975) reported the incidence of collar rot caused by *R. solani* in amaranthus. Leaf blight caused by *R. solani* was reported in other vegetables (Bandyopathyay and Khauta, 1985) and in ornamentals (Jana *et al*., 1990). Nayar *et al*. (1996) reported severe infection in *A. tricolor* by *R. solani* during the post monsoon period of 1994 (August – September) in Kerala. Presence of cream coloured spots on leaves was observed which spread rapidly and resulted in extensive damage and economic losses. Priya (1998) reported that the tetraploid amaranth, *A. dubis* is the only species resistant to leaf blight caused by *R. solani* among the different species studied. The efficacy of the plant activator, acibenzolar-S-methyl and rhizobacteria in the management of foliar blight disease of amaranth was studied. It was reported that combined application of PGPR,

Pseudomonas fluorescens PN026R and Acibenzolar-S-Methyl was effective in the control of foliar blight disease of amaranth (Nair and Anith, 2009).

Sheela (2015) reported that the foliar application of combination of turmeric and sodium carbonate in the ratio 5:1 significantly suppressed the leaf blight pathogen of amaranthus by 68.38% over the untreated control. The same study reported that mancozeb @ 3g/litre was more effective in controlling the disease than the biocontrol treatments. Gireesh (2016) reported that soil solarization for 31 days followed by the application of tebuconazole (0.1%) was effective in controlling the Rhizoctonia leaf blight of amaranthus.

There are numerous sound evidences that endophytes can contribute to the control of plant diseases (Kloepper *et al*., 1992b). Despite this, only limited attempt has been made to use endophytic bacteria as biocontrol agents of leaf spot and leaf blight diseases (Manjula *et al*., 2002). The possibility of using the plant's own defence mechanisms induced by bacterial endophytes in the management of pests and diseases is a matter of interest (Rajendran *et al*., 2006). Endophytic microorganisms play a key role in the promotion of plant growth, control of pests and diseases, and thus help in preserving the environment (Azevedo and Araujo, 2007).

Till date, a number of endophytic bacteria have been reported from a wide variety of plants. However, plant growth promoting activity is known for a limited number of isolates. Isolation and characterization of endophytic bacteria from unexplored sources is advantageous in order to explore more potent endophytic bacteria possessing plant growth promoting traits.

2.1 BACTERIAL ENDOPHYTES

Plants can be considered as a complex micro-ecosystem where "different habitats can be exploited by a wide variety of bacteria" (McInroy and Kloepper, 1994). These habitats are not only represented by plant external surfaces - where epiphytic bacteria predominate - but also by internal tissues where many microorganisms penetrate and

survive. Endophyte is a composite Greek word that means "inside the plant". In the literature the word is utilized to define those microorganisms - mostly bacteria and fungi - which are able to enter plant tissues, and establish themselves inter and intracellularly (Di Fiore and Del Gallo, 1995).

Although the term “endophyte” is most commonly associated with fungi, there is a considerable amount of literature pertaining to bacteria as endophytes as well, some of which are assumed to impart beneficial effects, whereas others are regarded to have a neutral effect on plants. Endophytic bacteria can be defined as those bacteria capable of colonizing the internal tissue of the plant without showing any external sign of infection or negative effect on their host (Schulz and Boyle, 2005). For more than 50 years, bacteria have been observed to exist inside plants without causing apparent disease symptoms (Tervet and Hollis, 1948). Various reports indicate the presence of bacteria in a variety of tissue within numerous plant species which suggests the ubiquitous existence in most of the higher plants. (Lodewyckx *et al*., 2002)

Of the nearly 300,000 plant species that exists on earth, every individual plant is host to one or more endophytes (Strobel, 2002). Most endophytic bacteria possess biphasic life cycle that alternates between plant and soil environments. Relationship between bacterial endophytes and its host plants can be either obligate or facultative. Bacterial endophytes which are strictly dependent on the host plant for their growth and survival are obligate bacterial endophytes whereas bacterial endophytes which have a stage in their life cycle in which they exist outside the host plants are facultative bacterial endophytes (Marella, 2014).

Generally, the Phylum Proteobacteria (Classes α, ß and γ-Proteobacteria) are reported to be dominant in diversity analysis of endophytes (Hallmann *et al*., 1997). However, members of the Firmicutes, Actinobacteria and Bacteroidetes are also among the classes most commonly found as endophytes. Other Classes such as, Acidobacteria, Planctomycetes and Verrucomicrobia are the less commonly found endophytes. The most common genera of bacterial endophytes are *Bacillus, Burkholderia, Microbacterium,*

Micrococcus, Pantoea, Pseudomonas and *Stenotrophomonas* (Romero *et al*., 2014; Shi *et al*., 2014).

2.2 Colonization of plants by the endophytic bacteria

Distinct microbial communities have been found in various plant organs such as roots, stem, leaves, flowers as well as fruits and seeds or even during plant development (Okunishi *et al.,* 2005) which indicates different capacities of bacterial strains to colonize various plant compartments. A complex communication is involved between the two partners in the colonization process. Through root and through aerial plant parts endophytic bacteria get entry into and colonize themselves inside the host (Zinniel *et al*., 2002).

2.2.1 Rhizosphere colonization by the endophytic bacteria

Colonization in the rhizosphere is a highly competitive task for the endophytic bacteria to occupy spaces and get nutrients. The bacterial population in the rhizosphere can range between 10^7-10^9 $cfu^{-1}g$ fresh weight of the rhizosphere soil and rhizoplane population ranges from 10^5 -10^7 $cfu^{-1}g$ fresh weight (Benizri *et al*., 2001). Bacterial traits like motility and polysaccharide production are important in the colonization of plant rhizosphere, as demonstrated for endophytic *Alcaligenes faecalis* and *Azospirillum brasilense* (Santoyo *et al*., 2016).

2.2.2 Root colonization by the endophytic bacteria

Bacterial endophytes enter into the plant root and start colonization after establishment in the rhizosphere and rhizoplane, with sub-populations ranging from 10^5-10^7 cfu/g fresh weight (Hallmann, 2001). The process of penetration into the host root can be active or passive. Active penetration by the competent endophytic bacteria involves presence of lipopolysaccharides, flagella, pili, twitching motility, and quorum sensing, which can affect endophytic colonization and bacterial movement inside the host

plants (Bohm *et al*., 2007). Passive penetration can occur at cracks present at root emergence areas, root tips, or through damaged tissues (Hardoim *et al*., 2008).

After penetrating the root, the primary site of bacterial colonization is intercellular spaces in plant tissue, as reported in *Acetobacter diazotrophicus* in Brazilian sugarcane (James *et al*., 1994) and intracellular spaces as reported in *Azoarcus* sp in grasses (Hurek *et al*., 1994). Some bacterial endophytes have been shown to colonize inter as well as intracellular spaces e.g., *Burkholderia* sp. strain PsJN in grapevines (Compant *et al*., 2005).

2.2.3 Systemic colonization of aerial plant tissues by the endophytic bacteria

Only a few bacteria can colonize aerial vegetative parts of their host plants due to the specific physiological requirements needed to occupy the plant niches (Hallmann, 2001). Their movement inside plant is mainly supported by bacterial flagella and plant transpiration stream. Motility along intercellular spaces requires the secretion of cell-wall degrading enzymes like cellulases and pectinases (Compant *et al*., 2010). But movement through xylem element occurs through perforated plates that allow movement of bacteria through large pores without requiring cell-wall degrading enzymes (Compant *et al*., 2010).

2.2.4 Entry through aerial plant parts

Aerial portion of the plant also excretes some exudates on its surface that attracts microbes (Compant *et al*., 2010). Endophytic bacteria colonize the leaf tissues by entering into the leaves via stomata, hydathodes or damaged tissues (Senthilkumar *et al*., 2011).

2.3 PLANT GROWTH-PROMOTING ENDOPHYTIC BACTERIA

Beneficial plant–microbe interactions that enhance plant health and development have been the subject of extensive study. Endophytes colonization in a plant is considered as a sign of a healthy plant system since this interaction between endophytes and host

plant offers benefit to both the partners and it helps to develop a closer biological relationship between these organisms and their respective hosts (Taghavi, 2010).

By increasing certain growth attributes such as root number, dry-matter weight, leaf area and seed germination (Frommel *et al*., 1991), endophytes are found to be associated with growth promotion of many cultivated plants, such as tomato, lettuce, potato, and corn (Hallmann *et al*., 1997).

Nejad and Johnson (2000) observed a significant increase in plant height and shoot dry weight in tomato and rape by endophytic bacteria. Endophytic bacterial colonization offers several advantages to the host plant by producing various secondary metabolites which can be directly or indirectly described as plant growth-promoting agents. They possess vital ability to aid plant growth by various mechanisms; including production of indole 3-acetic acid (IAA), siderophore, ACC (1-aminocyclopropane-1-carboxylate) deaminase and solubilization of mineral phosphate (Jasim, 2014).

Uppala *et al.* (2010) reported that the application of several bacterial endophytes (EB-20, EB-43 and EB-45) was found to improve the biometrics of amaranth. Silva *et al*. (2012) isolated several strains of endophytic bacteria (217) and fungi (17) from coffee tissues and evaluated their potential to control coffee leaf rust (*Hemileia vastatrix*) and to promote the growth of coffee seedlings. None of the fungal strains induced plant growth or reduced disease severity. Bacterial strains 85G (*Escherichia fergusonii*), 161G, 163G, 160G, 150G (*Acinetobacter calcoaceticus*) and 109G (*Salmonella enterica*) increased plant growth; the maximum increase was induced by strain 85G. This strain under *in vitro* conditions produced phosphatase and indole acetic acid.

Mechanisms of plant growth promotion by endophytes include production of plant hormones or their analogs, nitrogen fixation, increasing nutrient availability through phosphorus solubilization and mineralization of organic matter (Melnick *et al*., 2008).

Various plant hormones released by endophytic bacteria have been reported to result in enhanced plant nutrient uptake and biomass (Shi *et al*., 2014). Indole-3-acetic

acid (IAA), ethylene, cytokinins and gibberellins are important phytohormones regulated or produced by endophytic bacteria. The endophytic bacteria *Sphingomonas* sp LK11 was found to produce 11.23 μM^{-1}ml of IAA. Tomato plants inoculated with this endophytic bacterium significantly increased growth parameters (shoot length, shoot, and root dry weights) compared to the control (Khan *et al*., 2014).

Abdallah *et al*. (2016) screened seven endophytic bacterial isolates recovered from native *Nicotiana glauca* plants for their ability to suppress tomato *Fusarium* wilt caused by *Fusarium oxysporum* f. sp. *lycopersici*, and to enhance plant growth. A significant improvement in growth parameters was reported when tomato plants were inoculated with pathogen and treated with endophytes *Alcaligenes faecalis* S18 and *Bacillus cereus* S42. This study clearly described that *N. glauca* is a potential source for the isolation of potent endophytic bacteria exhibiting *Fusarium* wilt-suppressive and plant growth-promoting effects on tomato.

Andreolli *et al* (2016) conducted an investigation on ecology of endophytic bacteria isolated from three and 15 year-old vine stems of *Vitis vinifera* cv. *corvina*. Among isolated strains, from 3 and 15 year-old stems, respectively, 34 and 39% produce siderophores; 22 and 15% secrete ammonia; 22 and 21% produce indole-3-acetic acid; 8.7 and 41% solubilize phosphate. In addition, two strains obtained from 15 year-old grapevines showed ACC deaminase activity.

Vyshakhi (2016) conducted an experiment to study the effects of bacterial root endophytes isolated from tomato, brinjal and chilli. Endophytes with plant growth promoting ability were selected through *in vitro* studies and were also assessed for their compatibility with *Piriformospora indica.* Seed treatment with bacterial endophytes alone and in combination with *P. indica* were found to significantly promote the growth of tomato, brinjal and chilli.

A total of 49 bacterial endophytic strains, identified as members of the phyla of Firmicutes and Proteobacteria were isolated from tomato roots on selected media (Tian *et*

al., 2017). In an experiment to evaluate the ability of endophytes to promote the growth of wheat plants *in vivo*, almost all the isolated tomato root endophytic strains showed ability to promote plant growth. Four strains were shown to produce IAA when grown in medium supplemented with L-tryptophan. The results showed that among the tomato root endophytes, most of the tested plant host-benefiting traits were derived from bacteria of three genera, *Pseudomonas*, *Bacillus* and *Rhizobium*. *Bacillus* strains have great taxonomic and functional potential to explore as biological agents that contribute diverse nutrient acquisition and growth promotion activities to their hosts (Tian *et al*., 2017).

Screening of 84 endophytic bacteria isolated from nodules and roots of *Cicer arietinum* and *Pisum sativum* plants for siderophore production was carried out by Maheshwari *et al*. (2019). Of 84 endophytic bacteria, 14 produced siderophore and when analysed most of them produced hydroxomate and carboxylate type of siderophores.

2.4 BACTERIAL ENDOPHYTES AS POTENTIAL BIOCONTROL AGENTS

There are reported evidences for endophytes contributing largely to the management of plant diseases (Kloepper *et al*., 1992a). According to Backman *et al*. (1997), the biocontrol activity of endophytes depends on many factors such as host specificity, the population dynamics and pattern of host colonization, the ability to move within host tissues, and the ability to induce systemic resistance.

Rosales *et al*. (1993) made *in vitro* and *in vivo* studies at International Rice Research Institute, Philippines, to know the effect of 23 selected endophytic bacterial strains isolated from paddy in controlling rice sheath blight pathogen. They observed that all strains inhibited mycelial growth of *Rhizoctonia solani* under *in vitro* condition, whereas seed bacterization with the 23 strains provided upto 73 per cent protection against sheath blight in glass house trials.

Jetiyanon (1994) established that cabbage colonized by endophytes in the greenhouse had season-long reduced black rot in the field due to induction of defense mechanisms. Several studies have reported the effective colonization of *Bacillus* in the

rhizosphere and control root diseases (Kim *et al*., 1997; Ryder *et al*., 1998). Recently they have gained increasing scientific interest as these bacterial strains in addition to their rhizosphere competence exhibit the ability to colonize the internal tissue of plants. Because of the endophytic colonization, bacterial biocontrol agents may not be affected by detrimental abiotic factors and may represent a second line of defense against soil-borne pathogen.

Colonization of multiple hosts has been observed with other species of endophytes and plants. *Pseudomonas putida* 89B-27 and *Serratia marcescens* 90-166 reduced anthracnose and Fusarium wilt in cucumber (Liu *et al*., 1995) as well as Cucumber Mosaic Virus in tomatoes and cucumbers (Raupach *et al*., 1996).

Barka (2002) explained the divergent host colonization of an onion endophyte, *Pseudomonas* sp. strain PsJN, in grape. They inhibited *Botrytis cinerea* Pers. and promoted vine growth in the colonized grapevines. Rangeshwaran *et al.* (2002) reported that out of the twenty-five endophytic bacteria isolated from internal tissues of root and stem portions of chickpea, sunflower and chilli plants, ten isolates were found to inhibit the pathogens *viz*. *Fusarium oxysporum f. sp. ciceris*, *F. udum*, *R. solani* and *Sclerotium rolfsii*.

Mercado-Blanco *et al*. (2004) obtained a bacterial endophyte *Pseudomonas fluorescens* PICF7 from the roots of olive trees and tested it for the disease suppression against of Verticillium wilt. Treatment of olive tree roots with this strain in greenhouse conditions significantly delayed the onset of symptoms, and reduced Verticillium wilt incidence and severity by 82% and 96%, respectively.

Sessitsch *et al.* (2004) isolated and screened 35 plant associated bacteria from potato to study their antagonistic activity. Only seven endophytes were found to antagonize fungal pathogens like *V. dahliae*, *R. solani*, *Sclerotinia sclerotiorum*, *Phytophthora cactorum* and bacterial pathogens like *Erwinia carotovora*, *Streptomyces scabies* and *Xanthomonas campestris*.

Erdogan and Benlioglu (2010) isolated four *Pseudomonas* strains FP22, FP23, FP30 and FP35 from the roots of *Xanthium strumarium*, *Portulaca* sp., *Gossypium hirsitum*, and *Convolvulus arvensis*, respectively, and tested them for their effect on Verticillium wilt and growth attributes in cotton. The disease severity was found to be reduced by the seed bacterization with these strains compared to non-bacterized control (from 39.2% to 50.9% in 2005 trials and from 22.1% to 36.8% in 2006 trials).

A bacterial endophyte isolated from mangrove, identified as *Bacillus amyloliquefaciens* Bg-C31, was found to be effective in the biocontrol of *Ralstonia solanacearum* causing Capsicum bacterial in pot and field trials. The antimicrobial substance produced by Bg-C31 was identified as a protein encoded by *LCI* gene (Hu *et al*., 2010).

Uppala *et al*. (2010) reported the antagonistic action of endophytes isolated from amaranth against *R. solani* and it was studied using dual culture method and pot culture method. The study revealed that bacterial endophytes significantly suppressed the pathogen in amaranth with varying degrees of inhibition under both the conditions.

Kollakkodan (2017) reported that wild relative of black pepper, *Piper colubrinum* as a good source for isolation of bacterial antagonist against *Phytopthora capsici.* Application of plant associated bacteria from *Piper colubrinum* and *Piper nigrum* suppressed the development of foliar infection of *P. capsici*.

Athira (2018) reported the suppression of bacterial wilt incidence by the bacterial endophytes, rhizobacteria and root endophytic fungus, *Piriformaspora indica.* Individual and combined application of bacterial bioagents and fungal endophytes were found to be significantly suppressing wilt incidence in tomato.

2.5 PHYLLOPLANE BACTERIA

Phylloplane is an important matrix for microbial life, as it is directly exposed to frequent environmental factors, like temperature, moisture, and solar radiation. However,

the microbial population is higher in the phyllosphere than in any other ecological niche (Dubey *et al*., 2017).

Plant modulates bacterial community by altering leaf exudates through stomatal opening. Some microorganisms live on the leaf surface under limited moisture (Beattie and Lindow, 1999), while others invade the leaf tissue to cope with moisture stress (Mercier and Lindow, 2000). The phylloplane comprises carbon sources like glucose, fructose, and sucrose leached from the interior of the plant (Mercier and Lindow, 2000). This may be the intrinsic feature of the plant that mainly shapes its epiphytic microbial community. Most nutrients on the phylloplane are transient, isolated, or infrequent. Hence most of the phyllospheric microbes are oligotrophic (Lindow and Brandl, 2003). Epiphytic microbes are gaining attention as they are the key players for plant growth promotion. Phyllosphere and phylloplane microbial interactions enhance the productivity of agricultural crops and improve sustainability of crop in its habitat (Whipps *et al*., 2008). Phyllospheric bacteria help plants by acting as biostimulator, bio-protectants and biofertilizers.

2.6 PHYLLOSPHERIC PLANT GROWTH PROMOTING BACTERIA

Many evidences support the functional roles played by phyllosphere complex microbial communities. N_2 fixing bacteria have been reported to be present in the phyllosphere of many crop plants (Miyamoto *et al*., 2004). Mwajita *et al*. (2013) evaluated the rhizosphere, rhizoplane and phyllosphere bacteria from rice fields in Kenya for PGPB and reported that over 50% of bacterial isolates from phyllosphere were able to solubilize phosphates.

Production of indole-3-acetic acid (IAA) is common among many phyllospheric bacteria (Brandl *et al*., 2001). Marques *et al*. (2010) assessed plant growth promoting (PGP) abilities of six bacterial isolates on *Zea mays* plant and reported that all isolates produced IAA, hydrogen cyanide (HCN) and ammonia *in vitro* as well as in green house experiments.

Kishore *et al*. (2005) reported that the bacterial isolates from habitats like phylloplane and endosphere were also effective, like rhizobacteria, in plant growth promotion when applied as seed treatment. Seed bacterization with phylloplane isolates significantly promoted groundnut growth.

Batool *et al*. (2016) conducted experiments to compare plant growth promoting (PGP) phylloplane bacteria of three commercially popular wheat varieties, namely Sehar, Faisalabad and Lasani, in order to assess whether better wheat variety harbours better PGP bacteria that play part in its superior yield. Phylloplane bacteria were isolated from the three wheat varieties thrice during the plant growth. Isolates belonging to Sehar phylloplane produced auxin in highest amounts (52.95 μg ml^{-1}) during second sampling when the plant was showing rapid growth. Many isolates from all three varieties fixed nitrogen, solubilized phosphates and some isolates also produced hydrogen cyanide. The results clearly indicated that the beneficial bacteria associated with phylloplane of better yielding variety were showing better PGP abilities when compared to their counterparts from low yielding varieties.

Inoculation of culturable phylloplane bacteria isolated from *Jatropha curcas* significantly increased shoot and root length of maize seedlings. Isolates exhibited ACC (1-aminocyclopropane-1-carboxylic acid) deaminase, phosphatase, potassium solubilization, and indole acetic acid (IAA) production activities. The phosphate-solubilizing capacity (PO_4 solubilized by 10^8 cells) varied from 0.04 to 0.21 mg. The IAA production potential (IAA produced by 10^8 cells in 48 h) of the isolates varied from 0.41 to 9.29 μg. A linear regression model of the plant-growth-promoting activities significantly correlated with the growth parameters (Dubey *et al*., 2017).

Application of pink-pigmented facultative methylotrophic (PPFMs) bacteria isolated from cotton phylloplane significantly increased plant height, plant dry weight, leaf area, boll number, and boll dry weight. PPFMs from sugarcane when applied as foliar application, significantly increased plant height and specific leaf area of sugarcane and led to a cane yield increase of 9.8% over control plants (Madhaiyan *et al*., 2006b).

Nysanth *et al*. (2019) carried out the screening of pink pigmented facultative methylotrophs isolated from the phyllophere of rice plants for growth enhancement in paddy. The isolates PPFM11, PPFM16 and PPFM19 showed efficient growth promotion by increase in growth and yield attributes of paddy.

2.7 BIOCONTROL POTENTIAL OF PHYLLOPLANE BACTERIA

The antagonistic activity of bacterial strains isolated from phylloplane on phytopathogens is well documented (Haidar *et al*., 2016). Their presence on plant surfaces affects the development of plant diseases since they interact directly with pathogens through inhibitory activities and/or triggering plant defense responses (Dukare *et al*., 2018; Carmona-Hernandez *et al*., 2019).

Halfeld-Vieira (2008) observed that treatment of tomato plants with *Novosphingobium capsulatum* isolated from the tomato phylloplane led to a profound decrease in the percentage of fruits with late blight symptoms caused by *Phytophthora infestans*. Late blight intensity was reduced by 55% in the middle leaves of the plant and 62% in the upper leaves.

Foliar application of 36h old culture of phylloplane bacterium BMO-111 (1×10^8 colony-forming units ml^{-1}) considerably reduced the blister blight disease incidence in tea. The culture of BMO-111 as well as its culture filtrate significantly inhibited the mycelial growth of various fungal plant pathogens (Sowndhararajan *et al*., 2012).

The effective phylloplane bacterial isolates obtained from healthy and sheath blight infected leaf samples of rice plants have been used for the management of important soil borne fungal pathogen, *Rhizoctonia solani* (Akter *et al*., 2014).

Seven isolates of *P. fluorescens* (BPf, TPf, VPPf, MEPf, MKPf, MOPf and SAPf) were isolated from jasmine phyllosphere and evaluated to test the antagonism against *Alternaria jasmini* under *in vitro* conditions. Of the seven antagonists tested, VPPf-isolate recorded the highest inhibition of mycelial growth (22.60 mm) of *A. jasmini* over

control by recording 74.70 per cent reduction of mycelial growth over control. The effect of different concentration of culture filtrate of bacterial isolates on the mycelial growth of *A. jasmini* under laboratory conditions revealed that the culture filtrate of the isolate VPPf totally inhibited the mycelial growth of *A. jasmini* at 25% concentration under *in vitro* conditions followed by the isolate SAPf (Hemanandhini *et al*., 2019).

Islam *et al*. (2019) isolated and identified the plant growth promoting bacteria from rice phylloplane and rhizosphere that antagonistic *to Xanthomonas oryzae* pv. *oryzae*. Rice phylloplane and rhizosphere bacteria were isolated from the surface of rice leaves and stem as well as from the soil attached to the roots of rice plants, respectively. The antagonistic activity of these isolated bacteria was determined by dual culture method. Sixteen bacterial isolates were identified as antagonist to *X. oryzae* pv. *oryzae* out of 300 bacterial isolates by dual culture method.

Lorenzini and Zapparoli (2020) isolated fifty epiphytic bacterial strains from withered grape berries. The obtained isolates were identified and characterized. Several Bacillus strains displayed antagonistic effects on grape-rotting fungi such as *Botrytis cinerea, Penicillium expansum* and *Aspergillus uvarum*. The other strains were weakly or non-antagonistic on these fungi. Assay on antagonistic interactions among bacteria was also carried out. The occurrence of these isolates could reduce the contamination of fungal pathogens during grape withering. Epiphytic antagonistic bacteria could potentially be of interest for fungal biocontrol in the post-harvest processing of fruit and vegetables.

2.8 ENDOSPORE FORMING ENDOPHYTIC BACTERIA AS PLANT GROWTH PROMOTERS AND BIOCONTROL AGENTS

The genus *Bacillus* comprises an important group of low G+C Gram positive endospore forming bacteria found within the phylum *Firmicutes* (Class *Bacilli*; Order *Bacillales*; Family *Bacillaceae*) (Priest, 1993; Logan and De Vos, 2009; Maughan and Van der Auwera, 2011). Members of this genus are aerobic or facultatively anaerobic,

rod-shaped bacteria that can differentiate into endospores. Till date, 88 species and five sub-species are documented within the genus (Borriss *et al.*, 2011; Yi *et al*., 2014). Overall, aerobic endospore-forming bacteria found within the order *Bacillales* account for some 25 different genera incorporating over 200 species.

Bacillus spp. are spore-forming, Gram positive, rod shaped bacteria that consists one of the most common soil bacterial groups and they are commonly isolated from the rhizosphere of plants. *Bacillus* species are also common endophytes (Kobayashi and Palumbo, 2000; Araujo *et al*., 2001).

Due to their endospore forming nature they are readily adaptable to different field applications (Liu and Sinclair, 1993). The ability to form endospores is an important survival strategy which allows vegetative *Bacillus* cells to differentiate into resting structures when nutrient depletion condition prevails, critical cell population density thresholds exceeds or other stress factors are encountered (Stragier and Losick, 1996). Dormant endospores are extremely resistant structures which can tolerate a wide range of environmental stresses for long periods. This adaptation has enabled bacilli to become widely dispersed, to the extent that they are considered to be ubiquitous. Soil environments are important habitats for many bacilli; here they function as saprophytes and are able to utilize a wide range of organic materials derived from plants and animals (Garbeva *et al*., 2003). There are many species of Bacillus that are well known as plant growth-promoters (Kumar *et al*., 2011).

Endophytic bacteria colonize within plant tissues and have been found to promote plant growth. Three Gram-positive spore-forming rods, two *Bacillus subtilis* strains and a *Bacillus thuringiensis* strains were found to increase soybean weight when co-inoculated with one of the isolates and *Bradyrhizobium japonicum* under nitrogen-free conditions, compared with plants inoculated with *B. japonicum* alone (Bai *et al*., 2002).

Endophyte *Bacillus amyloliquefaciens* YN201732 isolated from tobacco seeds exhibited significant increase in seed germination, seedling plant growth, and improve plant photosynthesis when compared to control (Jiao *et al*., 2020).

Melnick *et al*. (2011) illustrated that endospore forming endophytic bacteria isolated from *Theobroma cacao* trees possessed the potential of antagonists to cacao pathogens and harbors a broad range of bacterial endophytes. Sixty-nine endospore forming endophytic bacteria were selected as potential biocontrol agents against cacao pathogens. Among the selected isolates 42% inhibited *Moniliopthora roreri,* 33% inhibited *Moniliopthora perniciosa* and 49% inhibited *Phytopthora capsici.*

Bacillus species, as a specialist group, may offer several advantages over fluorescent pseudomonads and other bacteria in terms of biocontrol activity for protection against root pathogens. They can be formulated rapidly because they are able to form resistant endospores that are tolerant to heat and desiccation and also produce a broad spectrum of antibiotics (Handelsman *et al*., 1990). Bacillus species have been first regarded as less effective in colonization of the rhizosphere compared to fluorescent pseudomonads. However, several studies have reported that *Bacillus* species may effectively colonize the rhizosphere and control root diseases (Kim *et al*., 1997; Sari *et al*., 2006, 2007).

One of central characteristics of the genus *Bacillus* is that they form endospores, which are a resistant structure that helps in the survival of the bacteria under stressed conditions. This property can be exploited in bio-inoculation technologies, as it results in long shelf-life of the organisms in bioformulation before application.

Materials and Methods

3. MATERIALS AND METHODS

The experiment on "Plant associated endospore forming bacteria from amaranthus as growth promoters and biocontrol agents against Rhizoctonia leaf blight" was carried out in the Department of Agricultural Microbiology, College of Agriculture, Vellayani during the period 2018-2020.

The details of the materials used and methods followed during the course of study are mentioned below.

3.1 ISOLATION OF ENDOSPORE FORMING ENDORHIZOSPHERE AND PHYLLOSPHERE BACTERIA FROM *Amaranthus* spp.

3.1.1 Isolation of Endospore Forming Endorhizosphere Bacteria from *Amaranthus* spp.

3.1.1.1 Isolation of Endospore Forming Endorhizosphere Bacteria from Red Amaranthus variety Arun

Healthy plants (Plate 1a) from the Instructional Farm of the College of Agriculture, Vellayani were selected and uprooted for root samples. The root system was washed in running tap water to get rid of the adhering soil particles and separated from the shoot portion. Samples were cut into small bits of 4 cm and four prewashes were given with sterile distilled water. Surface sterilization was carried out by soaking the sample bits in 4% sodium hypochlorite for three minutes. Then they were rinsed four times in sterile distilled water (SDW) to clear them off with sodium hypochlorite. To monitor the efficiency of the disinfestation procedure sterility checks were carried. For the checks, 0.1 ml of the last wash was transferred to Nutrient Agar and spread plated and another 0.1 ml of the final wash was transferred to 0.9 ml of Nutrient Broth and incubated at 28^0C temperature. The bacteria recovered during the isolation process were considered to be endophytes if no bacterial growth occurred in the sterility check after 48 hrs. For the isolation of endospore forming endorhizosphere bacteria, the surface sterilized root bits

were initially blot dried using sterile tissue paper. The root bits were then placed inside sterilized Petri plates and kept for drying at 35^0C consecutively for two days. The dried root bits were triturated in 1 ml phosphate buffered saline solution (PBS with pH 7.4) with mortar and pestle under aseptic condition. The obtained macerated root tissue was heated in a hot water bath at 80^0C for 10 minutes. Nutrient agar medium was spread plated with 0.1ml of tissue macerate. Tissue macerate of 0.1 ml was mixed with 0.9 ml of sterile water and vortexed to get 10^{-1} dilution. Diluted suspension of 0.1 ml was spread plated on NA. With the same procedure, dilution was made upto 10^{-3} and plated on NA agar plates. The agar plates were incubated at 28^0C. Bacterial colonies that appeared frequently, morphologically different and endospore forming were selected for further studies. Each isolate was sub-cultured and checked for purity. For short-term storage, the isolates were preserved on NA slants under refrigerated condition. They were preserved in sterile glycerol by mixing 750 µl bacterial suspension with 250 µl sterile glycerol and stored at -80^0C in a deep freezer for long term storage.

3.1.1.2 Isolation of Endospore Forming Endorhizosphere Bacteria from Green Amaranthus variety CO-1

The procedure described in *3.1.1.1* was carried with the variety CO-1 (Plate 1b) for the root samples.

3.1.1.3 Isolation of Endospore Forming Endorhizosphere Bacteria from Wild Relative (Amaranthus viridis)

The procedure described in *3.1.1.1* was carried with wild relative (*Amaranthus viridis*) (Plate 1c) for the root samples.

3.1.2 Isolation of Endospore Forming Phyllosphere Bacteria from *Amaranthus* spp.

3.1.2.1 Isolation of Endospore Forming Phyllosphere Bacteria from Red Amaranthus variety Arun

Leaf samples were collected from healthy amaranthus plants from the Instructional farm of the College of Agriculture, Vellayani, and thoroughly washed with running tap water. Leaf samples were rinsed once with sterile distilled water and were placed inside sterile petriplates followed by dried at 35^0C consecutively for two days. The dried leaf samples were placed in 50 ml sterile distilled water and kept in a shaker for 15 minutes. The obtained leaf wash was heated in a hot water bath at 80^0C for 10 minutes. The heated leaf wash was directly plated on Nutrient Agar. Leaf wash of 0.1 ml was added to 0.9 ml of sterile water to make 10^{-1} dilution and 0.1 ml from 10^{-1} was added to 0.9 ml of sterile water for 10^{-2} dilution and spread plated on NA. The agar plates were incubated at 28^0C. Bacterial colonies that appeared frequently, morphologically different and endospore forming were selected for further studies. Each isolate had been subcultured and checked for purity. For short-term storage, the isolates were preserved on NA slants under refrigerated condition. They were preserved in sterile glycerol by mixing 750 µl bacterial suspension with 250 µl sterile glycerol and stored at -80^0C in a deep freezer for long term storage.

3.1.2.2 Isolation of Endospore Forming Phyllosphere Bacteria from Green Amaranthus variety CO-1

The procedure described in *3.1.2.1* was carried with CO-1 variety for the leaf samples.

3.1.2.3 Isolation of Endospore Forming Phyllosphere Bacteria from Wild Relative (Amaranthus viridis)

The procedure described in *3.1.2.1* was carried with Wild relative (*Amaranthus viridis*) for the leaf samples.

3.2 CHARACTERIZATION OF ENDOSPORE FORMING ENDORHIZOSPHERE AND PHYLLOSPHERE BACTERIAL ISOLATES

3.2.1 Morphological Characterization of Bacterial Isolates

Isolated bacteria were streak purified on NA plates to get single well isolated colonies and colony morphology of selected bacteria was studied. The cell shape, colour, colony morphology, cell arrangement, Grams reaction and Endospore formation were also carried out and results were recorded.

3.2.1.1 Gram reaction of the Bacterial Isolates

The Gram Staining was carried out for all the isolates. The heat fixed bacterial smear was prepared for every isolate. Firstly, primary stain (crystal violet) was added to bacterial smear, followed by the addition of a mordant (Gram's Iodine), a rapid decolourization with alcohol and lastly, counterstaining with safranin. Except safranin every stain was retained over the smear for one minute and was washed before the application of next stain. The later was kept for 30 seconds and excess stain was removed by washing. The stained bacterial smear was observed under oil immersion (100X) objective.

3.2.1.2 Endospore staining of the Bacterial Isolates

Endospore staining was carried out for all the bacterial isolates for testing the presence of endospores. From three days old bacterial cultures single well isolated colonies were taken and smear was prepared on a clean glass slide. Smear was allowed to air dry and was heat fixed. The smear was flooded with malachite green and was placed on a steamed water bath for 10 minutes. Precaution was taken to avoid drying up of stain. Slides were carefully removed and were washed with running tap water. Counter staining was done with safranin for 30 seconds followed by washing with running tap water. Slides were air dried and observed under oil immersion (100X) objective for the presence of endospores.

3.3 PATHOGEN

3.3.1 Isolation

Amaranthus leaves (var. Arun) showing symptoms of leaf blight disease were collected from the Instructional Farm of the College of Agriculture, Vellayani. The advancing portion of the cream coloured lesion on leaves was cut into smaller pieces and washed thoroughly in sterile distilled water for five times. Plates of Potato Dextrose Agar (PDA) were poured and the smaller bits of infected leaves were then kept on the media. Plates were kept for incubation at 28^0C for mycelial development on the media. Smaller bits of mycelial growth were cut and placed on PDA plates to get a pure culture of the pathogen. Mycelium was aseptically transferred to sterile PDA slants and then maintained in a refrigerator for further use.

3.3.2 Testing for Pathogenicity

The isolated pathogen was artificially inoculated on detached healthy leaves of Amaranthus (var. Arun) and tested for its pathogenicity. The obtained *R. solani* was pure cultured on PDA plates. Mycelial discs (8 mm dia) was taken from the pure cultured PDA plates were placed on the lower leaf surface and were covered with moist cotton. To avoid drying of leaves the proximal ends of leaves were covered with moist cotton. The inoculated leaves were placed inside the sterile Petri plates. Development of leaf lesion was observed 24-48h after pathogen inoculation.

3.4 CHARACTERIZATION FOR PLANT GROWTH PROMOTION

The endospore forming endorhizosphere bacteria were screened for their effect on plant growth promotion in Amaranthus (var. Arun).

3.4.1 Estimation of Indole Acetic Acid Production

Estimation of Indole Acetic Acid was carried out as per the standard procedure described by Gordon and Weber (1951).

Nutrient broth of 100 ml was prepared in 250 ml Erlenmeyer flasks and sterilized by autoclaving at 121^0C for 20 min. Bacterial isolates were aseptically inoculated to the medium. Flasks were incubated for 7 days at 28^0C. After incubation, the bacterial cultures were centrifuged at 4500 rpm for 20 minutes. For 10 ml of culture supernatant 2 ml of the Salkowski reagent was added and incubated at room temperature for 25 minutes. Salkowski reagent was prepared by mixing 2 ml 0.5M FeCl3 and 49 ml water and 49 ml 70% perchloric acid in the fume hood. Absorbance was read at 530 nm. Using the standard curve for IAA, the amount of IAA was calculated.

3.4.2 Seedling Vigour index

Roll towel method was used for the assessment of seedling vigour index (Abdul-Baki and Anderson, 1973) Amaranthus seeds (var. Arun) were surface sterilized with 4% sodium hypochlorite for 3 minutes followed by washing in sterile distilled water thrice and blot dried using sterile tissue paper. Each of the bacterial root isolates was heavily cross streaked on nutrient agar plates and incubated at 28^0C for 24 hours. The culture plates were drenched with 10 ml sterile distilled water under aseptic conditions and the respective bacterial suspension was obtained. Surface sterilized seeds (30 number) were soaked in freshly prepared bacterial suspension of 8 isolates individually for 30 minutes. The bacterized seeds were placed in a row of 10 numbers per moist towel paper. The rolled paper towels were placed at 28^0C and saturated moisture condition was maintained by watering the paper towel every day. Three replications were maintained for each treatment. After 10 days the rolled towel papers were removed and observations were recorded.

3.4.2.1 Observations

3.4.2.1.1 Percentage Seed Germination

The number of seeds germinated was recorded. Percentage seed germination was calculated using the formula,

$$\text{Seed Germination (\%)} = \frac{\text{Number of germinated seeds}}{\text{Total number of seeds}} \text{X100}$$

3.4.2.1.2 Root Length

After 10 days the root length was measured from the base of the stem to the tip of the root. It was expressed in cm.

3.4.2.1.3 Shoot Length

After 10 days the shoot length was measured from the base of the stem to the tip of the longest leaf. It was expressed in cm.

3.4.2.1.4 Seedling Vigour Index

Root length and shoot length of the seedlings measured were used for the calculation of seedling vigour index using the formula mentioned below.

Seedling Vigour Index = Germination Percent x (Shoot length + Root length) (Abdul-Baki and Anderson, 1973).

3.4.3 Plant Growth Promotion in Protrays

Vermiculite was used as the planting material for raising amaranthus seedlings (var. Arun). Moistened planting material was packed in polypropylene bags and sterilized for 3 consecutive days by autoclaving at 121^0C for 1 hour. Amaranthus seeds were surface sterilized using sodium hypochlorite 4% for 3 minutes followed by three sterile distilled water washes. The bacterial suspension for seed treatment was carried out as mentioned in section *3.2.1.2* above. Protray cavities were filled with the sterilized planting medium. Two seeds were sown in each cavity of protray and were kept in a naturally ventilated green house. An even foliar spray of 0.5 % NPK (19:19:19) was given at 15 days after sowing. Plants were watered two times a day. After 20 days the plants were carefully uprooted and the observations were recorded.

3.4.3.1 Observations

3.4.3.1.1 Root Length

Root length was measured from the base of the stem to the tip of the root and expressed in cm.

3.4.3.1.2 Shoot Length

Shoot length was measured from the base of the stem to the tip of the longest leaf and expressed in cm.

3.4.3.1.3 Seedling Vigour Index

Measurement of the seedling root length and shoot length were used for calculaing the seedling vigour index. Seedling vigour index was calculated using the formula mentioned below.

Seedling Vigour Index = Germination Percent x (Shoot length + Root length) (Abdul-Baki and Anderson, 1973).

3.4.3.1.4 Root Fresh Weight

Fresh weight of the root (mg) of the uprooted plants was measured.

3.4.3.1.5 Shoot Fresh Weight

Fresh weight of the shoot (mg) of the uprooted plants was measured.

3.4.3.1.6 Root Dry Weight

The dry weight of root (mg) samples was taken after drying constantly at 60^0C in a drying oven.

3.4.3.1.7 Shoot Dry Weight

The dry weight of shoot (mg) samples was taken after drying constantly at 60^0C in a drying oven.

3.5 ASSESSMENT OF *IN VITRO* ANTAGONISM AGAINST *RHIZOCTONIA SOLANI* BY ENDOSPORE FORMING PHYLLOSPHERE BACTERIA.

3.5.1 Direct Antagonism

Dual culture plate assay was carried out to assess the antagonistic activity of bacterial isolates against leaf blight pathogen *Rhizoctonia solani*.

3.5.1.1 Dual culture plate assay

PDA plates were prepared. Bacterial isolates were streaked on NA media for getting isolated single colonies. Mycelial plug (8 mm dia) was taken from 5 days old culture of *R. solani* grown on PDA plates. The mycelial plug was placed at the centre of fresh PDA plate and two streaks of 1 cm length with fresh bacterial culture were done on two opposite edges of the plate. *R. solani* alone in PDA plates were maintained as control. Inoculated plates were incubated at 28^0C for 2 days. Observations were recorded by measuring the zone of inhibition if any (mm).

3.5.2 Indirect Antagonism

Culture filtrate of the bacterial bioagents was tested for their antagonistic activity against the leaf blight pathogen *R. solani*.

3.5.2.1 Extraction of culture filtrate

The bacterial bioagents were purified and a loopful of cells from single isolated bacterial colonies was transferred to Nutrient broth of 100 ml prepared in 250 ml Erlenmeyer flask. Inoculated flasks were incubated overnight in incubator shaker (100 rpm) at 28°C. From the broth culture of each bacterial bioagents, 10 ml of the broth

culture was centrifuged at 4,500 rpm for 15 minutes in a sterile polypropylene tube. The supernatant of each bacterial bioagents was collected aseptically. Using a 0.2 µ nitrocellulose bacteriological filter the supernatant was filter sterilized. The filtrate was aseptically collected and stored at 4^0C for further use.

3.5.2.2 Agar Well Diffusion Method

R. solani was grown on PDA plates. Mycelial plug (8 mm dia) was cut from 5 days old culture of *R. solani* and was transferred to the centre of fresh PDA plate. The PDA plates were incubated at 28^0C for one day. After incubation at two opposite edges of the plate, wells (8 mm dia) were cut using a sterile cork borer. Initially, the wells were filled with 100 µl of 1% agar to avoid the spread of culture filtrate. As the agar solidified, 100 µl each of the culture filtrate of each bacterial bioagents was added to the wells. Plates were incubated for 48 h at 28^0C. Each of the organisms were maintained with four replications. Observations were recorded and the zone of inhibition from the well was measured.

3.5.3 Screening by Detached Leaf Assay

Bacterial isolates were cross streaked on NA medium. After one day of incubation at 28^0C, suspension of bacterial isolates was obtained by drenching the plates with sterile distilled water. Detached leaves of Amaranthus (var. Arun) were treated with individual bacterial isolates by briefly dipping them in the bacterial suspension. Leaves dipped with sterile water served as control. The treated leaves were kept inside sterile Petri dishes. From the fully grown *R. solani* plates, mycelial discs of size 4 mm were used for the assay. 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. The appearance of leaf lesions was observed from the first day of inoculation. The antagonistic activity of bacterial isolates was assessed by measuring the diameter of the lesion developed (cm). Each treatment was maintained with four replications.

3.6 BIOCHEMICAL CHARACTERIZATION OF BACTERIAL ISOLATES

Biochemical characterization of endospore forming endorhizospheric and phyllospheric bacterial isolates was done by performing various biochemical tests and carbohydrate utilization tests by using readymade HiMedia© kits (KB001 HiIMViC Biochemical Test Kit). Colour change was observed on the biochemical amended media of the kit after spot inoculating culture suspensions of the isolates followed by incubation for 24h. The reaction concerning different biochemicals or carbohydrates as positive or negative was observed. Various biochemical tests performed were Indole, Methyl red, Voges Proskauer and Citrate utilization. Different carbohydrate utilization tests performed were Glucose, Adonitol, Arabinose, Lactose, Sorbitol, Mannitol, Rhamnose and Sucrose.

3.7 MOLECULAR CHARACTERIZATION OF BACTERIAL ISOLATES

Molecular characterization of bacterial isolates was done by 16S rRNA cataloging using universal primers at Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram.

3.7.1 Genomic DNA Isolation

Genomic DNA was isolated from the tissues using NucleoSpin® Tissue Kit (Macherey-Nagel) following manufacturer's instructions.

A part of culture is taken in a microcentrifuge tube. 180 µl of T1 buffer and 25 µl of proteinase K were added and incubated at 56^0C in a water bath until it was completely lysed. After lysis, 5 µl of RNase A (100 mg/ml) was added and incubated at room temperature for 5 minutes. 200 µl of B3 buffer was added and incubated at 70^0C for 10 minutes. 210 µl of 100% ethanol was added and mixed thoroughly by vortexing. The mixture was pipetted into NucleoSpin® Tissue column placed in a 2 ml collection tube and centrifuged at 11000 x g for 1 minute. The NucleoSpin® Tissue column was transferred to a new 2 ml tube and washed with 500 µl of BW buffer. Wash step was

repeated using 600 µl of B5 buffer. After washing the NucleoSpin® Tissue column was placed in a clean 1.5 ml tube and DNA was eluted out using 50 µl of BE buffer.

3.7.2 Agarose Gel Electrophoresis for DNA Quality and Quantity check

The quality of the DNA isolated was checked using agarose gel electrophoresis. 1µl of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0) was added to 5µl of DNA. The samples were loaded to 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/ml ethidium bromide. Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 75 V until bromophenol dye front has migrated to the bottom of the gel. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

3.7.3 PCR Analysis

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, 4% DMSO, 5pM of forward and reverse primers and FTA disc as template.

Primers used

Target	Primer Name	Direction	Sequence (5’ → 3’)
16S rRNA	16S- RS-F	Forward	CAGGCCTAACACATGCAAGTC
	16S- RS-R	Reverse	GGGCGGWGTGTACAAGGC

The PCR amplification was carried out in a PCR thermal cycler

(GeneAmp PCR System 9700, Applied Biosystems).

3.7.4 PCR amplification profile

The cycle conditions for the PCR amplification were as follows: initial denaturation at 94^0C for 3 min, followed by 30 cycles of 30 s at 95^0C, 30 s at 56^0C and 90 s at 72^0C, with a final extension step at 72^0C for 10 min.

3.7.4.1 Agarose Gel electrophoresis of PCR Products

The PCR products were checked in 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide. 1 µl of 6X loading dye was mixed with 5 µl of PCR products and was loaded and electrophoresis was performed at 75V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 hours, until the bromophenol blue front had migrated to almost the bottom of the gel.

The molecular standard used was a 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

3.7.4.2 ExoSAP-IT Treatment

ExoSAP-IT (USB) consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications.

Five micro litres of PCR product is mixed with 2 µl of ExoSAP-IT and incubated at 37^0C for 15 minutes followed by enzyme inactivation at 80^0C for 15 minutes.

3.7.4.2.1 Sequencing Using BigDye Terminator v3.1

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufactures protocol.

The PCR mix consisted of the following components:

PCR Product (ExoSAP treated)	-	10-20 ng
Primer	-	3.2 pM (either Forward or Reverse)
Sequencing Mix	-	0.28 µl
Reaction buffer	-	1.86 µl
Sterile distilled water	-	make up to 10µl

The sequencing PCR temperature profile consisted of a 1st cycle at 96^0C for 2 minutes followed by 30 cycles at 96^0C for 30 sec, 50^0C for 40 sec and 60^0C for 4 minutes.

3.7.4.2.2 Post Sequencing PCR Clean up

1. Master mix I of 10µl milli Q and 2 µl 125mM EDTA per reaction and master mix II of 2 µl of 3M sodium acetate pH 4.6 and 50 µl of ethanol were prepared.
2. 12µl of master mix I was added to each reaction containing 10µl of reaction contents and was properly mixed.
3. 52 µl of master mix II was added to each reaction.
4. Contents were mixed by inverting and incubated at room temperature for 30 minutes

5. Spun at 14,000 rpm for 30 minutes
6. Decanted the supernatant and added 100 µl of 70% ethanol
7. Spun at 14,000 rpm for 20 minutes.
8. Decanted the supernatant and repeated 70% ethanol wash
9. Decanted the supernatant and air dried the pellet.

The cleaned up air dried product was sequenced in ABI 3730 DNA Analyzer (Applied Biosystems).

3.7.4.3 Sequence Analysis

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.6.

The nucleotide sequence of 16S rRNA was compared with the sequence available in the database using the BLAST tool offered by National Centre for Biotechnology Information (NCBI). BLASTn provided by NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was carried for homology search.

3.8 *IN VIVO* STUDIES

3.8.1 Study on Plant Growth Promotion

An experiment was conducted in completely randomized design to evaluate the efficacy of selected endospore forming endorhizosphere bacteria for their plant growth promoting activity in Amaranthus (var. Arun). The study was carried out in rain shelter structure of Instructional Farm, College of Agriculture, Vellayani.

3.8.1.1 Raising of Amaranthus Seedlings

The seedlings were raised in the protrays. Sterilized vermiculite was used as planting medium for raising seedlings. Vermiculite was sterilized by autoclaving for three consecutive days at 121^0C for 1 h each. Pro-trays having 40 cells, each cell measuring diameter of 5 cm were filled with the sterile potting mixture. Seeds of amaranthus were surface sterilized in 4% sodium hypochlorite aqueous solution for 3 minutes in an aseptic condition. The seeds were further washed thrice with sterile distilled water to wash off the sodium hypochlorite.

The selected endospore forming endorhizosphere bacterial isolates were heavily cross streaked on Nutrient Agar medium and plates were incubated at 28^0C. After 24 h of incubation, the culture plates were drenched with 10 ml sterile distilled water and the respective bacterial suspension was obtained aseptically. The surface sterilized seeds were soaked for a period of 2h in the bacterial suspension and bacterization of seeds was carried out.

Two bacterized seeds were planted per cell of protray and after germination further thinning was carried to single seedling per cell. Seedlings were irrigated with tap water twice a day. After 15 days of seeding, at four leaf stage the seedlings were transplanted to plastic pots (15 cm dia) filled with one kg each of potting mixture (soil, coir pith and cow dung in the ratio 2:1:1).

3.8.1.2 Seedling Dip Method

Fifteen days old healthy amaranthus seedlings were selected for transplanting. The seedlings were carefully uprooted from each portray cell and dipped in the respective bacterial suspension for 30 min and transplanted into individual pots. Control plants were dipped in sterile distilled water and planted. The pots were drenched with 50 ml of 1% NPK (19:19:19), after 10 days of transplanting.

Design : CRD

Treatments : 7

Replication : 4

Number of plants/replication : 5

3.8.1.3 Treatments

T_1 : *Bacillus* sp AR1

T_2 : *Bacillus siamensis* AR2

T_3 : *Bacillus amyloliquefaciens* GR1

T_4 : *Bacillus amyloliquefaciens* GR2

T_5 : *Bacillus* sp WR1

T_6 : *Bacillus amyloliquefaciens* WR2

T_7 : Control

3.8.1.4. Pathogen Inoculation on to Treated Plants

Virulent strain of *Rhizoctonia solani* was used for inoculation on amaranthus plants 25 days after transplanting. Inoculation was carried out to three healthy intact leaves of three plants of all treatments. 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 of the plants of all the treatments. The mycelial plugs were covered with thin layer of moist cotton to provide humid condition for the growth of the pathogen.

3.8.1.5 Biometric Observation

Biometric observations of plants were recorded at an interval of ten days. The observations recorded were; plant height (cm), number of leaves plant^{-1}, number of

branches plant^{-1} and pest and disease occurrence, if any. Destructive sampling was done after 30 days of transplanting to assess fresh shoot weight (g), dry shoot weight (g), fresh root weight (g) and dry root weight (g). The dry weights of samples were taken after drying at 60^{0}C in a hot air oven.

3.8.1.5.1 Number of Leaves

Number of leaves of treated amaranthus plants was counted and recorded at 5 DAT, 15 DAT and 25 DAT.

3.8.1.5.2 Number of Branches

Number of branches in each of the treated amaranthus plants was counted and recorded at 15 DAT and 25 DAT.

3.8.1.5.3 Plant height (cm)

Height of the plant was measured from the base to the growing tip of the shoot in cm at 15 DAT and 25 DAT.

3.8.1.5.4 Fresh Weight of Roots

Fresh weight of roots (g) was taken immediately after uprooting the plants and weighed using an electronic single pan balance.

3.8.1.5.5 Dry Weight of Roots

Dry weight of roots (g) was taken after drying the samples at 60^{0}C in a drying oven.

3.8.1.5.6 Fresh Weight of Shoots

Fresh weight of shoots (g) was taken immediately after uprooting the plants and weighed using an electronic single pan balance.

3.8.1.5.7 Dry Weight of Shoots

Dry weight of shoots (g) was taken after drying the samples at 60^0C in a drying oven.

3.8.1.5.8 Disease Severity

Disease severity was calculated for each plant inoculated with pathogen. Severity of disease was graded by using the 0-9 scale (KAU, 1996) (Plate 2).

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:

$$PDI = \frac{(\text{Sum of individual ratings})}{\text{Number of leaves assessed X Maximum grade used}} \text{ X } 100$$

3.8.2 Testing for Disease Suppression

An experiment was conducted to evaluate the efficacy of selected endospore forming phyllosphere bacteria in controlling the disease caused by *R. solani* in red amaranthus variety Arun. The study was carried out in rain shelter structure of Instructional Farm, College of Agriculture, Vellayani.

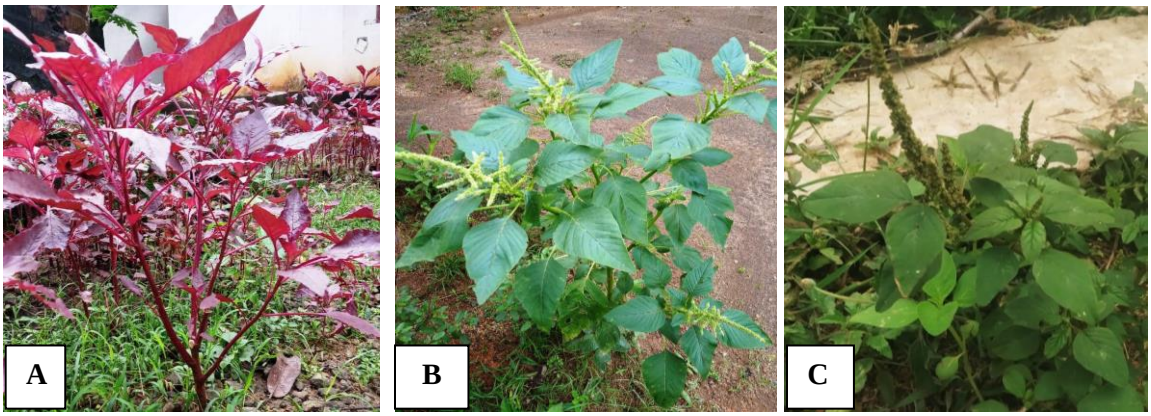


Plate 1. Varieties of Amaranthus used in the study

A) Red Amaranthus (Arun) **B) Green amaranthus (CO-1)**

C) Wild relative (*Amaranthus viridis*)

Plate 2. Scale for the scoring of foliar blight of amaranthus

3.8.2.1 Raising of seedlings

Seedlings were raised in the pro-trays with sterilized vermiculite as planting medium. Vermiculite was sterilized by autoclaving for three consecutive days at 121^0C for 1 h each. Protrays having 40 cells, each cell measuring diameter of 5 cm were filled with the sterile potting mixture. Seeds of amaranthus were surface sterilized in 4% sodium hypochlorite aqueous solution for 3 minutes in an aseptic condition. The seeds were further washed thrice with sterile distilled water to wash off the sodium hypochlorite.

Two surface sterilized seeds were sown per cell of pro-tray and after germination further thinning was carried out to single seedling per cell. Seedlings were irrigated with tap water twice a day. After 15 days of seeding, at four leaf stage, the seedlings were transplanted to plastic pots (15 cm dia) filled with one kg each of potting mixture (soil, coir pith and cow dung in the ratio 2:1:1).

After 20 days of transplanting, foliar spray of six promising endospore forming phyllosphere bacteria selected from the *in vitro* antagonism tests was carried out. Plants sprayed with Mancozeb (0.3 %) were taken as chemical control. Pathogen inoculated and absolute (uninoculated) controls were also maintained.

Design : CRD

Treatments : 9

Replications : 4

Number of plants/replication : 5

3.8.2.2 Treatments

T_1 : *Bacillus* sp AL1

T_2 : *Bacillus* sp AL3

T_3 : *Bacillus subtilis* GL2

T_4 : *Bacillus* sp GL3

T_5 : *Bacillus subtilis* WL1

T_6 : *Bacillus amyloliquefaciens* WL2

T7 : Pathogen inoculated control

T8 : Chemical control (0.3% Mancozeb)

T9 : Absolute control

3.8.2.3 Inoculation of Bacterial Cultures on to Amaranthus Plants

The selected endospore forming phyllosphere bacterial cultures were cross streaked on Nutrient Agar plates and kept for incubation at 28^0C for one day. The cultures plates were drenched with 10 ml of sterile distilled water and scraped off to obtain bacterial suspension. The obtained bacterial suspension was vortexed to get a uniform suspension. The suspension was then sprayed on to the intact leaves of plants after 20 days of transplanting.

3.8.2.4 Application of Mancozeb

Mancozeb (0.3%) was sprayed onto the foliage one week before pathogen inoculation. The sprayed plants were maintained has chemical control.

3.8.2.5 Pathogen Inoculation on to Treated Plants

Virulent strain of *Rhizoctonia solani* was used for inoculation on amaranthus plants. Inoculation was carried out to three healthy intact 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 for the growth of the pathogen.

3.8.2.6 Observations

Observations on disease incidence and disease severity were recorded on 3rd and 5th DAI of the pathogen.

3.8.2.6.2 Disease Severity

Disease severity was calculated as per *3.8.1.5.8*

3.8.2.6.3 Biometric Observation

Biometric observations of plants were recorded at the time of harvest. After the disease scoring, the plants were harvested and destructive sampling was done to assess fresh shoot weight (g), dry shoot weight (g), fresh root weight (g) and dry root weight (g). The dry weights of samples were taken after drying at 60^0C in a hot air oven.

3.9 STATISTICAL ANALYSIS

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

Results

4. RESULTS

The present investigation on "Plant associated endospore forming bacteria from amaranthus as growth promoters and biocontrol agents against Rhizoctonia leaf blight" was carried out in Department of Agricultural Microbiology during 2018-2020. The experimental data from the above mentioned investigation were analyzed and the results are presented in this chapter.

4.1 ISOLATION OF ENDOSPORE FORMING ENDORHIZOSPHERE AND PHYLLOSPHERE BACTERIA FROM *Amaranthus* spp.

4.1.1 Isolation of Endospore Forming Endorhizosphere Bacteria from *Amaranthus* spp.

4.1.1.1 Isolation of Endospore Forming Endorhizosphere Bacteria from Red Amaranthus variety Arun

Endospore forming endorhizosphere bacteria from healthy dried roots were isolated by enrichment method on NA medium. Three bacterial isolates were obtained from the endorhizosphere of red amaranthus variety Arun (Plate 3a). The isolates were given abbreviation as AR indicating Arun root. All the isolates had single rod shaped cells. Regarding colony morphology, all the isolates showed irregular form with undulated margin. However, one isolate was flat and other two isolates exhibited raised elevation. Colonies of these three isolates were creamish white in colour and were positive for Grams reaction and all were endospore formers (Table 1)

4.1.1.2 Isolation of Endospore Forming Endorhizosphere Bacteria from Green Amaranthus variety CO-1

Three endospore forming endorhizosphere bacteria from healthy dried roots of Green amaranthus variety CO-1 were obtained by enrichment method on NA medium (Plate 3b). The isolates were given with the abbreviation GR indicating Green-root. All isolates were endospore formers and showed Gram's positive reaction. All the three

isolates were single rod and showed creamy white coloured colonies. The isolates differed in their colony morphology, with irregular form and undulate margin of which one isolate showed raised elevation and other two were flat (Table 1)

4.1.1.3 Isolation of Endospore Forming Endorhizosphere Bacteria from Wild Relative (Amaranthus viridis)

Two endospore forming endorhizosphere bacteria were obtained from Wild Relative of amaranthus (*Amaranthus viridis*) (Plate 3c). Isolation was carried from healthy dried roots by enrichment method on NA medium. The isolates were given the abbreviation WR, indicating Wild root. All the isolates were single rods and the isolates showed irregular form, umbonated elevation and undulated margin as their colony characters. Every isolate showed creamish white coloured colonies, positive Gram's reaction and were endospore formers (Table 1).

4.1.2 Isolation of Endospore Forming Phyllosphere Bacteria from *Amaranthus* spp.

4.1.2.1 Isolation of Endospore Forming Phyllosphere Bacteria from Red Amaranthus variety Arun

Three isolates were obtained by enrichment method on NA medium and were given the abbreviation AL indicating Arun Leaves (Plate 4a). All the isolates were single rod and showed positive for Grams' reaction and were endospore formers. All the isolates were creamy white coloured colonies. Every isolate showed similar colony morphology with irregular form, raised elevation and undulate margin (Table 2).

4.1.2.2 Isolation of Endospore Forming Phyllosphere Bacteria from Green Amaranthus variety CO-1

Three isolates were obtained and were given with the abbreviation GL indicating Green Leaves (Plate 4b). The isolates single rod shaped, Gram positive cells and all were endospore formers. Among the three isolates, two showed raised elevation

and creamy white colony colour while the other isolate was flat and the colonies were brownish creamy white coloured. Colonies were creamy white in colour, irregular form and undulate margin (Table 2).

4.1.2.3 Isolation of Endospore Forming Phyllosphere Bacteria from Wild Relative (Amaranthus viridis)

Two isolates were obtained from the enrichment method on NA medium (Plate 4c). The isolates were abbreviated with WL indicating Wild Leaves. All the isolates showed single rod shaped cells and positive for Grams' reaction. Colonies with irregular margin, raised elevation and undulate margin were observed. Every isolate showed creamy white coloured colonies and and all were endospore formers (Table 2).

Different endospore forming endorhizosphere bacterial isolates obtained from red amaranthus variety Arun (Plate 5), green amaranthus (Plate 6) and wild relative *Amaranthus viridis* (Plate 7) and endospore forming phyllosphere bacterial isolates obtained from red amaranthus variety Arun (Plate 8), green amaranthus (Plate 9) and wild relative *Amaranthus viridis* (Plate 10) by enrichment method on NA medium were purified by the streak plate method.

4.2 PATHOGEN

4.2.1 Isolation

Virulent strain of leaf blight pathogen *Rhizoctonia solani* was isolated from naturally infected leaves of Red amaranthus variety Arun on PDA medium. Colour, shape, texture and growth characters of pathogen *Rhizoctonia solani*, were observed. Mycelial growth was visible after two days on PDA medium. Initially the mycelium was creamish in colour, however, later the colour changed to light hyaline-brown, with aerial mycelium. As growth progressed, alternating dark and light concentric rings were

Table 1. Morphological and colony characteristics of endospore forming endorhizosphere bacterial isolates

Isolates*	Cell shape and arrangement	Colony characters				Gram's reaction	Endospore formation
		Colour	Form	Elevation	Margin		
AR1	Single rod	Creamy white	Irregular	Raised	Undulate	G^+	+
AR2	Single rod	Creamy white	Irregular	Raised	Undulate	G^+	+
AR3	Single rod	Creamy white	Irregular	Flat	Undulate	G^+	+
GR1	Single rod	Creamy white	Irregular	Raised	Undulate	G^+	+
GR2	Single rod	Creamy white	Irregular	Flat	Undulate	G^+	+
GR3	Single rod	Creamy white	Irregular	Flat	Undulate	G^+	+
WR1	Single rod	Creamy white	Irregular	Umbonate	Undulate	G^+	+
WR2	Single rod	Creamy white	Irregular	Umbonate	Undulate	G^+	+

*AR – Arun root *GR – Green root *WR – Wild root

Table 2. Morphological and colony characteristics of endospore forming phyllosphere bacterial isolates

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 – Arun leaves *GL – Green leaves *WL – Wild leaves

a. Arun variety **b. CO-1 variety** **c. Wild Relative**

Plate 3. Endospore forming endorhizosphere bacteria isolated on NA medium

a. Variety Arun **b. CO-1 variety** **c. Wild Relative**

Plate 4. Endospore forming phyllosphere bacteria isolated on NA medium

AR1 **AR2** **AR3**

Plate 5. Pure culture of Endospore forming Endorhizosphere Bacteria isolated from red amaranthus variety Arun

GR1 **GR2** **GR3**

Plate 6. Pure culture of Endospore forming Endorhizosphere Bacteria isolated from green amaranthus variety CO-1

WR1 **WR2**

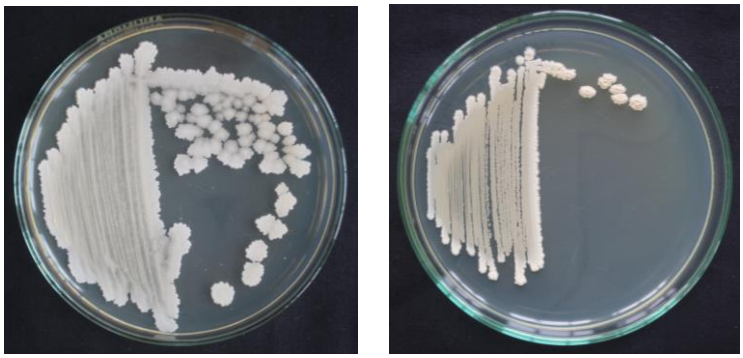
Plate 7. Pure culture of Endospore forming Endorhizosphere Bacteria isolated from wild relative (*Amaranthus viridis*)

AL1 **AL2** **AL3**

Plate 8. Pure culture of endospore forming phyllosphere bacteria isolated from red amaranthus variety Arun

GL1 **GL2** **GL3**

Plate 9. Pure culture of endospore forming phyllosphere bacteria isolated from green amaranthus variety CO-1



WL1 **WL2**

Plate 10. Pure culture of endospore forming phyllosphere bacteria isolated from wild relative (*Amaranthus viridis*

observed (Plate 11). On microscopic observation, formation of young branches, approximately right angles to the main hyphae was seen. At the branching point right angle branching and cross septa of mature hyphae was observed (Plate 12). The sclerotia became brown in colour after 7 days of incubation. Mature sclerotia were dark brown in colour without definite form and were scattered over the Petri plate.

4.2.2 Testing for Pathogenicity

The isolated pathogen was artificially inoculated with pinprick on detached healthy leaves of red amaranthus variety Arun under *in vitro* conditions. Symptoms initiated as translucent irregular green patches on the inoculated areas of leaves after two to three days of inoculation (Plate 13). Gradually the patches enlarged and covered the entire leaf lamina. Later, the leaves turned creamy and dried up.

4.3 CHARACTERIZATION FOR PLANT GROWTH PROMOTION

In vitro evaluation of the endospore forming endorhizosphere bacteria for plant growth promoting activities was carried out and their results are given below.

4.3.1 Estimation of Indole Acetic Acid Production

The endospore forming endorhizosphere bacterial isolates obtained from the red amaranthus variety arun, green amaranthus variety CO-1 and from the wild relative *Amaranthus viridis* were screened for *in vitro* production of IAA. The isolates produced IAA ranging from 4.09 µg mL^{-1} to 9.73 µg mL^{-1}.

There was a significant difference between the isolates in IAA production and the results are represented in Table 3. Maximum IAA production of 9.73 µg mL^{-1} of the culture was reported by the isolate AR3 and the minimum was reported by the isolate AR2 (4.09 µg mL^{-1}).

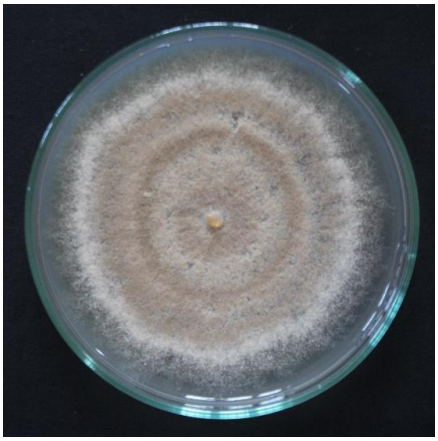


Plate 11. Growth of *Rhizoctonia solani* on potato dextrose agar medium

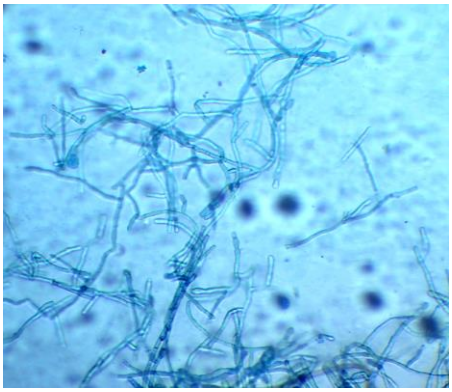


Plate 12. Microscopic view of mycelia of *Rhizoctonia solani*

Plate 13. Appearance of foliar blight symptom on amaranthus leaves on artificial inoculation

Table 3. Indole acetic acid production by endospore forming endorhizospheric bacterial isolates

Isolates	**IAA (µg mL^{-1})***
AR1	5.37 ± 0.29^{f}
AR2	4.09 ± 0.15^{g}
AR3	9.73 ± 0.09^{a}
GR1	7.72 ± 0.30^{d}
GR2	8.80 ± 0.04^{b}
GR3	7.56 ± 0.17^{d}
WR1	6.93 ± 0.16^{e}
WR2	8.36 ± 0.27^{c}

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

4.3.2 Seedling Vigour Index

The endospore forming endorhizosphere bacterial isolates were tested for their influence on germination percentage and seedling vigour in red amaranthus variety Arun using roll towel assay. The isolates showed significant difference in germination percentage. The isolate WR1 showed maximum germination percentage (43.33%) whereas the minimum (20%) was reported by AR1 and GR3 isolates which were found to be statistically on par with each other (Table 4).

There was significant influence of isolates on root length, shoot length and seedling vigour index and the details are represented in Table 5.

The root length of plants treated with different isolates ranged between 1.35 to 2.87 cm. Maximum root length was reported by isolate AR1 (2.87 cm) followed by WR2 (2.84 cm) which were found to be statistically on par with each other and isolate GR1 showed minimum root length (1.35 cm) (Plate 14).

In the study, shoot length of plants treated with different isolates ranged between 2.46 to 3.55 cm which were reported by the isolates GR1 and AR3 respectively. There was a significant difference in seedling vigour index between the isolates. Isolate WR1 showed highest seedling vigour index (223.66) and the least was observed in isolate GR1 (90.99) (Plate 14)**.**

4.3.3 Plant Growth Promotion study in Protray

In the portray study, the effect of endospore forming endorhizosphere bacterial isolates on number of leaves, root length, shoot length, germination percentage and seedling vigour index are presented in Table 6.

Observations on the number of leaves showed no significant difference between the treatments and the values ranged between 3.69 to 4.33.

Table 4. Effect of endospore forming endorhizosphere bacterial isolates on amaranthus seed germination (var. Arun)

Treatments	**Germination (%)***
AR1	20.00 ± 0.00^{b}
AR2	23.33 ± 5.77^{b}
AR3	23.33 ± 5.77^{b}
GR1	23.33 ± 5.77^{b}
GR2	26.67 ± 5.77^{b}
GR3	20.00 ± 0.00^{b}
WR1	43.33 ± 11.55^{a}
WR2	23.33 ± 5.77^{b}
Water	30.00 ± 0.00^{b}

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

Table 5. Effect of endospore forming endorhizosphere bacterial isolates on root length, shoot length and seedling vigour index of Amaranthus seedlings (var. Arun) in paper towel assay.

Treatments	Root length (cm)/plant*	Shoot length (cm)/plant*	Seedling Vigour Index*
AR1	2.87 ± 0.63^{a}	3.02 ± 0.21^{b}	117.67 ± 10.02^{b}
AR2	1.5 ± 0.57^{bc}	3.17 ± 0.19^{ab}	109.66 ± 31.86^{b}
AR3	2.28 ± 0.08^{abc}	3.55 ± 0.05^{a}	135.67 ± 30.60^{b}
GR1	1.35 ± 0.48^{c}	2.46 ± 0.11^{c}	90.99 ± 37.31^{b}
GR2	1.79 ± 0.75^{abc}	2.71 ± 0.52^{bc}	117.32 ± 12.65^{b}
GR3	2.53 ± 0.85^{ab}	2.73 ± 0.46^{bc}	105.33 ± 16.62^{b}
WR1	2.24 ± 0.66^{abc}	2.85 ± 0.17^{bc}	223.66 ± 81.14^{a}
WR2	2.84 ± 0.20^{a}	2.72 ± 0.20^{bc}	129.18 ± 33.81^{b}
Control	1.69 ± 0.55^{bc}	2.86 ± 0.22^{bc}	136.53 ± 11.24^{b}

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

Table 6. Effect of endospore forming endorhizosphere bacteria on growth of amaranthus seedlings in portray method

Treatments	No. of leaves/plant*	Root length (cm)/plant*	Shoot length (cm)/plant*	Germination (%)*	Seedling Vigour Index*
AR1	3.94 ± 0.24^{a}	4.17 ± 0.78^{ab}	5.95 ± 0.52^{ab}	80.00 ± 0.00^{bc}	809.60 ± 101.75^{a}
AR2	3.78 ± 0.21^{a}	3.78 ± 0.42^{abc}	5.98 ± 0.37^{ab}	85.00 ± 7.07^{b}	828.96 ± 51.06^{a}
AR3	3.69 ± 0.55^{a}	3.32 ± 0.58^{bc}	5.61 ± 0.60^{ab}	80.00 ± 0.00^{bc}	714.60 ± 78.83^{b}
GR1	4.33 ± 0.24^{a}	4.76 ± 0.66^{a}	6.26 ± 0.58^{ab}	45.00 ± 7.07^{e}	496.01 ± 51.70^{d}
GR2	3.81 ± 0.46^{a}	4.06 ± 0.45^{ab}	5.48 ± 0.60^{b}	65.00 ± 7.07^{d}	620.26 ± 40.53^{bc}
GR3	3.75 ± 0.62^{a}	3.61 ± 0.55^{bc}	5.70 ± 0.44^{ab}	62.50 ± 3.54^{d}	582.19 ± 58.50^{cd}
WR1	3.75 ± 0.35^{a}	3.73 ± 0.82^{abc}	6.34 ± 0.61^{a}	80.00 ± 0.00^{bc}	805.40 ± 64.39^{a}
WR2	3.75 ± 0.44^{a}	2.99 ± 0.49^{c}	5.85 ± 0.17^{ab}	100.00 ± 0.00^{a}	884.50 ± 38.73^{a}
Control	3.69 ± 0.24^{a}	3.91 ± 0.90^{abc}	5.59 ± 0.49^{ab}	70.00 ± 0.00^{cd}	665.35 ± 48.65^{bc}

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

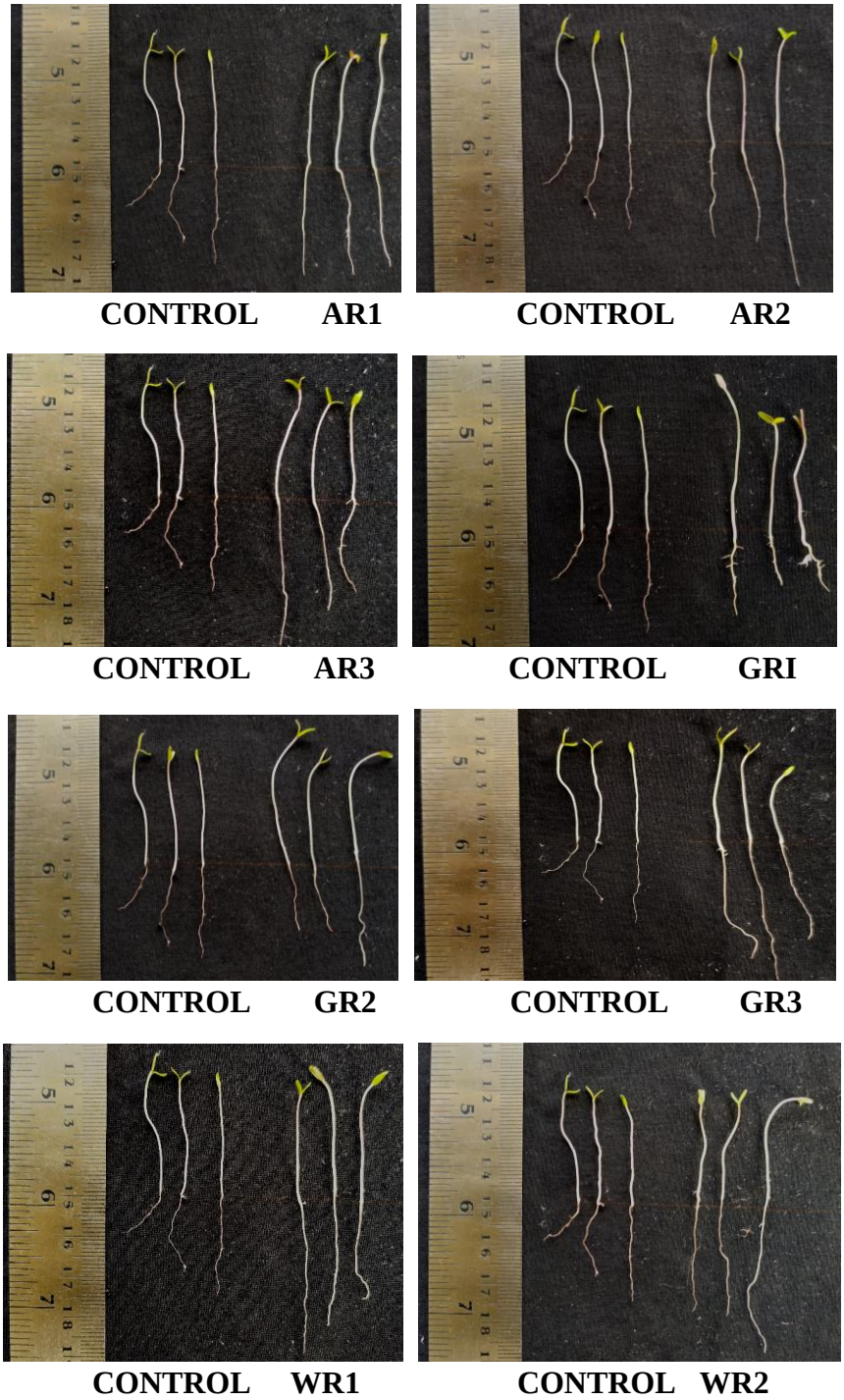


Plate 14. Influence of endospore forming endorhizosphere bacteria on growth of amaranthus in roll towel assay

There was significant influence of different isolates on root length of the seedlings. The highest root length was observed in the seedlings treated with isolate GR1 (4.76 cm) and the least was observed in seedlings treated with isolate WR2 (2.99 cm). The values of shoot length ranged between a minimum of 5.4 cm to maximum of 6.34 cm representing seedlings treated with isolate GR2 and WR1 respectively.

Isolates were also checked for their effect on germination of amaranthus seeds and it was found that the isolate significantly influenced it. 100 percent germination was obtained in seeds treated with the isolate WR2 whereas the isolate GR1 showed the minimum percent of seed germination (45 %).

Seedling vigour index was calculated by using the root length, shoot length and germination percentage. When the data were analyzed, significant influence of the bacterial isolates on seedling vigour index was observed. The values ranged from 496.01 (GR1) to 884.50 (WR2).

In the portray study, the seedlings treated with different bacterial isolates had influence on fresh and dry weights of shoot and roots. There was significant improvement in root and shoot fresh weight, root dry weight of the seedlings treated with different isolates (Table 7). There was no significant influence of bacterial isolates on shoot dry weight.

Highest root fresh weight was recorded with the plants treated with isolate GR1 (8.12 mg/plant) followed by AR2 (8.03 mg/plant) which were found on par with each other. The lowest root fresh weight of 3.56 mg/plant was recorded with the seeds treated with water (control plants).

There was significant difference between the plants treated with different bacterial isolates and control plants in shoot fresh weight. The values of weight ranged from the minimum 35.92 mg/plant (control) to maximum 83.54 mg/plant (AR2) (Plate 15).

Table 7. Effect of isolates of endospore forming endorhizosphere bacteria on root and shoot fresh weight and dry weight of Amaranthus seedlings (var. Arun) in protray experiment

Treatments	Root fresh weight(mg)/plant*	Shoot fresh weight(mg)/plant*	Root dry weight(mg)/plant*	Shoot dry weight(mg)/plant*
AR1	4.62 ± 1.01^{bc}	74.33 ± 20.15^{ab}	2.51 ± 0.10^{bc}	14.81 ± 1.11^{a}
AR2	8.03 ± 0.82^{a}	83.54 ± 9.53^{a}	2.32 ± 0.12^{cd}	14.10 ± 0.62^{a}
AR3	4.82 ± 0.78^{bc}	54.32 ± 20.32^{bc}	2.25 ± 0.12^{cd}	14.69 ± 1.06^{a}
GR1	8.12 ± 0.91^{a}	68.36 ± 17.58^{ab}	2.49 ± 0.32^{bc}	15.75 ± 0.97^{a}
GR2	5.96 ± 2.20^{b}	48.51 ± 25.34^{bc}	2.65 ± 0.11^{b}	14.60 ± 1.91^{a}
GR3	5.65 ± 1.64^{b}	53.93 ± 21.15^{bc}	2.11 ± 0.04^{d}	14.25 ± 1.09^{a}
WR1	5.87 ± 1.87^{b}	74.36 ± 15.22^{ab}	2.99 ± 0.23^{a}	14.27 ± 1.08^{a}
WR2	4.20 ± 0.62^{bc}	59.12 ± 13.98^{abc}	2.15 ± 0.10^{d}	14.75 ± 0.65^{a}
Control	3.56 ± 0.21^{c}	35.92 ± 4.37^{c}	2.46 ± 0.15^{bc}	13.98 ± 0.88^{a}

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

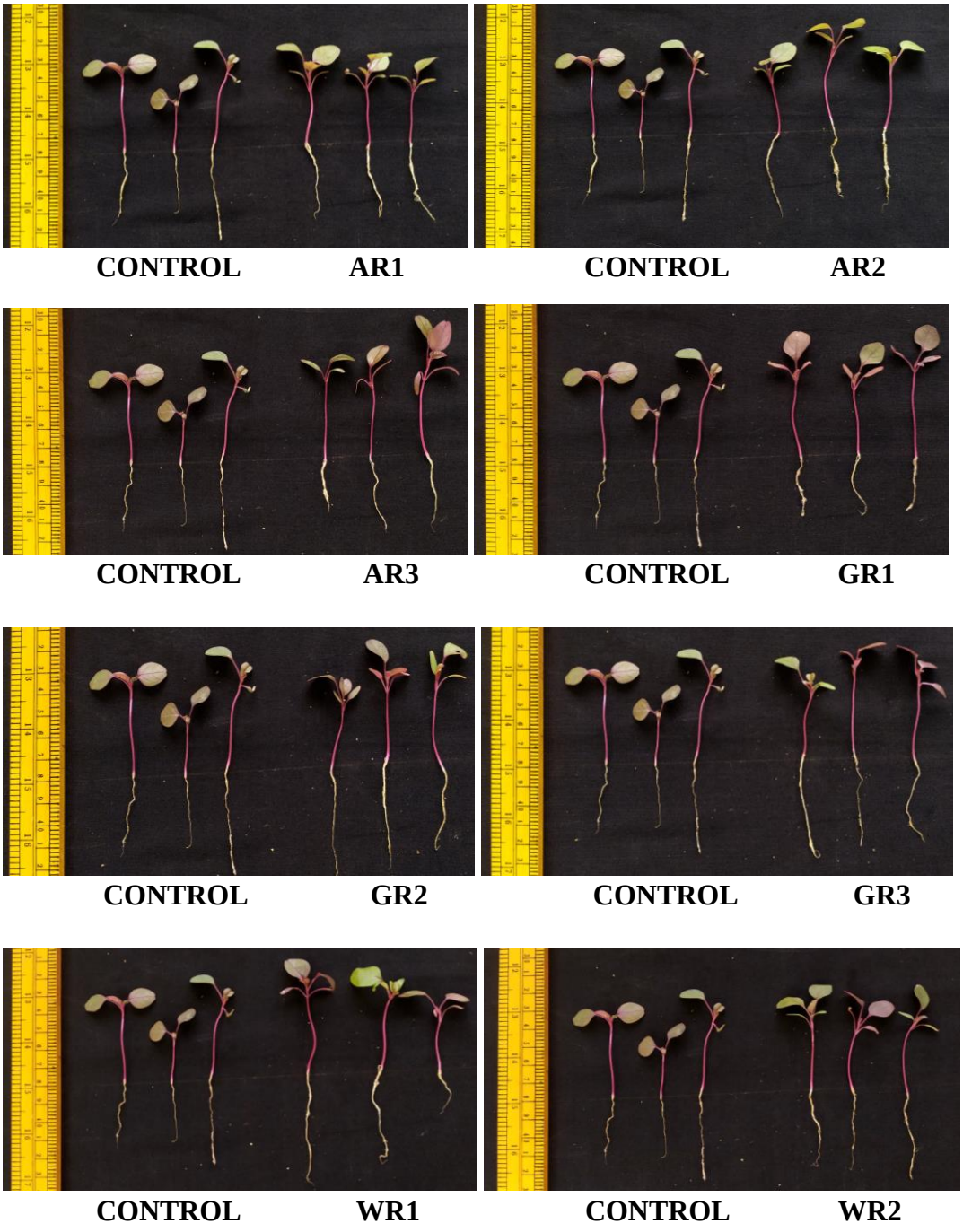


Plate 15. Influence of endospore forming endorhizosphere bacteria on growth of amaranthus in portray method

After drying of fresh root samples, the root dry weight was recorded and the analysis of data was carried out. It was found that highest root dry weight was found in plants treated with isolate WR1 (2.99 mg/plant). The least root dry weight was observed in plants treated with isolate GR3 (2.11 mg/plant). There was no significant influence of the treatments in shoot dry weight.

4.4 ASSESSMENT OF *IN VITRO* ANTAGONISM AGAINST *RHIZOCTONIA SOLANI* BY ENDOSPORE FORMING PHYLLOSPHERE BACTERIA.

4.4.1 Direct Antagonism

4.4.1.1 Dual culture plate assay

Screening of endospore forming phyllosphere bacterial isolates was performed using dual culture plate assay to assess the antagonistic potential against *Rhizoctonia solani*. Interaction of endospore forming phyllosphere bacteria against *R. solani* in dual culture plate assay is shown in Table 8.

The presence of zone of inhibition in dual culture plate was considered as a sign of antagonism of endospore forming phyllosphere bacterial isolates against *R. solani*. Inhibition zone was measured in mm. The maximum inhibition zone against *R. solani* was shown by isolate GL3 (8.17 mm) followed by the isolate WL2 (7.67 mm) which were found to be significantly on par with each other. The least inhibition zone was exhibited by isolate GL1 (1.00 mm) (Table 9) (Plate 16)**.**

4.4.2 Indirect Antagonism

Inhibition of pathogen growth with culture filtrate was carried out to check the indirect antagonism activity of endospore forming phyllosphere bacterial isolates against *R. solani*. All the 8 isolates of endospore forming phyllosphere bacteria were tested for their antagonistic activity.

Table 8. Interaction of endospore forming phyllosphere bacterial isolates against *Rhizoctonia solani* in dual culture plate assay

Isolates	**Presence of inhibition**
AL1	++
AL2	+
AL3	++
GL1	+
GL2	++
GL3	+++
WL1	++
WL2	+++

+ : < 2 mm, ++ : < 5 mm, +++ : < 9 mm

Table 9. Antagonistic activity of endospore forming phyllosphere bacterial isolates in dual culture plate assay

Isolates	Zone of inhibition (mm)*
AL1	3.17 ± 0.75^{c}
AL2	2.00 ± 0.63^{d}
AL3	4.83 ± 0.75^{b}
GL1	1.00 ± 0.63^{e}
GL2	3.00 ± 0.63^{c}
GL3	8.17 ± 0.75^{a}
WL1	4.50 ± 1.05^{b}
WL2	7.67 ± 1.03^{a}

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

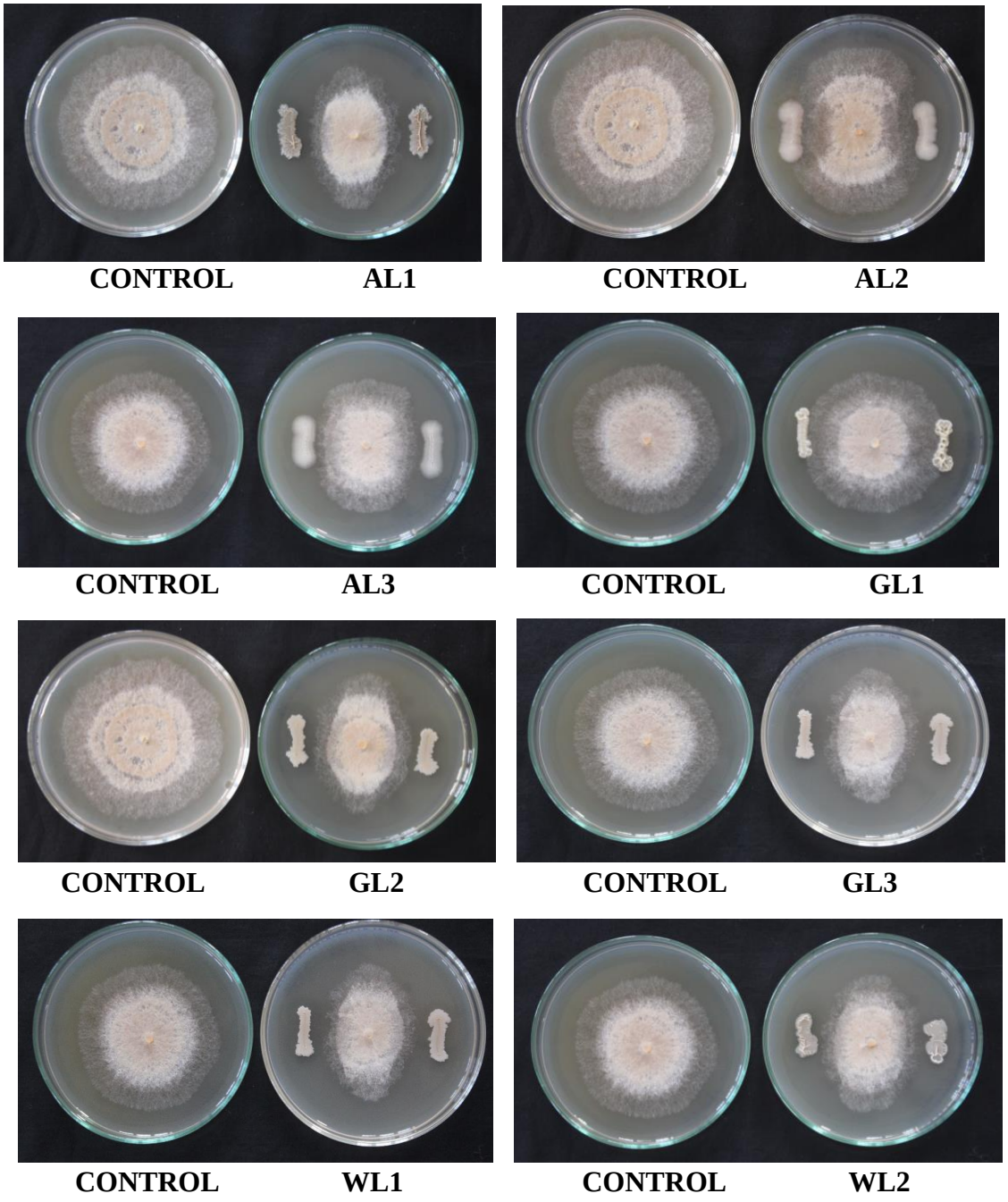


Plate 16. Dual culture plate assay for checking the direct antagonism of endospore forming phyllosphere bacterial isolates against *Rhizoctonia solani*

The zone of inhibition was measured in millimeter produced by the bacterial isolates against *R. solani* were analyzed (Table 10). The culture filtrate obtained from the isolate GL3 (2.88 mm) isolated from green amaranthus showed maximum zone of inhibition against *R. solani*. This was followed by the inhibition zone produced by isolates WL1 (2.25 mm) and AL3 (2.12 mm). The minimum zone of inhibition was exhibited by the culture filtrate obtained from the isolate GL1 (0.50 mm) (Plate 17).

4.4.3 Screening by Detached Leaf Assay

Detached leaf assay was performed on the leaves of red amaranthus variety Arun. All the isolates of endospore forming phyllosphere bacteria were used for testing their antagonistic activity against *R. solani*. The observations of lesion development on the detached leaf assay were taken on 3rd and 4th day after inoculation. The lesion area was calculated by measuring the length and breadth of the lesion. The obtained data was analyzed and represented in Table 11.

All the isolates of endospore forming phyllosphere bacteria tested showed antagonistic activity against *R. solani*. There was significant difference between lesion development on the leaves treated bacterial isolates and the leaves treated with water as control. On the 3rd day of pathogen inoculation, the maximum lesion area was exhibited by the uninoculated detached leaves (control) (3.49 cm^2) and which was followed by GL1 (2.77 cm^2), isolate AL2 (2.62 cm^2) and isolate GL2 (2.52 cm^2), were found to be on par with each other. The least lesion area was measured in the leaves treated with WL2 isolate (0.42 cm^2), hence the maximum biocontrol activity. When the lesion area was measured on 4th day after pathogen inoculation, there was a significant difference between the detached leaves treated with bacterial isolates and with water. The leaves without bacterial treatment (control) exhibited maximum lesion area of 12.23 cm^2. This was followed by the leaves treated isolates GL1 (9.59 cm^2). The minimum lesion area was developed on the leaves treated with isolate WL2 (3.11 cm^2) (Plate 18).

Table 10. Mycelial growth inhibition of *Rhizoctonia solani* by culture filtrate of endospore forming phyllosphere bacteria

Isolates	**Zone of inhibition (mm)***
AL1	1.37 ± 0.52^{cd}
AL2	1.12 ± 0.64^{cde}
AL3	2.12 ± 0.64^{b}
GL1	0.50 ± 0.53^{e}
GL2	1.75 ± 0.71^{bc}
GL3	2.88 ± 0.64^{a}
WL1	2.25 ± 0.71^{ab}
WL2	0.87 ± 0.64^{de}

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

Table 11. 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 ± 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. Values in a column followed by same letter do not differ significantly according to Duncan's Multiple Range Test ($p \leq 0.05$)

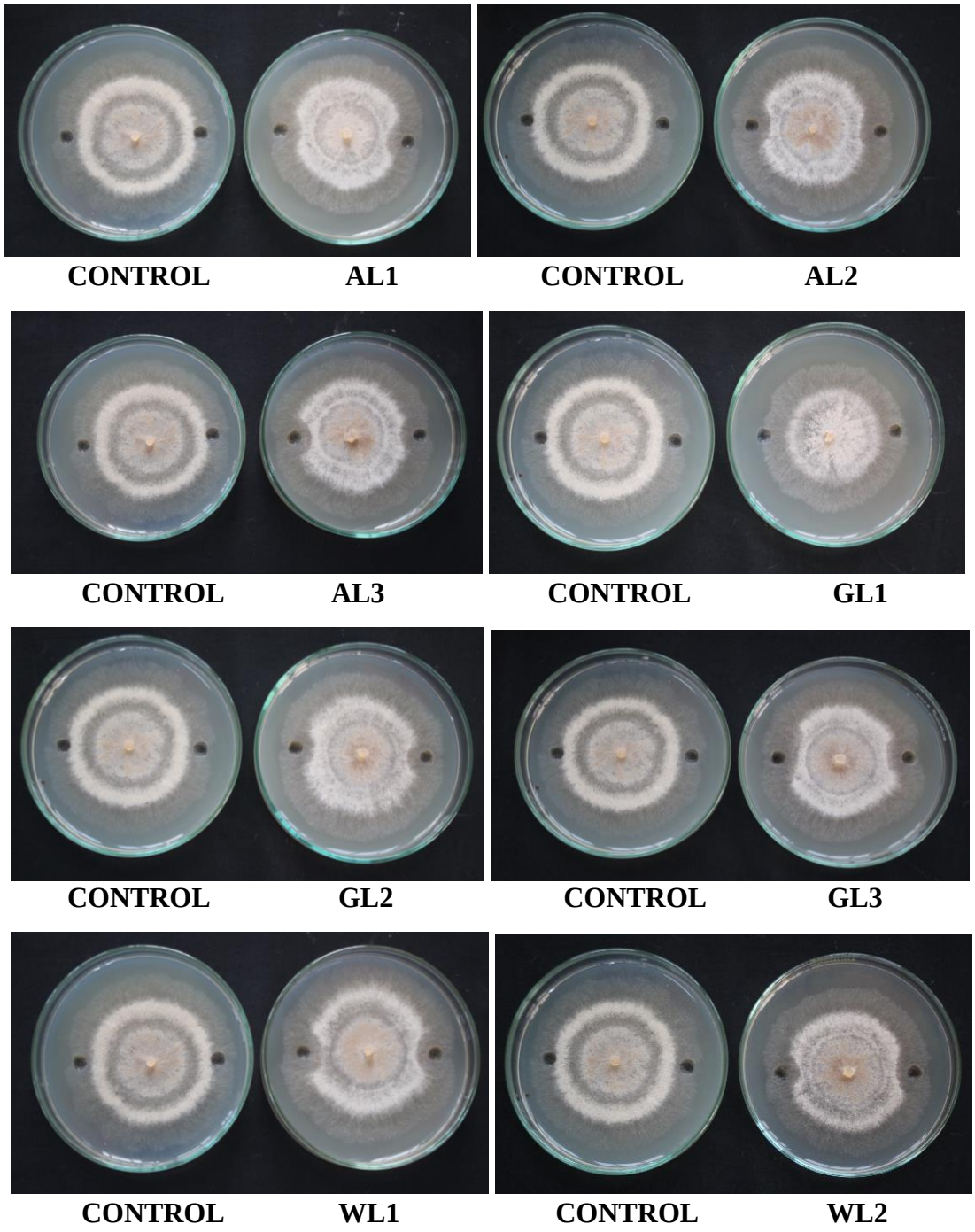


Plate 17. Indirect antagonism by culture filtrate of endospore forming phyllosphere bacterial isolates against *Rhizoctonia solani*

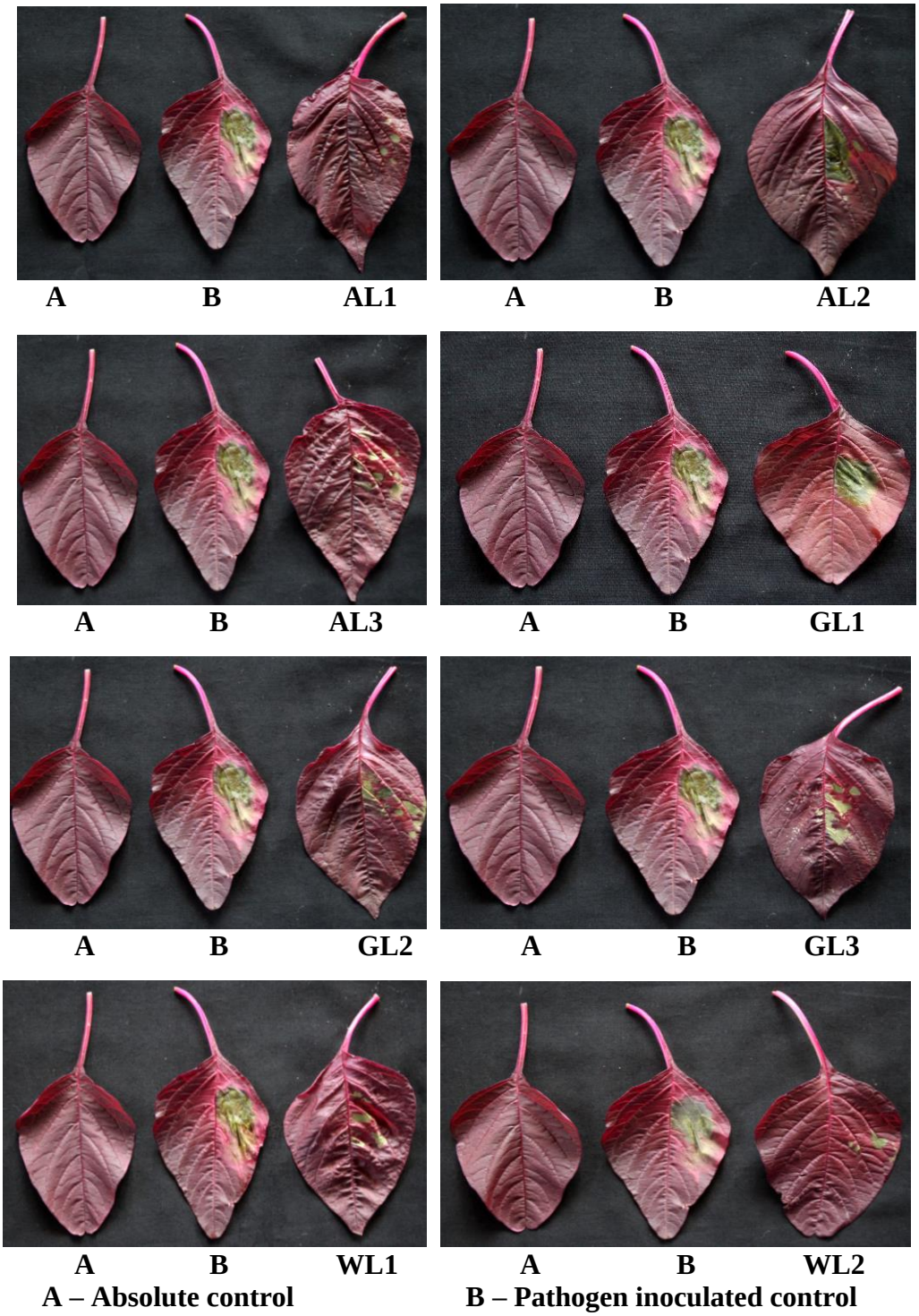


Plate 18. Screening of endospore forming phyllosphere bacterial isolates against *Rhizoctonia solani* by detached leaf assay

4.5 BIOCHEMICAL CHARACTERIZATION OF BACTERIAL ISOLATES

Various biochemical and carbohydrate utilization tests were carried out with every isolate of endospore forming endorhizosphere and endospore forming phyllosphere bacteria.

Isolate AR2 showed positive for Voges proskauer's and Mannitol utilization test, isolate AR3 found to utilize the carbohydrate Glucose and Arabinose. Positive results were obtained for Voges proskauer's biochemical test and Sorbitol, Mannitol carbohydrate utilization by isolate WR1 whereas isolate WR2 found positive for utilization of Voges proskauer's biochemical test. Negative results were obtained for all the biochemical and carbohydrate utilization test for isolates AR1, GR1, GR2 and GR3 (Plate 19). The results obtained on different biochemical test and carbohydrate utilization are represented in Table 12.

All the isolates of endospore forming phyllosphere bacteria were positive for Voges proskauer's biochemical test. Out of 8 isolates, isolates AL1, AL2, GL2, GL3 and WL2 found to be positive for Methyl Red test. Isolate GL2 and GL1 was found to be positive for citrate utilization and Glucose utilization. Utilization of carbohydrate Arabinose was found in isolate GL2 and Sorbitol by isolate GL2 and GL3. All the isolates showed negative results for Indole biochemical test, Adonitol, Lactose and Mannitol utilization (Plate 20). The various results for biochemical test and different carbohydrate utilization of endospore forming phyllosphere bacterial isolates are represented in Table 13.

4.6 MOLECULAR CHARACTERIZATION OF BACTERIAL ISOLATES

16S rRNA sequence of endospore forming endorhizosphere and phyllosphere bacterial isolates obtained are presented in table 14. The BLAST details of the most matching sequence are presented in Table 15.

Table 12. Biochemical characterization of endospore forming endorhizosphere bacterial isolates

Biochemical test	**Isolates**							
	AR1	**AR2**	**AR3**	**GR1**	**GR2**	**GR3**	**WR1**	**WR2**
Indole	-	-	-	-	-	-	-	-
Methly red	-	-	-	-	-	-	-	-
Voges proskauer's	-	+	-	-	-	-	+	+
Citrate utilization	-	-	-	-	-	-	-	-
Glucose utilization	-	-	+	-	-	-	-	-
Adonitol utilization	-	-	-	-	-	-	-	-
Arabinose utilization	-	-	+	-	-	-	-	-
Lactose utilization	-	-	-	-	-	-	-	-
Sorbitol utilization	-	-	-	-	-	-	+	-
Mannitol utilization	-	+	-	-	-	-	+	-
Rhamnose utilization	-	-	-	-	-	-	-	-
Sucrose utilization	-	-	-	-	-	-	-	-

*AR – Arun root *GR – Green root *WR – Wild root

Table 13. Biochemical characterization of endospore forming phyllosphere bacterial isolates

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

*AL – Arun leaves *GL – Green leaves *WL – Wild leaves

CONTROL AR1 AR2 AR3 GR1 GR2 GR3 WR1 WR2

Plate 19. Biochemical characterization of endospore forming endorhizosphere bacteria

CONTROL AL1 AL2 AL3 GL1 GL2 GL3 WL1 WL2

Plate 20. Biochemical characterization of endospore forming phyllosphere bacteria

Table 14. 16S rRNA sequence of the endospore forming endorhizosphere and phyllosphere bacterial isolates obtained with universal primer

Isolates	Sequence
AR1	GCACGGAAGGTGGGGGCTTGCTCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGAGGATAA CTCCGGGAAACCGGGGCTAATACCGGATGGTTGTCTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACAGA TGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCA CACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAA CGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCAAATAGCGGCAACCCTTG ATCCGGTCTAACAGAAAGCACCGCTAACTAACGTGCCAGCAGCCGCGGTAATACGTAGGTGGAGCGTTGTCCGGAATATTGGGCG TAAAGGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCCGGCTCAACCCGGGGAGGGTCATTGGGAAACTGGGGAAC TTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGAC TCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGAT GAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTTCGCAAGA CTTGAAACTTCAAAGATTGGACGGGGGCCCGCACACGGGTGAGCATGTTGGGTTTTAATTTGCCGAAAGTCAACGCGAAGAACCT TACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCA GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGG TGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAA TGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGA CTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCCGGATGGCCGGCCGATGCGGG
AR2	GGAAGAAGGGGGTGCTTGCTCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTC CGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACAGATGG ACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACAC TGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGC CGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCAAATAGGGCGGACTTGGAACG GTACTACAGAAAGGCACGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAAGGTGGCAAGCGTTGTCGGAATTATTGGGCGTAA AGGGGCTCCGCAGGGCGGGTTTCTTAAGTCCTGATGTGAAAGCCCCCGGGCTCAAACCGGGGAGGGTCATTGGAAACTGGGGAAC TTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGAC TCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGAT GAGTGCTAAGTGTTAGGGGTTCCGCCCCTTTAGTGCTGCAGCTAACGCATAAGCACTCCCGCCTGGGGGAGTACGGTTCGCAAGA ACTGGAAACTTCAAGGAATTGACTGGGGCCCGCACACAGCGTGGGAAGACATGGGTGGGTTTAAAGTTCGAAGCAACGCGAAGA ACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTC GTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCT AAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCT ACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAAC TCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGGTCAGGATGGCCGCCCGAATGCCGG

Table 14 continued

Isolates	Sequence
AR3	GACGGGTTATACCTTGACGGTTATCTTACCAGAAAGGCCACGGCTAACTTACGTGCCAGCAAGCGGCGGTATTACGTAGGTGGCA AGCGTGTTCGGAATTTATTTGGGCGTAAAGGGCTTCGCAGGGCCGGTTTTCTTTAAGTCTTGATGTGAAAGCCCCCCGGCTCAACC CGGGAGGGTCATTGGAAAGGGGCACCTCCCTTGATCGGGAGTGTAAGGGGGGAAACGTGTCGTGATGCGGAGAGGTGGAGAAC ACCAGTGGCGAGGCGACTCTCTGGCTGTAACTGACGCTGAGGAGCGAAGCGGGAGCACGGTAGATACCGGTGGTGTCCACGGTA ACGCATGAGTCAACTAGGGGTTCGCTACCACTTAGTGACCGCGCATTAAGCACTCGCTGGGAGGTAACGGCTCACAAGGCAGAC AGATGAGGGGTGCGCCACACGGGAGACAGTGATCGAAGCACGGAGACTTAAGGTCTTGACATCTCGACAATGAAGGACGGACCC GGGCAGGATGACAGGTGTCATGGTTTCGTAAGCTCGTGTGTAGAGGAAGTCCGTTCGAATGGCGACCCTTGATCTTAGTCCAGAC AGGCACGCTAAGTTGCCGGGACACGCGGTAATAGAGGTGGAGGGTCCGAATATCATGCTAGCTCGCAGGCGGTTCAGTGCTACA TGTGAAGACGGGCACGAACGGAGGTTAAGCAATCAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTTGAAGCT GGAATCGCTAGTAATCGCGGATCAAGGAAGGCCCGGCCGCAACCCTTGG
GR1	AGAGGCAAGTGGGGGAGCTGGCTTCCTGATGTTAGCGGCGGACGGGTGGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGA TAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTAC AGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCG GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGA GCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCAAATAGCGGGCAC CCTTGGATCGGTCTAACCAGAAAGCCACGGCTAACTACCGGTGCCAGCAGCCGCGGTAATACGTAGGGTGGCAAGCGTTGTCCG GAATTATTGGGCGTAAAGGGGCTCGCAGGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGGCTCAACCCGGGGAGGGTCATTG GAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAAATGCGTAGAGATGTGGAGGGAACAC CAGGTGGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGGAGCGAAAAGCGTGGGGGAGCGAACAGGATTAGGATACCC TGGTAGTCCCACGCCGTAAACGATGAGTGCTAAGTTGTTAGGGGTTTCCGCCCCTTATGCTGCAGCTTAACGCATTAAGCACTCCG GCCTGGGGAGTACGGGTCCGCAAGAACTTGAAAACCTTCAAAGGAAATTTGAAACGGGGGCCCGCACAAGCGGTGGAGCATGTG GTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCA GAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTA GTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCC CCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCT GTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGGATGGCCGCCATTGGG CG

Table 14 continued

Isolates	Sequence
GR2	GCCAGAAAGTGGGGAGCTTGCTCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAA CTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACAGA TGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCC ACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCA ACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTCATCATATTTAAGGGCGGC ACTGACGGTACCTACCAGGAAAAGGCATGGCTAACTAACGTGCCAGCCAGCCGCGGTATACGTAGGTGGCAAGCGTTGTCGGAA TTATTGGGCGTAAAGGGCTCGCAGGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCCGGCTCAACCGGGGAGGGTCATTGGAAAC TGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGG CGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGGTAGTCCAC GCCGTAAACGATGAGTGCTAAGTGTTATGGGGGTTTCCGCCCCTTAGTGCTGCAGGCTAACGCAATTAAGCACTCCGGCCTGGGG AGTACGGTCGCCAAGGACTGAAACTCAARGGAATTGGAAGGGGCCGCACAAGCGGTTGGGAGCCAATGTGGATTTTAATTCGGC AACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGT GCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAG TTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCT ACACACGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATC GCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGGATGTGCCGCCGAATGTTCCG
GR3	GGAGCAAGGGGGAGCTTGCTCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACT CCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACAGATG GACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCAC ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAAC GCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCAAATAGGGCGRCACCCTTG ATCGGTTACTACAGAAAGCAGCTAACTACGTGCCAGCAGCCGCGGTATACGTAGGGTGGCAAGCGTTGTCGGAATTATGGCGTA AAAGGGCTCGCAGGGCGGTTCTTAAGTCTGATGTGAAAGCCCCCCGGCTCAACCCGGGGAGGGTCATTGGAAACTGGGGAACTT GAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACT CTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGAT GAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGGAGTACGGTCGCAACT GAAACTCAAAGAWTTGACGGGGGCCCGCACAGCGGTGAGCATGTGGTTTATCGAAGGCAAGCGAAGAACCTTACCAGGTCTTGA CATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTG AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGT GACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAAC AAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAA GCTGGAATCGCTAGTAATCGCGGATCAGGATGGCCGCCCGAGGGCCCG

Table 14 continued

Isolates	Sequence
WR1	GCCCACCAAGTGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAA CTCCGGGAAACCGGGGCTAATACCGGATGCTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGA TGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCC ACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCA ACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGCGTTACCTTG ACCGGTCTAACAGAAAGCACGGCTAACTAACGTGGCCAAGCCAGCGCGGTAATACGTAGGTGGCAAGCCGTTGTCCGGAATTTA TTGGGGCGTAAGGGCTCGCAGGCCGGTTTCTTTAAGTCTGATGTGAAAGCCCCCCGGCTCAACCCGGGGAGGGTCATTTGGAAAC TGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAATGCGTAGAGATGTGGAGGAACACCAGTGGCG AAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAAACAGGATTAGATACCCTGGGTAGTCCACG CCGTAAACGATGAGTGCTAAGTGTTRGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACG TCGCAAGACTGAAAACTCAAAAGGAATTGACGGGGGCCCGCACAAGCGGGTGGAGCCATGTTGGGTTTTAAATGTCGAAGCAAC GCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCA TGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTG GGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTAC ACACGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGC AGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGGATGGCCGCCCCATGCCGG
WR2	GGCGGCAAAGGGGGGAGCTTGCTCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATA ACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACAG ATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGC CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGC AACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCAAATAGGGCGTRCAAC CCTGATCGGTAACCTAACCAGAAAGCACGGCTAACTACGTGCCAGCAGCCGCGGTAATTACGTAAGGTGGCAAGCGTTTGTCCG GAATTATTGGCGTAAAAGGGCTCGCAGGCGGTTTCTTAAAGTCTGATGTGAAAGCCCCCCGGCTCAAACCGGGGAGGGTCATTGG AAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGGAGGAACACC AGTGGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGGTA GTCCACGCCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCGCACGCATTAAGCACTCCGCCCTGGG GAAGTACGGTCGCACAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAA CGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGC ATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTT GGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTA CACACGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCG CAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCCGCATGGCCCGCCCGAATCCCGCGCG

Table 14 continued

Isolates	Sequence
AL1	GGCAGAAGTGGGACTTGCTCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTC CGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGG ACCCGCGGCGCATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACA CTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACG CCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTCTCGAAATAGGGCGGTACTTGA CGGTACCTAACCAGAAAGGCCACGGCCTACTACGTGCCAGCAGCCGCGGTATACGTAGGTGGAGCGTTGTCGGAATTATTGGGC GTAAAGGGGCTCGCAGGGCGGTTCTTAAGTCTGATGTGAAAGCCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAAC TTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGA CTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACG ATGAGTGCTAAGTGTTAGGGGGTTTCCCGCCCCTTTAGTGCTGCAGCTAACGCATTAAGCACTCCGGCCCTGGGGGAGTTACTGT CGGCAGACTGGAAACTCAAAGGAATTGACGGGGCCCGCACACGGTGGAGCATTGTTGGTTTTAATTCGGAAGCGAGAACCTTAC CAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGC TCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGT GACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAA TGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCG ACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAAGGATGGCCGCCGATGGCCG
AL2	CACCGGAAGGGGACTTGCTCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTC CGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGG ACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACA CTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACG CCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCCTTTG GACGGTACTACCAGAAAGGCACGCTAACTACGTGCCAGCAGGCCGCGGTAATACGTAAGGTGGCAAGCGTTGTCMGGAATTATT GGGCGTAAAGGGGCTCCGCAGGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTG GGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGA ARGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGGAGCGAACAGGATTAGATACCCTGGGTAGTCCACGC CGTAAACGATGAGTGCTAAGTGTTAAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTTAACGCATTAAGCACTCCGCCCTGGGGGAG TTACCGGTTCGCAACTGGAAACTAAGATTGACGGGCCCGCACAAGCGTGGAGCATGTTGGTCTTTTAATTCGAGCAAGAAAACCT TACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTC AGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAA GGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTA CAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAAC TGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGGATGGCGGCCCAGGACGG

Table 14 continued

Isolates	Sequence
AL3	CGGACAAGGGGGGGCTTGCTCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACT CCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATG GACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCAC ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAAC GCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATGGGCGGGTTAACCTT GAACCGGTACTACCAGAAAGCACGGCTAACTACGTGGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTAT TGGGCGTAAAGGGCTCGCAGGCCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGGCTCAACCGGGGAGGGTCATTGGGAAACTG GGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGA AGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCG TAAACGATGAGTGCTAAGTTGTTAGGGGGTTTCCGCCCCTTTAGTGCTGCAGCTAACCGCATTAAAGCACTCGCCTGGGGGAGTA CGGTCGCAGACTGAAACTCAAAGATTGACGGGGCCCGCAACAAGCCGGTGGAAGCATGTGTGGTTTATTGACGAAGACCTTACC AGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCT CGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTG ACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAAT GGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGA CTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGGATGCCGCCCCATGCCGG
GL1	GGACAAATGGGACTTGCTCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCC GGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACAGATGG ACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACA CTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACG CCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGACAAGTGCTCAAATGGGCGTGCACCTTGAACGTA CCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTATACGTAGGTGGCCAAGCGTTGTCCGGAATTATTGGGCGT AAAGGGGCTCGCAGGGCGGTTTCTAGTCTGATGTGAAAGCCCCCGGCTCAACCCGGGGAGGGTCATTGGAAACTGGGGAACTTG AGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTC TCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGAT GAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATAGCACTTCCGCCTGGGGAGTACGGTTCGCCAAGA ACTTGAAACTAAGGAATTTGGACGGGGGCCCGCACAAGCGGTGGAAGCAATTGTGTTATTCGAACAACGGAGAGACCTTACCAG GTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCG TGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGAC TGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGG ACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACT GCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGGATGCCGCCCGATTGCGGC

Table 14 continued

Isolates	Sequence
GL2	GGAACGAGGTGGTGCTTGCTCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACT CCGGGAAACCGGGGCTAATACCGGATGCTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATG GACCCGCGGCGCATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCAC ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAAC GCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTTACCTTG GACGGTACTACCAGAAAGCCACGGCTAACTACCGTGCGCAGCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGAATTATTGGGC GTAAACGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCCGGGCTCAAACCGGGGAGGGTCATTGGGAAACTGGGG AACTTGAGTGCAGAAGAGGAGAGTGGAATTCCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAG GCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGGTAGTCCACGCCGT AAACGATGAGTGCTAAGTGTTAGGGGGTTTCCCGCCCCTTAGTGCTGCAGCTAACGGCATTAAGCACTCCGCCTGGGGAGTACGG TCCGCAAGACTGAACTCAAAGGAATGACGGGGCCCGCACAAGCGGTGGAGCATGGTTTATATTCGAAGCACAAGAACCTTACCA GGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTC GTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGA CTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATG GACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGAC TGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGGATGCCGGCGATGGGCG
GL3	AGCGCCATGGGGAGCTTGCTCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACT CCGGGAAACCGGGGCTAATACCGGATGCTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATG GACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCAC ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAAC GCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGCGGTTAACCCTT GAACCGGTACTACGAAAGCACGGCTACTACCGTGCCAGCAGCCCGCGGTTATACCGTAGGGTGGCAAGCGTTGTCGGAATTATTG GGCGTAAAAGGGCTCGCAGGGCGGTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAAACCGGGGAGGGTCATTGGAAACTGGGG AACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGC GACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAA GATGAGTGCTAAGTGTTAGGGGGTTTTCCGGCCCCTTTAGTGCTGCAGCTAACCGCATTAAGCACTCCGGCCCTGGGGAGTACGG GTCGGCAACTGAACTCAAAGGATGACTGGGCCCGCCACACGGTGGAGCCATGGTGGTTTTTAATTTTCCTGAAGGCCACGCGAAG AACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTG TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACT CTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGT GCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTG CAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATTCCGGCGAATGGCGG

Table 14 continued

Isolates	Sequence
WL1	GCACCGGGTGGGTGCTTGCTCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTC CGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGG ACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACA CTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACG CCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATGRGGGCCGGTTACCTG AGTACTAACCAGAAAGGCCACGGCTAACTACGTGCCAGCAGCCGCGGTATACGTAGGTGGCAAGCGTTGTCCGGAATTATGGGC GTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCCGGGGAGGGTCATTGGGAAACTGGGGAAC TTGAGTGCAGAAGAGGAGAGTGGAATTTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCG ACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC GATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCCATTAAGCACTCCGCCTGGGGGAGTTACGGTCG CAGACTGAACTCAAAGGATTGACGGGGGCCCACACGTGGAGCATGGTAGGTTTAATTTCGCAAAGCAACGCGAAGAACCTTACC AGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCT CGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTG ACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAAT GGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGA CTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAAGGATGCCGCCCGATGGGCGG
WL2	GGACAATGTAGCTTGCTCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGG GAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACC CGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTG GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCG CGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTCATAGGCGGACCTTGACGTACCTAA CCAGAAAGTCAGCTAACTACGTGCCAGCCAGCCGCGGTTATACGTAGGTGGAGCGTTGTCGGAATTATTGGGCGTAAAGGGGCT CGCAGGGCGGTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGA AGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTG TAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAG TGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCCTGGGGGGAGTACGGTCGCAAGACTGAAACT CAAAGGAATTGACGGGGCCGCACAAGCGTGGAGCCATGGTGGTTTAATTAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCC TCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATG TTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAA ACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGG GCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGA ATCGCTAGTAATCGCGGATCAAGGATGGCCGGCCGATTGGGCG

Table 15. BLAST search details of the sequences producing most significant alignment of the plant associated bacterial isolates

Isolate	Max score	Total score	Query cover (%)	Best match in Genbank data base	Accession no.
AR1	2218	2218	98%	*Bacillus* sp strain 26LG	MT762874.1
AR2	2209	2209	98%	*Bacillus siamensis* strain SZ004	MK788212.1
AR3	1013	1013	91%	*Bacillus subtilIs* strain S-11	KF830999.1
GR1	2206	2206	98%	*Bacillus amyloliquifaciens* strain QT-36	MT065711.1
GR2	2189	2189	97%	*Bacillus amyloliquifaciens* strain I2ScCTM4	MH985211.1
GR3	2270	2270	98%	*Bacillus amyloliquifaciens* strain GC25	KF158226.1
WR1	2237	2237	98%	*Bacillus* sp strain 4N	MF765317.1
WR2	2268	2268	97%	*Bacillus amyloliquefaciens* strain Rab5	MK782759.1
AL1	2239	2239	98%	*Bacillus* sp T4a	JX155393.1
AL2	2206	2206	98%	*Bacillus subtilIs* strain MES146	MN960406.1
AL3	2239	2239	99%	*Bacillus* sp RC29	KJ669214.1
GL1	2248	2248	99%	*Bacillus amyloliquefaciens* strain NCCP-49	AB547229.1
GL2	2268	2268	98%	*Bacillus* sp strain 4N	MF765317.1
GL3	2161	2161	98%	*Bacillus* sp RC29	KJ669214.1
WL1	2268	2268	98%	*Bacillus subtilIs* strain LX1	HE985180.1
WL2	2261	2261	98%	*Bacillus amyloliquefaciens* strain NCCP-49	AB547229.1

The bacterial isolates AR1, AR2, AR3, GR1, GR2, GR3, WR1, WR2, AL1, AL2, AL3, GL1, GL2, GL3, WL1 and WL2 were identified as *Bacillus* sp., *Bacillus siamensis, Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus amyloliquefaciens, Bacillus amyloliquefaciens, Bacillus* sp., *Bacillus amyloliquefaciens, Bacillus* sp., *Bacillus subtilis, Bacillus* sp., *Bacillus amyloliquefaciens, Bacillus* sp., *Bacillus* sp., *Bacillus subtilis* and *Bacillus amyloliquefaciens* respectively.

4.7 *IN VIVO* STUDIES

4.7.1 Study on Plant Growth Promotion

Endospore forming endorhizosphere bacterial isolates selected based on the *in vitro* plant growth promotion studies were tested for their efficacy of plant growth promotion activities in red amaranthus variety Arun (Plate 21)**.** The results obtained from the study are presented below.

4.7.1.1 Treatment Effect on Number of Leaves

The observations on the number of leaves were recorded on 5 DAT, 15 DAT and 25 DAT. The treatments showed significant influence on the number of leaves (Table 16). On 5th day of transplantation, the plants treated with *Bacillus amyloliquefaciens* GR2 (5.68) showed maximum number of leaves. The minimum number of leaves was observed in plants treated with *Bacillus amyloliquefaciens* WR2 (4.82). On 15 DAT, the maximum number of leaves per plant was observed in *Bacillus siamensis* AR2 (25.50). Treatment *Bacillus amyloliquefaciens* WR2 showed least influence on number of leaves per plant (14.43) and which was followed by control plants (16.09 leaves per plant), found to be on par with each other.

Observations at 25 DAT showed significant influence of the treatments on the number of leaves. Plants treated with *Bacillus* sp AR1 showed the maximum number of leaves (47.73), followed by plants treated with *Bacillus siamensis* AR2, *Bacillus* sp WR1

and *Bacillus amyloliquefaciens* GR2 with 44.66, 44.17 and 39.89 respectively. The least number of leaves was found in plants treated with *Bacillus amyloliquefaciens* WR2 (27.78).

4.7.1.2 Treatment Effect on Number of Branches

Treatments showed significant influence on the number of branches (Table 17). Observations on 15 DAT showed that the maximum number of branches was recorded in plants treated with *Bacillus amyloliquefaciens* GR1 (2.77), which was followed by the plants treated with *Bacillus siamensis* AR2, *Bacillus* sp AR1, *Bacillus amyloliquefaciens* GR2 and *Bacillus* sp WR1 with 2.66, 2.62, 2.35 and 2.22 respectively. The least number of branches was seen in control plants (1.07).

At 25 DAT, treatment *Bacillus* sp WR1 (6.30) showed the highest number of branches, followed by plants treated with *Bacillus amyloliquefaciens* GR1 (6.10), found to be on par with each other. The least number of branches per plant of 3.75 was recorded in control plants.

4.7.1.3 Treatment Effect on Plant Height

Data given in Table 18 shows the influence of different treatments on the plant height. Observations on plant height were recorded on 15 DAT and 25 DAT. At 15 DAT, the maximum plant height was observed in plants treated with *Bacillus siamensis* AR2 (33.42 cm), which was followed by plants treated with *Bacillus amyloliquefaciens* GR1 (32.03), *Bacillus* sp AR1 (31.66) and *Bacillus amyloliquefaciens* GR2 (31.28). The least plant height was exhibited by plants in the control (27.78 cm) and those treated with *Bacillus amyloliquefaciens* WR2 (28.28 cm) (Plate 22).

The observations on 25 DAT showed that treatment with *Bacillus siamensis* AR2 showed the maximum influence on plant height (60.11 cm) and which was significantly superior to all other treatments. The least was observed in control plants (48.77 cm).

Table 16. Number of leaves produced by amaranthus plants on treatment with selected endospore forming endorhizosphere bacteria

Treatments		Number of leaves/plant*		
		5 DAT	**15 DAT**	**25 DAT**
T1	*Bacillus* sp AR1	5.03 ± 0.34^{bcd}	20.60 ± 2.29^{b}	47.73 ± 8.08^{a}
T2	*Bacillus siamensis* AR2	5.41 ± 0.43^{ab}	25.50 ± 3.70^{a}	44.66 ± 1.82^{ab}
T3	*Bacillus amyloliquefaciens* GR1	4.97 ± 0.28^{bcd}	21.82 ± 3.72^{ab}	37.37 ± 2.09^{abc}
T4	*Bacillus amyloliquefaciens* GR2	5.68 ± 0.14^{a}	20.42 ± 1.88^{b}	39.89 ± 9.98^{ab}
T5	*Bacillus* sp WR1	5.34 ± 0.30^{abc}	18.21 ± 2.54^{bc}	44.17 ± 6.86^{ab}
T6	*Bacillus amyloliquefaciens* WR2	4.82 ± 0.39^{d}	14.43 ± 0.85^{c}	27.78 ± 3.75^{c}
T7	Control	4.93 ± 0.21^{cd}	16.09 ± 1.80^{c}	35.26 ± 5.09^{bc}

*DAT – Days after transplantation. Mean ($\pm$ SD) of 4 replications. Values in a column followed by same letter do not differ significantly according to Duncan's Multiple Range Test ($p \leq 0.05$)

Table 17. Number of branches produced by amaranthus plants on treatment with selected endospore forming endorhizosphere bacteria

Treatments		Number of branches/plant*	
		15 DAT	**25 DAT**
T1	*Bacillus* sp AR1	2.62 ± 0.37^{ab}	5.48 ± 0.53^{ab}
T2	*Bacillus siamensis* AR2	2.66 ± 0.68^{ab}	5.22 ± 0.56^{abc}
T3	*Bacillus amyloliquefaciens* GR1	2.77 ± 0.92^{a}	6.10 ± 1.87^{a}
T4	*Bacillus amyloliquefaciens* GR2	2.35 ± 0.38^{ab}	5.35 ± 1.33^{ab}
T5	*Bacillus* sp WR1	2.22 ± 0.29^{ab}	6.30 ± 0.65^{a}
T6	*Bacillus amyloliquefaciens* WR2	1.75 ± 0.71^{bc}	4.37 ± 0.20^{bc}
T7	Control	1.07 ± 0.08^{c}	3.75 ± 0.45^{c}

*DAT – Days after transplantation. Mean ($\pm$ SD) of 4 replications. Values in a column followed by same letter do not differ significantly according to Duncan's Multiple Range Test ($p \leq 0.05$)

Table 18. Plant height of amaranthus plants treated with selected endospore forming endorhizosphere bacteria

Treatments		Plant height (cm)*	
		15 DAT	**25 DAT**
T1	*Bacillus* sp AR1	31.66 ± 2.37^{ab}	52.23 ± 5.35^{bc}
T2	*Bacillus siamensis* AR2	33.42 ± 1.15^{a}	60.11 ± 7.01^{a}
T3	*Bacillus amyloliquefaciens* GR1	32.03 ± 2.01^{ab}	53.62 ± 4.24^{abc}
T4	*Bacillus amyloliquefaciens* GR2	31.28 ± 0.82^{ab}	50.91 ± 1.75^{bc}
T5	*Bacillus* sp WR1	29.26 ± 1.06^{bc}	56.43 ± 2.06^{ab}
T6	*Bacillus amyloliquefaciens* WR2	28.28 ± 1.87^{c}	49.86 ± 3.91^{bc}
T7	Control	27.78 ± 0.90^{c}	48.77 ± 3.59^{c}

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

Plate 21. General view of pot culture experiment

Control T1 T2 T3 T4 T5 T6

Control T1

Control T2

Control T3

Control T4

Control T5

Control T6

Plate 22. Effect of endospore forming endorhizosphere bacteria on growth of amaranthus

4.7.1.4 Treatment Effect on Root Fresh Weight

Analysis of data implied that there was a significant effect on root fresh weight of red amaranthus plants treated with endospore forming endorhizosphere bacteria (Table 19). The maximum mean root fresh weight was observed in plants treated with *Bacillus amyloliquefaciens* GR1 (13.64 g/plant). The lowest root fresh weight was observed in plants treated with *Bacillus siamensis* AR2 (9.33 g/plant), which was found to be on par with plants treated *Bacillus* sp AR1, control and *Bacillus amyloliquefaciens* WR2, recorded a root fresh weight of 9.42, 9.88 and 10.71 g/plant respectively (Plate 23)**.**

4.7.1.5 Treatment Effect on Root Dry Weight

Treatments had a significant effect on root dry weight of red amaranthus plants compared to control plants (Table 19). The plants treated with *Bacillus amyloliquefaciens* GR1 with the root dry weight of 1.98 g/plant was found to be significantly superior compared to other treatments. This was followed by the plants treated with *Bacillus amyloliquefaciens* GR2 (1.78 g/plant), *Bacillus* sp AR1 (1.74 g/plant), *Bacillus* sp WR1 (1.73 g/plant) and *Bacillus siamensis* AR2 (1.68 g/plant). The lowest root dry weight was observed in control plants of 1.37 g/plant which was found to be on par with the plants treated with *Bacillus amyloliquefaciens* WR2 (1.40 g/plant).

4.7.1.6 Treatment Effect on Shoot Fresh Weight

The effect of different isolates of endospore forming endorhzosphere bacteria on shoot fresh weight of red amaranthus plants was analyzed in Table 20. The maximum shoot fresh weight was observed in plants treated with *Bacillus amyloliquefaciens* GR1 with 54.30 g/plant, which significantly differed from other treatments. This was followed by the shoot fresh weight of the plants treated with *Bacillus siamensis* AR2 (50.44 g/plant). The minimum root fresh weight was observed in control plants of 40.24 (g/plant).

4.7.1.7 Treatment Effect on Shoot Dry Weight

Significant effect was observed in shoot dry weight of red amaranthus plants treated with endospore forming endorhizosphere bacteria (Table 20). *Bacillus siamensis* AR2 treated plants showed significantly highest shoot dry weight of 6.74 g/plant compared to other treatments. This was followed by the shoot dry weight of plants treated with *Bacillus amyloliquefaciens* GR1 and *Bacillus* sp WR1 which recorded 6.54 and 6.15 g/plant respectively. The lowest shoot dry weight was observed in plants treated with *Bacillus amyloliquefaciens* WR2 (4.32 g/plant).

4.7.1.8 Percent Disease Index

Treatments showed significant results on percent disease index calculated based on scoring the lesion size on inoculated leaves at three days after inoculation and five days after inoculation. At 3 DAI, no symptoms were developed on the absolute control plants. The plants treated with *Bacillus amyloliquefaciens* GR1 showed the least PDI 12.96 which accounts to 54.35% disease suppression over control plants (Table 21). The maximum PDI was observed in control plants (28.39).

Percent disease index on 5^{th} day after pathogen inoculation showed significant difference between the treatments and the control plants. 100% disease suppression was observed in absolute control plants. *Bacillus* sp WR1 treated plants showed the minimum PDI of 26.54 with 41.10% disease suppression over the pathogen control, which was followed by *Bacillus amyloliquefaciens* GR1 with PDI of 29.62 and percentage disease suppression of 34.25 over the pathogen control. The maximum percent disease index was observed with the pathogen control plants (45.06).

Bacillus amyloliquefaciens GR1 treated plants showed more biocontrol activity against *R. solani* on 3^{rd} DAI, whereas on 5^{th} DAI *Bacillus* sp WR1 treated plants showed the maximum biocontrol activity against *R. solani.*

Table 19. Effect of different treatments on root fresh weight and root dry weight of amaranthus plants treated with different endospore forming endorhizosphere bacteria

Treatments		**Root fresh weight (g)/plant***	**Root dry weight (g)/plant***
T1	*Bacillus* sp AR1	9.42 ± 1.95^{b}	1.74 ± 0.64^{ab}
T2	*Bacillus siamensis* AR2	9.33 ± 0.54^{b}	1.68 ± 0.13^{ab}
T3	*Bacillus amyloliquefaciens* GR1	13.64 ± 2.00^{a}	1.98 ± 0.10^{a}
T4	*Bacillus amyloliquefaciens* GR2	11.45 ± 0.83^{ab}	1.78 ± 0.10^{ab}
T5	*Bacillus* sp WR1	11.41 ± 1.93^{ab}	1.73 ± 0.26^{ab}
T6	*Bacillus amyloliquefaciens* WR2	10.71 ± 1.76^{b}	1.40 ± 0.21^{b}
T7	Control	9.88 ± 1.38^{b}	1.37 ± 0.37^{b}

* Mean ($\pm$ SD) of 4 replications taken 30 days after transplanting. Values in a column followed by same letter do not differ significantly according to Duncan's Multiple Range Test ($p \leq 0.05$)

Table 20. Effect of different treatments on shoot fresh weight and shoot dry weight of amaranthus plants treated with different endospore forming endorhizosphere bacteria

Treatments		**Shoot fresh weight (g)/plant***	**Shoot dry weight (g)/plant***
T1	*Bacillus* sp AR1	47.34 ± 3.61^{abc}	5.62 ± 1.42^{abc}
T2	*Bacillus siamensis* AR2	50.44 ± 11.26^{ab}	6.74 ± 1.66^{a}
T3	*Bacillus amyloliquefaciens* GR1	54.30 ± 7.20^{a}	6.54 ± 1.09^{ab}
T4	*Bacillus amyloliquefaciens* GR2	44.61 ± 3.99^{bc}	5.56 ± 1.13^{abc}
T5	*Bacillus* sp WR1	48.50 ± 3.45^{abc}	6.15 ± 0.43^{ab}
T6	*Bacillus amyloliquefaciens* WR2	41.34 ± 2.74^{bc}	4.32 ± 0.70^{c}
T7	Control	40.24 ± 4.04^{c}	4.76 ± 0.90^{bc}

* Mean ($\pm$ SD) of 4 replications taken 30 days after transplanting. Values in a column followed by same letter do not differ significantly according to Duncan's Multiple Range Test ($p \leq 0.05$)

Table 21. Effect of endospore forming endorhizosphere bacteria on the percent disease index at 3 DAI

Treatments		Percent Disease Index*	Disease suppression over the pathogen control (%)
T1	*Bacillus* sp AR1	20.99 ± 2.01^{cde}	26.07
T2	*Bacillus siamensis* AR2	24.07 ± 1.24^{def}	15.23
T3	*Bacillus amyloliquefaciens* GR1	12.96 ± 8.87^{b}	54.35
T4	*Bacillus amyloliquefaciens* GR2	25.30 ± 1.24^{def}	10.88
T5	*Bacillus* sp WR1	16.67 ± 4.22^{bc}	41.29
T6	*Bacillus amyloliquefaciens* WR2	27.16 ± 5.14^{ef}	4.34
T7	Pathogen Control	28.39 ± 4.03^{f}	-
T8	Chemical control (0.3% Mancozeb)	20.37 ± 3.70^{cd}	28.35
T9	Absolute control	0.00 ± 0.00^{a}	100

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

Table 22. Effect of endospore forming endorhizosphere bacteria on the percent disease index at 5 DAI

Treatments		Percent Disease Index*	Disease suppression over the pathogen control (%)
T1	*Bacillus* sp AR1	33.33 ± 5.34^{bcd}	26.03
T2	*Bacillus siamensis* AR2	37.65 ± 6.80^{cde}	16.44
T3	*Bacillus amyloliquefaciens* GR1	29.62 ± 5.88^{bc}	34.25
T4	*Bacillus amyloliquefaciens* GR2	40.12 ± 8.64^{de}	10.96
T5	*Bacillus* sp WR1	26.54 ± 5.09^{b}	41.10
T6	*Bacillus amyloliquefaciens* WR2	43.82 ± 4.21^{e}	2.75
T7	Pathogen Control	45.06 ± 5.47^{e}	-
T8	Chemical control (0.3% Mancozeb)	37.03 ± 5.88^{cde}	17.81
T9	Absolute control	0.00 ± 0.00^{a}	100

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

CONTROL T1 **CONTROL T2** **CONTROL T3**

CONTROL T4 **CONTROL T5** **CONTROL T6**

Plate 23. Rooting pattern of plants treated with endospore forming endorhizosphere bacterial isolates against control

4.7.2 Testing for Disease Suppression by Phyllosphere Isolates

4.7.2.1 Percent Disease Index

The endospore forming phyllosphere bacterial isolates were tested for their disease suppressive activity against *Rhizoctonia solani*. The percent disease index was calculated three and five days after pathogen inoculation. The treatments had significant effect on the PDI calculated by scoring the lesion size (Plate 24) on inoculated leaves at 3 DAI and 5 DAI.

On 3 DAI, absolute control showed no symptoms of disease (Table 23). The plants sprayed with *Bacillus* sp GL3 showed 32.92 % disease suppression over the pathogen with PDI of 11.80 which was followed by *Bacillus* sp AL3 with 32.86 % disease suppression and 11.81 PDI. These two were on par with each other. The maximum PDI was observed in pathogen inoculated control of 17.59.

Data in the Table 24 show that the percent disease index calculated based on scoring of lesion size of inoculated leaves differed significantly from each other. Absolute control plants showed no symptoms of disease. The maximum percentage disease suppression over pathogen inoculated control was observed in plants sprayed with *Bacillus* sp AL3 (44.51%) with the PDI of 18.75 (Plate 25)**.** The maximum percent disease index was observed in pathogen inoculated control of 33.79. Even though the *Bacillus* sp GL3 and *Bacillus* sp AL3 showed maximum biocontrol activity on 3 DAI, only *Bacillus* sp AL3 showed significant disease suppressing activity on 5 DAI. Development of symptom in different treatments are shown in plate 26.

4.7.2.2 Treatment Effect on Root Fresh Weight

Endospore forming phyllosphere bacterial isolates were tested for their influence on root fresh weight (Table. 25). Significant effect of different treatments over the fresh weight of roots was observed. Maximum fresh weight of 10.60 g/plant was recorded in

plants treated with *Bacillus* sp GL3 whereas the least was recorded in *Bacillus subtilis* WL1 of 7.30 g/plant.

4.7.2.3 Treatment Effect on Root Dry Weight

Significant effect on root dry weight of plants was observed with different treatments (Table 25). The plants treated with *Bacillus* sp GL3 showed maximum root dry weight of 2.07 g/plant, which was significantly superior compared to other treatments. The minimum root dry weight was observed with chemical control plants 1.50 g/plant, which was found to be on par with the root dry weight of absolute control, 1.54 g/plant and pathogen control, 1.56 g/plant.

4.7.2.4 Treatment Effect on Shoot Fresh Weight

There was significant influence of endospore forming phyllosphere bacteria over the shoot fresh weight of the treated plants (Table 26). *Bacillus* sp GL3 treated plants recorded highest shoot fresh weight of 60.93 g/plants whereas the least was observed in absolute control (40.13 g/plant).

4.7.2.5 Treatment Effect on Shoot Dry Weight

Influence of endospore forming phyllosphere bacteria on shoot dry weight was analyzed and represented in Table 26. Treatments showed significant effect on the shoot dry weight of the plants. The maximum shoot dry weight was recorded in plants inoculated with *Bacillus* sp GL3 (6.91 g/plant). The least shoot dry weight of 4.55 g/plant was observed in absolute control and which was found to be on par with pathogen inoculated control (4.75 g/plant).

Table 23. Effect of endospore forming phyllosphere bacterial treatments on the percent disease index at 3 DAI

Treatments		Percent Disease index*	Disease suppression over the pathogen control (%)
T1	*Bacillus* sp AL1	14.81 ± 2.93^{bc}	15.80
T2	*Bacillus* sp AL3	11.81 ± 3.41^{b}	32.86
T3	*Bacillus* sp GL2	13.66 ± 3.58^{bc}	22.34
T4	*Bacillus* sp GL3	11.80 ± 2.31^{b}	32.92
T5	*Bacillus subtilis* WL1	15.51 ± 3.57^{bc}	11.82
T6	*Bacillus amyloliquefaciens* WL2	12.26 ± 2.05^{bc}	30.30
T7	Pathogen control	17.59 ± 4.60^{c}	0
T8	Chemical control (0.3% Mancozeb)	14.25 ± 5.41^{bc}	18.99
T9	Absolute control	0.00 ± 0.00^{a}	100

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

Table 24. Effect of endospore forming phyllosphere bacterial treatments on the percent disease index at 5 DAI

Treatments		Percent Disease Index*	Disease suppression over the pathogen control (%)
T1	*Bacillus* sp AL1	28.70 ± 11.64^{bc}	15.06
T2	*Bacillus* sp AL3	18.75 ± 2.76^{b}	44.51
T3	*Bacillus* sp GL2	20.83 ± 7.80^{bc}	38.35
T4	*Bacillus* sp GL3	25.51 ± 10.48^{bc}	24.50
T5	*Bacillus subtilis* WL1	25.92 ± 1.07^{bc}	23.29
T6	*Bacillus amyloliquefaciens* WL2	20.60 ± 5.47^{bc}	39.03
T7	Pathogen control	33.79 ± 6.30^{c}	0
T8	Chemical control (0.3% Mancozeb)	26.85 ± 14.13^{bc}	20.54
T9	Absolute control	0.00 ± 0.00^{a}	100

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

Table 25. Effect of different treatments on root fresh weight and root dry weight of amaranthus plants treated with different endospore forming phyllosphere bacteria

Treatments		**Root fresh weight (g)/plant ***	**Root dry weight (g)/plant***
T1	*Bacillus* sp AL1	7.73 ± 0.30^{bc}	1.69 ± 0.22^{ab}
T2	*Bacillus* sp AL3	9.89 ± 2.21^{ab}	1.82 ± 0.43^{ab}
T3	*Bacillus* sp GL2	7.85 ± 1.39^{bc}	1.89 ± 0.16^{ab}
T4	*Bacillus* sp GL3	10.60 ± 2.97^{a}	2.07 ± 0.33^{a}
T5	*Bacillus subtilis* WL1	7.30 ± 1.02^{c}	1.70 ± 0.25^{ab}
T6	*Bacillus amyloliquefaciens* WL2	10.00 ± 1.38^{ab}	1.79 ± 0.27^{ab}
T7	Pathogen control	8.31 ± 1.18^{abc}	1.56 ± 0.26^{b}
T8	Chemical control (0.3% Mancozeb)	7.67 ± 0.87^{bc}	1.50 ± 0.14^{b}
T9	Absolute control	9.19 ± 0.58^{abc}	1.54 ± 0.18^{b}

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

Table 26. Effect of different treatments on shoot fresh weight and shoot dry weight of amaranthus plants treated with different endospore forming phyllosphere bacteria

Treatments		**Shoot fresh weight (g)/plant ***	**Shoot dry weight (g)/plant***
T1	*Bacillus* sp AL1	45.96 ± 3.77^{bc}	5.87 ± 0.50^{bc}
T2	*Bacillus* sp AL3	48.68 ± 8.74^{bc}	5.34 ± 0.74^{cd}
T3	*Bacillus* sp GL2	45.62 ± 3.03^{bc}	6.59 ± 1.14^{ab}
T4	*Bacillus* sp GL3	60.93 ± 5.05^{a}	6.91 ± 0.59^{a}
T5	*Bacillus subtilis* WL1	48.76 ± 11.02^{bc}	6.47 ± 0.61^{ab}
T6	*Bacillus amyloliquefaciens* WL2	53.07 ± 2.44^{ab}	6.04 ± 0.56^{abc}
T7	Pathogen control	48.07 ± 8.18^{bc}	4.75 ± 0.66^{d}
T8	Chemical control (0.3% Mancozeb)	50.53 ± 8.76^{abc}	5.45 ± 0.54^{cd}
T9	Absolute control	40.13 ± 3.69^{c}	4.55 ± 0.79^{d}

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

Plate 24. Symptom development in leaf inoculated with *Rhizoctonia solani*

T2 – AL3 T7 – Pathogen control T9 – Absolute control T8 – Chemical control

Plate 25. Incidence of foliar blight of amaranthus plants with different treatments

T1

T3

T4

T5

T6

T9- Absolute control

T7-Pathogen control

T8- Chemical control

Plate 26. Development of foliar blight symptoms in amaranthus plants with different treatments

Discussion

5. DISCUSSION

Amaranthus, the most commonly cultivated and consumed leafy vegetable crop of Kerala, can be considered as one of the cheapest sources of nutrients, vitamins and minerals. Leaves are the main economic part of amaranthus plant. Rhizoctonia leaf blight is a major threat to amaranthus crop production in Kerala, especially during the monsoon periods (Kamala *et al*., 1996). Even though both red and green amaranthus are used for culinary purpose, the people of Kerala prefer red amaranthus to the green one. But red amaranthus is more susceptible to Rhizoctonia leaf blight (Venuganan, 2003), which affects the marketability and thus reduces the economic value of the crop.

Foliar application of mancozeb (0.4 %) in cow dung supernatant at fortnightly intervals reduces the severity of disease (Nayar *et al*., 1996). Application of chemicals is an easy method and a quick solution for the control of the disease. As amaranthus is a leafy vegetable, use of fungicides is not advisable, as it poses serious health problems and is not an ecofriendly approach. Hence there is a need for effective biological control against the diseases.

Integrated disease management is an important strategy which includes cultural, biological and chemical methods for management of plant diseases. For the increase of crop production there is extensive use of agrochemicals, which pose health problems, deteriorates soil fertility, and creates imbalance in ecology. Thus, there is a need for biological substitute to chemicals. Thereby crop production can be increased with enhanced soil fertility and beneficial soil microflora. Now-a-days organic cultivation is gaining more importance in crop production, which mainly limits the use of chemicals. Microbial application in the form of biofertilizers and biocontrol agents is of predominant importance in organic crop production.

Application of plant growth promoting bacteria (PGPB) is promoted as a green technology which enhances crop productivity and nutrient content. They also represent potential alternatives for fungal disease management (Compant *et al*., 2010). Many of the

PGPB which have the ability to colonize and live inside plant tissues without causing any apparent symptoms of disease are referred as plant growth promoting bacterial endophytes (PGPE) (Reinhold-Hurek and Hurek, 2011). Among PGPE, *Bacillus* species are common endophytes. *Bacillus* species are considered to be safe microorganisms with the potential to produce a wide range of beneficial substances for agronomical purposes and are also reported as PGPE with a high potential as fungal antagonists (Hollensteiner *et al*., 2017). Various ecological and physiological traits which influence plant health and disease control of endospore forming bacteria make them potential candidates for biocontrol applications.

Several advantages conferred by *Bacillus* sp. such as production of endospores warrants their prevalence under adverse environmental conditions, its long-term storage, easy development of reliable formulations (Collins and Jacobsen, 2003), lower contamination rate, assure an easy usage and cheap treatment (Fravel, 2005). Considering their importance, an attempt for the isolation of endospore forming bacteria from endorhizosphere and phyllosphere of *Amaranthus* spp. was undertaken in the current study.

To start with, isolation of endospore forming bacteria from endorhizosphere and phylosphere from *Amaranthus* spp. by enrichment method on NA medium was carried out. The isolation was done from red amaranthus variety Arun, green amaranthus variety CO-1 and wild relative (*Amaranthus viridis*). A total of sixteen endospore forming bacterial isolates were obtained, of which eight were endospore forming endorhizosphere bacteria and other eight were endospore forming phyllosphere bacteria. Similar potential endophytic bacteria were isolated from root region of *Amaranthus* sp. (Uppala, 2007; Aparna, 2012). No similar reports pertaining particularly for the isolation of endospore forming bacteria from *Amaranthus* spp. is available which contributes for the novelty of the study.

Endophytes are microorganisms associated with plants and are found within the plant tissues with immense hidden potential. Isolation of endospore forming bacteria is a

relatively a direct and easy procedure which is achieved by heat treatment at 80^0C for 10 minutes, which eliminates the vegetative cells (Priest and Grigorova, 1990). There are reports regarding the isolation of endophytic bacteria from root region from the *Amaranthus* spp. using this strategy (Uppala, 2007; Aparna, 2012).

A new method was followed in the current investigation for the isolation of endospore forming endorhizosphere bacteria by a "double" enrichment strategy. Initially, surface sterilization of root samples was carried out in order to avoid the epiphytic microorganisms (Hallmann *et al*., 1997). Surface sterilized samples were allowed to dry at 35^0C consecutively for 2 days. Drying or reducing the water content of the samples facilitates the induction of endospore formation. The dried sample later was macerated in water under aseptic conditions and boiled at 80^0C for 10 minutes and when plated the total number of endospore formers obtained were high compared to other isolation procedures, which is the novelty of the method.

Phyllosphere refers to aerial or above ground parts of plants and this environmental niche supports the survival of large and complex microbial habitats (Inacio *et al*., 2002; Lindow and Brandl, 2003). Several reports on potential role of phylloplane bacteria in disease suppression and plant growth promotion are available (Lindow and Brandl, 2003; Madhaiyan *et al*., 2006a; Nysanth *et al*., 2019). Epiphytic bacteria present can be potentially used as bioinoculants for sustainable cultivation and biological control (Senthilkumar and Krishnamoorthy, 2017). Hence, an attempt for the isolation and characterization of endospore forming phyllosphere bacteria was done. Endospore forming phyllosphere bacteria were isolated by enrichment method on nutrient agar medium. The healthy leaf samples were kept for drying at 35^0C consecutively for 2 days, for induction of endospores. The leaf wash obtained was boiled at 80^0C for obtaining endospore formers. The procedure followed here shows the uniqueness of the study.

A total of eight endospore forming endorhizosphere bacteria were obtained, of which three were from red amaranthus variety Arun, three from green amaranthus variety

CO-1 and two from wild relative (*Amaranthus viridis*). Presence of diverse group of endophytic bacteria in amaranthus and their beneficial traits were reported (Uppala, 2007; Aparna, 2012). From the phyllosphere region, a total of eight isolates were obtained. From red amaranthus variety Arun and green amaranthus variety CO-1 three each and from the wild relative (*Amaranthus viridis*) two bacterial isolates were obtained. Several reports are in agreement with the presence of varied group of endospore froming phyllosphere bacteria (Swinburne, 1973; Swinburne *et al*., 1975; Kong *et al*., 1997; Pane and Zaccardelli, 2015). Cultural and morphological characters of all the bacterial isolates were studied. All the isolates were single rod shaped cells. Every isolate showed the colony colour of creamy white except isolate GL2, which showed brownish creamy white and all were with irregular form. They showed variation in elevation and margin of colonies on nutrient agar medium. As the isolates retained the primary stain, crystal violet, in the Gram staining they were considered positive for Grams reaction. All of them were found to be endospore formers. Hence based on the cultural, morphological characters, isolates were tentatively identified as *Bacillus* spp. (Harrigan and McCane, 1966; Bradshaw, 1973; Holt *et al*., 1994).

Virulent isolate of *Rhizoctonia solani* used in the study was isolated from naturally infected leaves of red amaranthus variety Arun by placing diseased portion of leaf bits on PDA plates. Pure culture of *R. solani* devoid of any contamination was obtained. Similarly, many others have isolated *R. solani* from infected leaves (Uppala, 2007; Gireesh, 2016).

Endophytic microorganisms play a key role in the promotion of plant growth (Bashan *et al*., 1989; Frommel *et al*., 1991; Azevedo and Araujo, 2007). Plant growth promotion is attributed to the production of phytoharmones like IAA, gibberellins and cytokinins. IAA produced by endophytic bacteria plays an important role in plant growth promotion. In the present study an attempt was made to study the ability of endospore forming endorhizosphere bacteria isolates to produce IAA. The isolates produced IAA ranging from 4.09 µg mL-1 to 9.73 µg mL-1 of culture filtrate (Figure 1) Maximum IAA

production was reported by the isolate AR3 and the minimum was reported by the isolate AR2. Similar reports of IAA production by endophytic bacteria were reported (Jasim *et al*., 2013; Kumar *et al*., 2020). Dhouib *et al*. (2019) reported the production of IAA by endophytic bacteria *Bacillus velezensis* isolated from the crown tissue of tomato.

Seed germination and seedling vigour index are two key components which need to be assessed to gain an insight into the performance of bioinoculation. Seeds start to lose their vigour before they lose their ability to germinate therefore, improvement of seed vigor is an important practice in sustainable crop production. Seedling vigor has always been an important parameter in crop establishment, especially in the early part of crop growth. Phaneendranath (1980) standardized roll towel assay as the best method for standard germination test results as it helps to maintain optimum moisture level for the germination of seeds. Hence, a roll towel assay was carried out to test the effect of endospore forming endorhizosphere bacterial isolates on germination percentage and seedling vigour in red amaranthus variety Arun. Seedling vigour index was calculated using the formula given by Abdul-Baki and Anderson (1973). Bacterization of seeds positively influenced the germination percentage and seedling vigour index which could bring substantial difference in the biometric parameters of plants compared to control plants. These results were strongly supported by previous studies (Sundaramoorthy and Balabaskar, 2012; Vyshakhi, 2016). In the present study, isolate WR1 showed maximum germination percentage whereas the minimum was reported by AR1 and GR3 isolates (Figure 2). Seedling vigour index between the isolates showed significant difference. Highest seedling vigour index was showed by isolate WR1 and the least was observed in plants treated with isolate GR1 (Figure 3, 4). *Bacillus* spp. can be applied as booster inoculants to enhance plant growth promotion as well as up regulation of systemic disease protection in the field (Kloepper *et al*., 2004). Three *Bacillus* spp. increased the weight of soybean plants when co-inoculated with one of the isolates and *Bradyrhizobium japonicum* under conditions devoid of nitrogen, compared with *B. japonicum* alone inoculated plants (Bai *et al*., 2002). In a study conducted by Thomas *et al*. (2010), out of 30 *Bacillus* isolates screened, 11 isolates were found to increase

seedling length and fresh and dry weight of cowpea seedlings than uninoculated control. Application of endospore forming *Bacillus* species to seeds or roots has been shown to cause alteration in the composition of rhizosphere leading to increase in growth and yield of different crops. The results of current study showed similar trend with other recent studies also (Uppala *et al*., 2010; Jasim *et al*., 2013).

Many of the previous laboratory experiments showed that introduction of plant growth promoting bacteria into the soil contributed to elevated content of auxins in the roots, increase in their mass and accumulation of phosphorus in the plants (Kudoyarova *et al*., 2017). However, direct introduction of bacteria into soil involves additional procedures like soil spraying, whereas after the presowing seed treatment, bacteria are introduced into the soil together with the planted seeds which positively influence the seed germination and seedling establishment (Arkhipova *et al*., 2019). Germination of the seeds, which is an important trait in crop production, has been found to be influenced by the bacterization with the bacterial isolates. Percentage of germination and early seedling establishment is a prerequisite for ensuing better plant growth and productivity (McDonald and Copeland, 1996). Bacterization of seeds with plant growth promoting endorhizosphere bacteria would help in increasing seed germination and enhanced seedling vigour index. Hence in the present study, to prove the potential of the seed bacterization with the isolates further, plant growth promotion in red amaranthus variety Arun was studied using a portray experiment. This experimentation would provide more of a natural simulation than the roll towel method. Significant difference was observed with biometric parameters like root length, shoot length and seedling vigour index in the portray experiment too. Seedling vigour index was calculated by using the root length, shoot length and germination percentage and the highest with isolate GR1 and the lowest with isolate to WR2. Besides, influence on fresh weight and dry weight of shoot and root was studied. Plants treated with isolate GR1 showed highest root fresh weight followed by AR2 which were found on par with each other. Control plants (treated with water) showed the lowest root fresh weight. In shoot fresh weight, isolate AR2 showed the maximum fresh weight, whereas control plants showed the minimum. Highest root dry

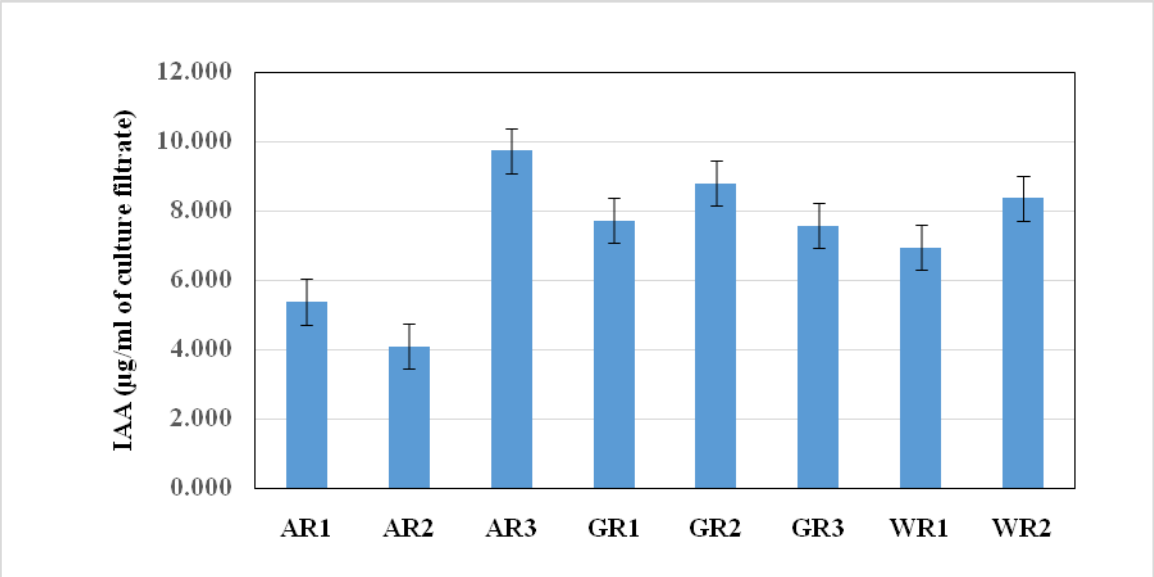


Figure 1. Indole Acetic Acid production by endospore forming endorhizosphere bacterial isolates

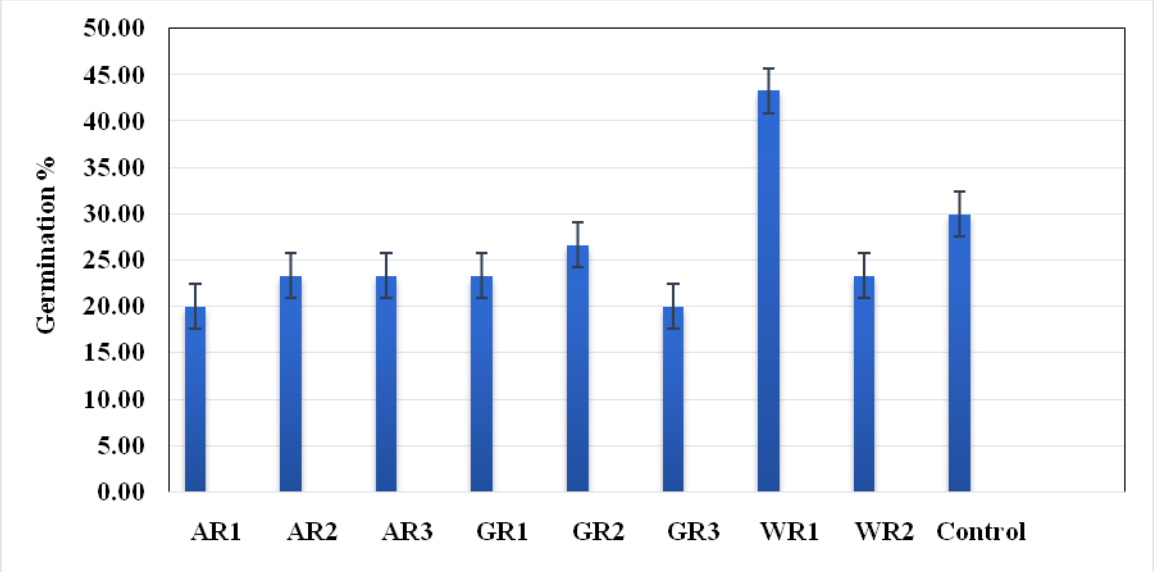


Figure 2. Effect of endospore forming endorhizosphere bacterial isolates on germination of amaranthus seeds (variety Arun)

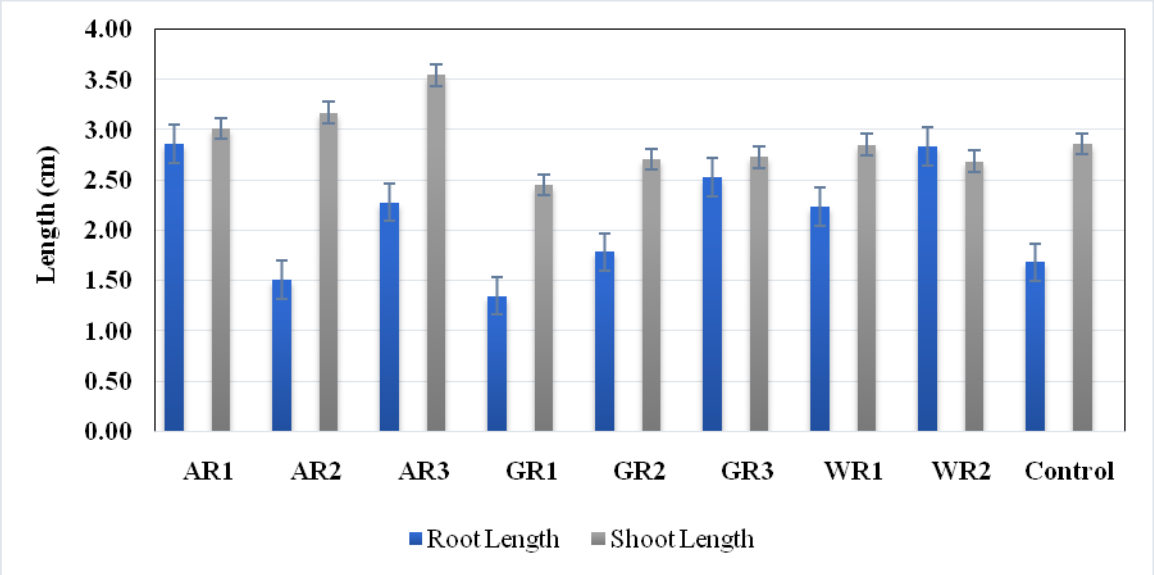


Figure 3. Effect of endospore forming endorhizosphere bacterial isolates on root and shoot length of amaranthus seedlings (variety Arun) in roll towel assay

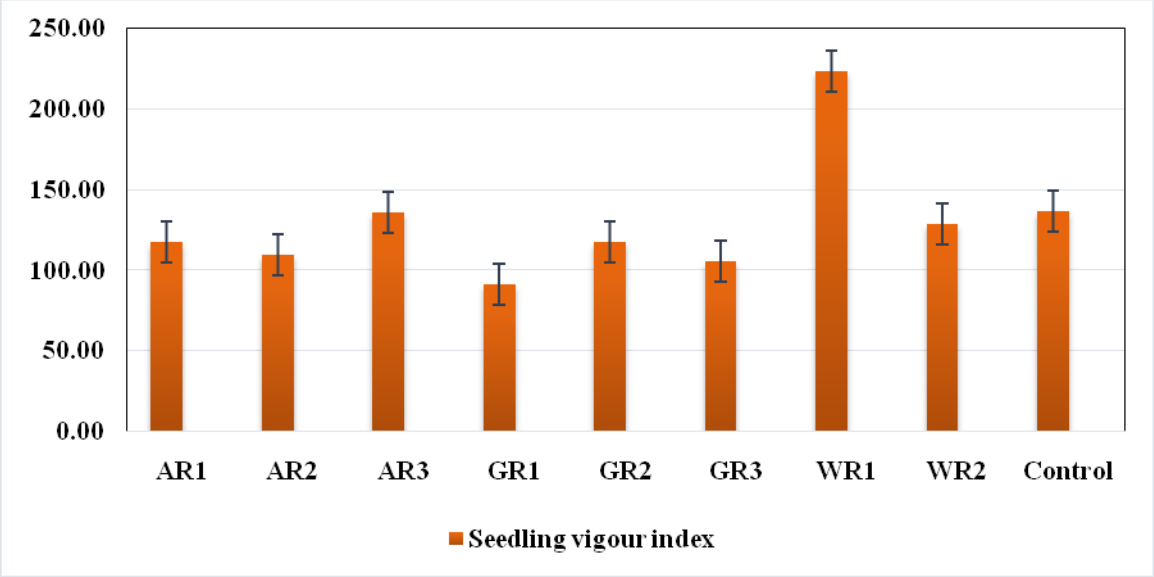


Figure 4. Effect of endospore forming endorhizosphere bacterial isolates on seedling vigour index of amaranthus seedlings (variety Arun) in roll towel assay

weight was observed with isolate WR1 treated plants and the least in plants treated with isolate GR3. Bacterial isolates had no significant influence in shoot dry weight. The application of *B. amyloliquefaciens* has been reported to increase root and shoot lengths, grain yield of rice by more than 50% (Preeti *et al*., 2002). Besides, Ng *et al*. (2012) reported that bacterization of rice seeds with *B. amyloliquefaciens, E. gergoviae* and *C. agropyri* increased shoot and root lengths, and total dry biomass of seedlings. Similar results in plant growth promotion were obtained in other studies also (Uppala *et al*., 2010; Thomas *et al*., 2010; Jasim *et al*., 2013). Here the data showing greater positive effects of bacterization on growth of amarathus treated with endsopore forming endorhizosphere bacterial isolates are in agreement with earlier reports.

Several bacterial strains isolated from phylloplane are reported for their biocontrol activity on phytopathogens (Akter *et al*., 2014; Haidar *et al*., 2016). However, there are no reports regarding the biocontrol activity of endospore forming phyllosphere bacterial isolates on Rhizoctonia leaf blight in amaranthus.

The inhibitory effect of the endospore forming phyllosphere bacterial isolates against the pathogen *Rhizoctonia solani* was carried out on PDA medium using a conventional method of assessing antagonism, dual plating technique (Dennis and Webster, 1971) which is relatively a simple and rapid method suitable for screening. The inhibition of mycelial growth of pathogen may be due to the production or 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). Production of antibiotics, cell wall-degrading enzymes and volatile compounds with antifungal activity by strains of *Bacillus* spp. is excellent biological control mechanisms against a many phytopathogens (Kim and Chung, 2004; Leelasuphakul *et al*., 2006). The bacterial isolates when tested showed antagonistic interaction against *R. solani* (Figure 5), which was measured as zone of inhibition in mm. The results obtained are in line with the earlier findings. Evident inhibition zone in dual culture plates of *Bacillus* strains against Alternaria early blight of tomato suggested an antibiosis like mechanism by Pane and Zaccardelli (2015). The strong antifungal activity

of the endospore forming phyllosphere bacterial isolates against the pathogen *Rhizoctonia solani* under *in vitro* conditions implied that their bioactive components might provide an alternative resource for the biocontrol of Rhizoctonia leaf blight in amaranthus.

To know if secreted metabolites are involved in the antagonism rather than the direct inhibition the culture filtrate of the isolates was also tested for their inhibitory potential. Culture filtrate obtained from the isolate GL3 isolated from green amaranthus showed maximum zone of inhibition against *R. solani*, which was followed by the inhibition zone produced by isolates WL1. The minimum zone of inhibition was exhibited by the culture filtrate obtained from the isolate GL1 (Figure 6). Production of antifungal volatiles by *Bacillus subtilis* resulted in hyphal deformation of *R. solani* (Fiddaman and Rossall, 1993). 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). The results showed that culture filtrate has strong *in vitro* inhibition activity against *R. solani*. Inhibition zone produced in the dual culture plate and culture filtrate assay was considered as an initial screening for the potential the isolates.

In order to confirm the antagonistic potential of the selected endospore forming phyllosphere bacterial isolates against Rhizoctonia leaf blight on host plant amaranthus, an assay need to be carried out that involves the interaction of pathogen, antagonist, and the host plant, which would provide an insight into the field level performance. Therefore, a detached leaf assay was carried out in red amaranthus variety Arun to evaluate the antaogonistic activity of endospore forming phyllosphere bacterial isolates against *R. solani*. The inoculated detached leaves showed lesions of various sizes. The data when analyzed showed significant difference between the treated and control leaves. The assay provides information on interaction between the pathogen, antagonist and the host plant, which is expected to resemble the field conditions and thus provides a rigid evidence for antagonism (Anith *et al*., 2003). On the 3rd day of pathogen inoculation, the maximum lesion area was exhibited by control leaves. The least lesion area was measured in the leaves treated with WL2 isolate, exhibiting the maximum biocontrol

activity. Significant difference in the lesion area was observed between the detached leaves treated with bacterial isolates and with water on 4th day after pathogen inoculation. Maximum lesion area was observed with control leaves (Figure 7). Endospore forming phyllosphere bacteria isolated from wild amaranthus treated leaves showed reduced lesion size. The isolates were successful in suppressing the pathogen *Rhizoctonia solani* due to direct antagonism. Some isolates failed in suppressing the pathogen establishment. Similarly, in a detached leaf assay, *Bacillus cereus* BT8 induced resistance against P. capsici in cacao (Melnick *et al*., 2008), *Bacillus subtilis* MBI 600, *B. subtilis* subsp *subtilis* AP 209 and AP 52, and *B. amyloliquefaciens* AP 219 significantly reduced the sheath blight lesions on detached rice leaves (Kumar *et al*., 2011).

In the present study all the endospore forming endorhizosphere and phyllosphere bacterial isolates were subjected to morphological and biochemical characterization. As per Bergey's Manual of Determinative Bacteriology all the isolates were tentatively identified as *Bacillus* sp. (Holt *et al*., 1994). Further, 16S rRNA gene phylogenetic analysis performed clearly showed the position of the isolates within the genus *Bacillus*. Molecular identification of the isolates was done at Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram. The isolates AR1, AR2, AR3, GR1, GR2, GR3, WR1, WR2, AL1, AL2, AL3, GL1, GL2, GL3, WL1 and WL2 were identified as *Bacillus* sp., *Bacillus siamensis, Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus amyloliquefaciens, Bacillus amyloliquefaciens, Bacillus* sp., *Bacillus* amyloliquefaciens, *Bacillus* sp., *Bacillus subtilis, Bacillus* sp., *Bacillus amyloliquefaciens, Bacillus* sp., *Bacillus* sp., *Bacillus subtilis* and *Bacillus amyloliquefaciens* respectively based on 16S rRNA sequence homology.

Neighbor-joining phylogenetic tree based on the sequence of the 16S rRNA gene of endospore forming endorhizosphere bacterial isolates obtained from *Amaranthus* spp. and related strains is depicted in Figure 8 to 12. Phylogenetic tree based on alignment of nucleotide sequences of the 16S rRNA genes of endospore forming phyllosphere bacterial isolates from *Amaranthus* spp. and related strain is shown in Figure 13 to 17.

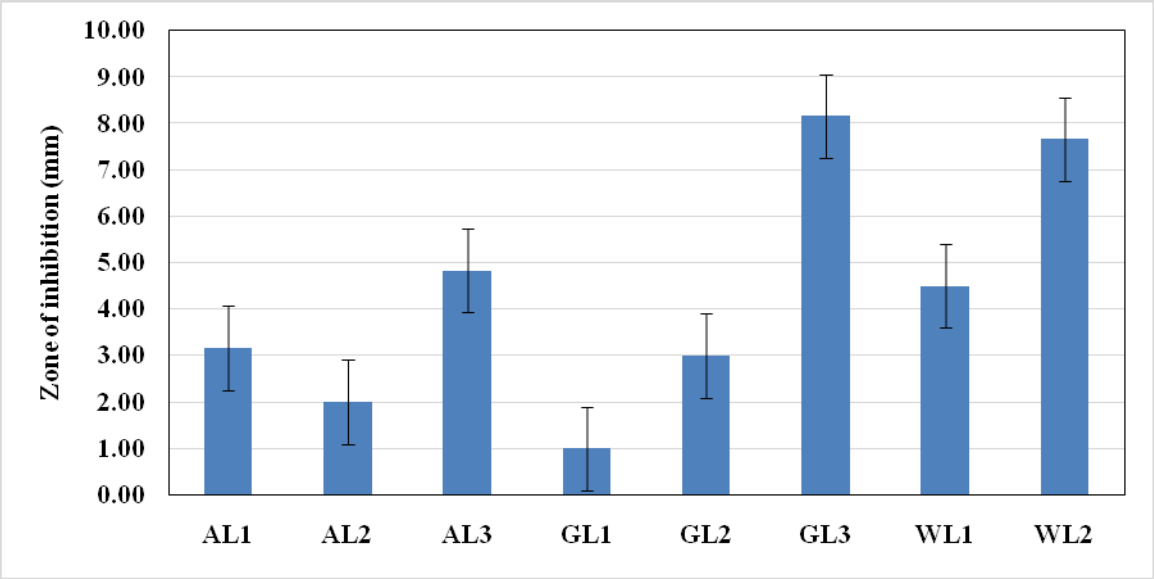


Figure 5. Antagonistic activity of endospore forming phyllosphere bacterial isolates in dual culture plate assay

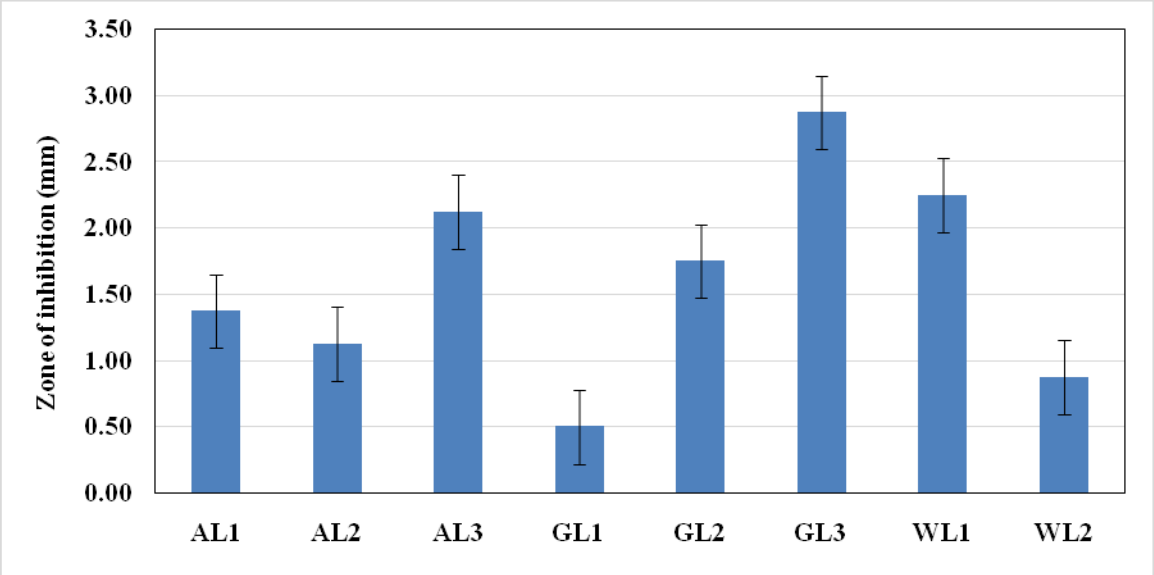


Figure 6. Effect of endospore forming phyllosphere bacterial culture filtrate in mycelial growth inhibition of *Rhizoctonia solani*

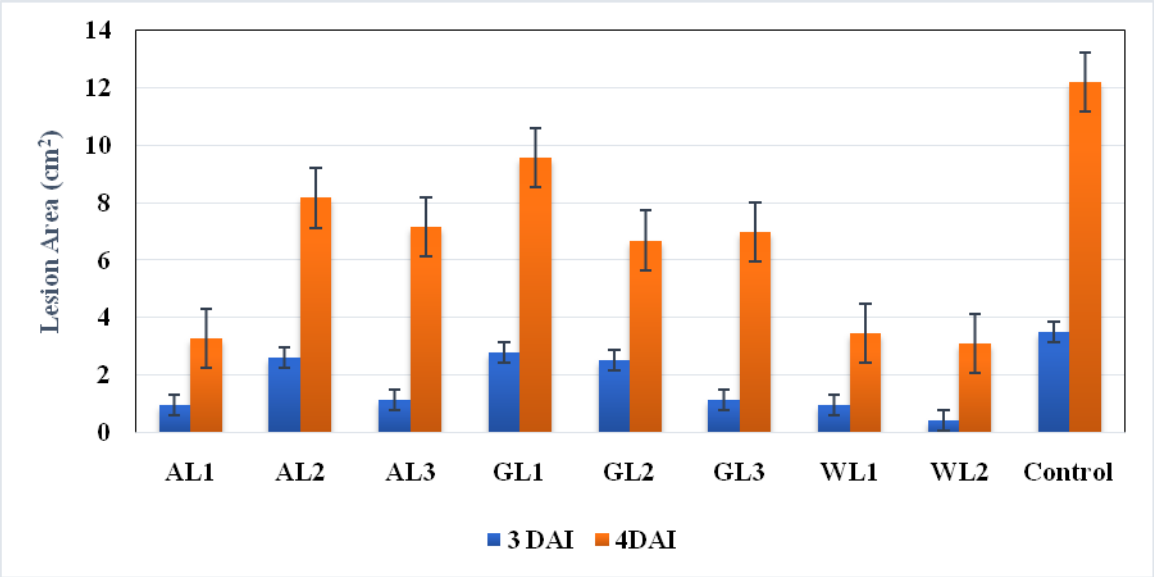


Figure 7. Disease suppression on leaves treated with endospore forming phyllosphere bacterial isolates in detached leaf assay over control

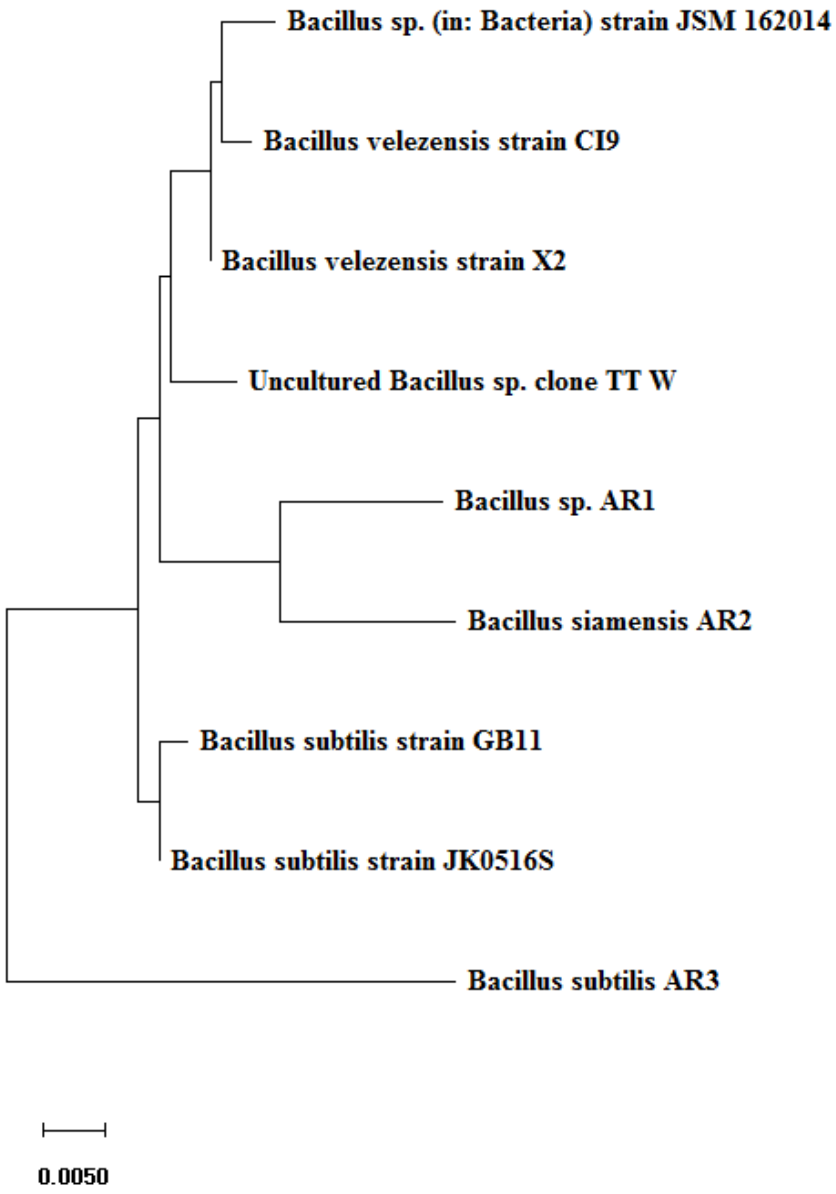


Figure 8. Phylogenetic tree of endospore forming endorhizosphere bacterial isolates from red amaranthus varity arun

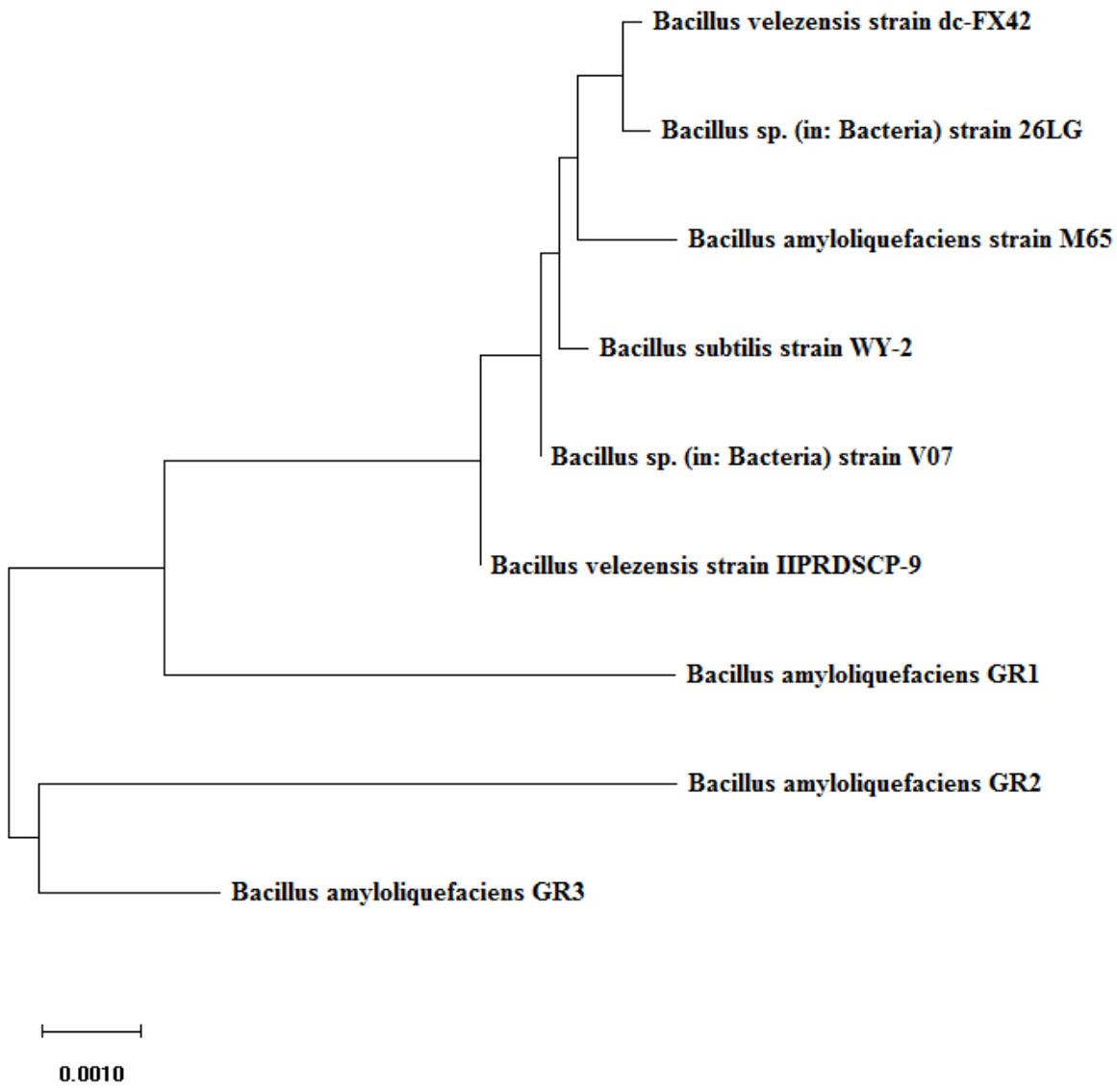


Figure 9. Phylogenetic tree of endospore forming endorhizosphere bacterial isolates from green amaranthus variety CO-1

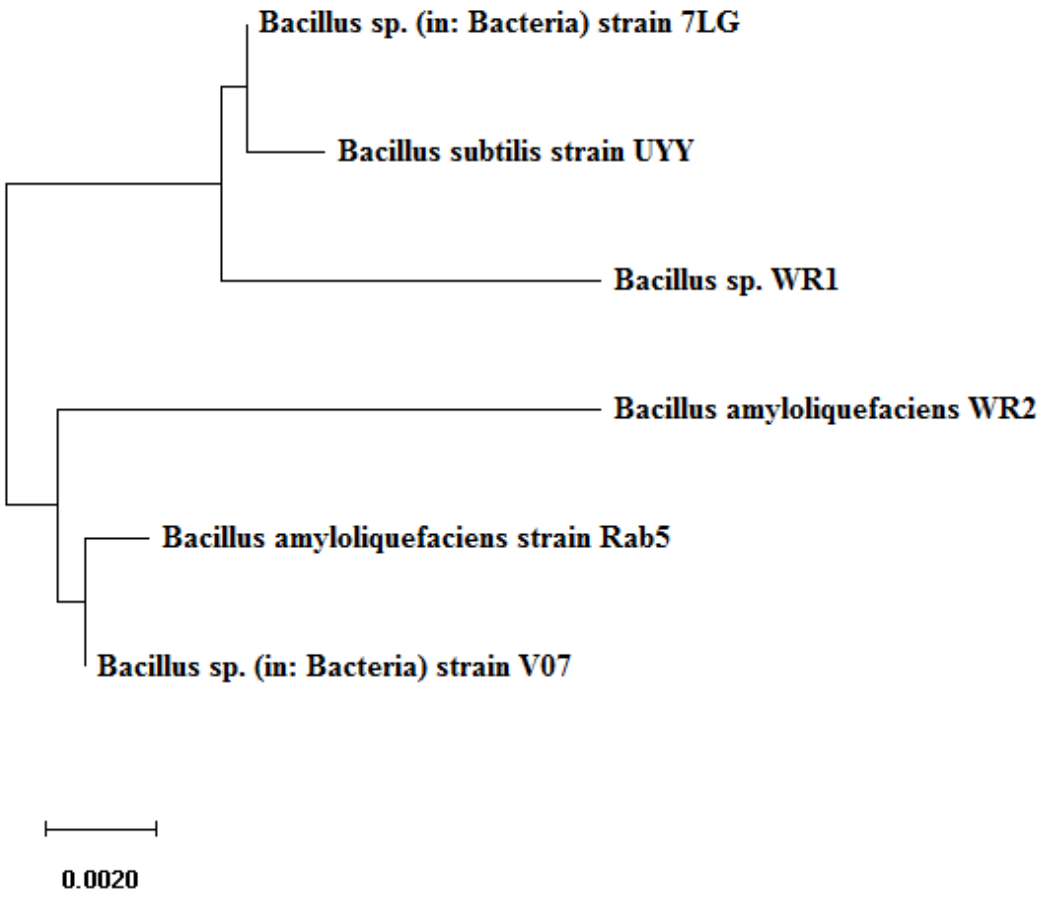


Figure 10. Phylogenetic tree of endospore forming endorhizosphere bacterial isolates from wild relative *Amaranthus viridis*

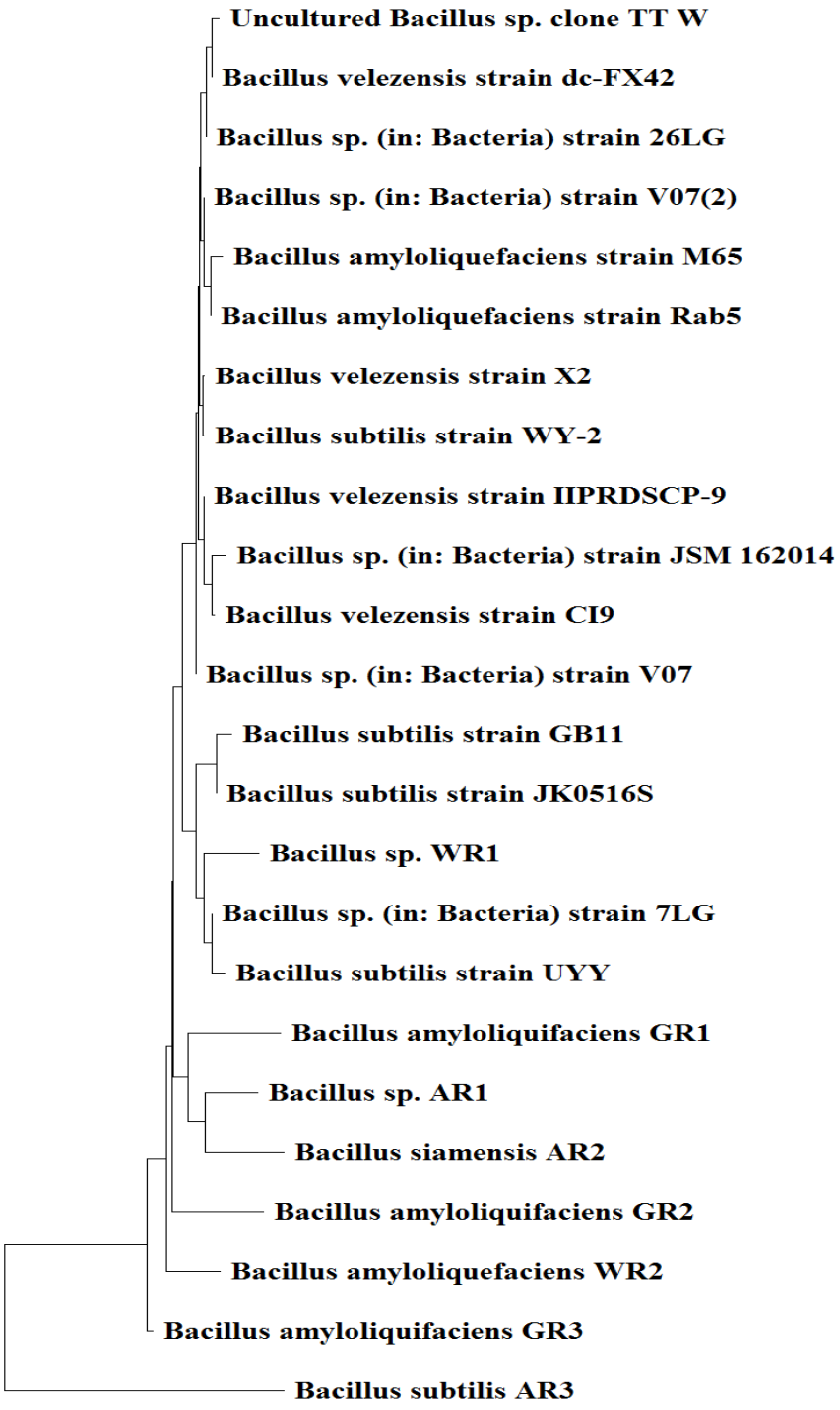


Figure 11. Phylogenetic tree of endospore forming endorhizosphere bacterial isolates from *Amaranthus* spp

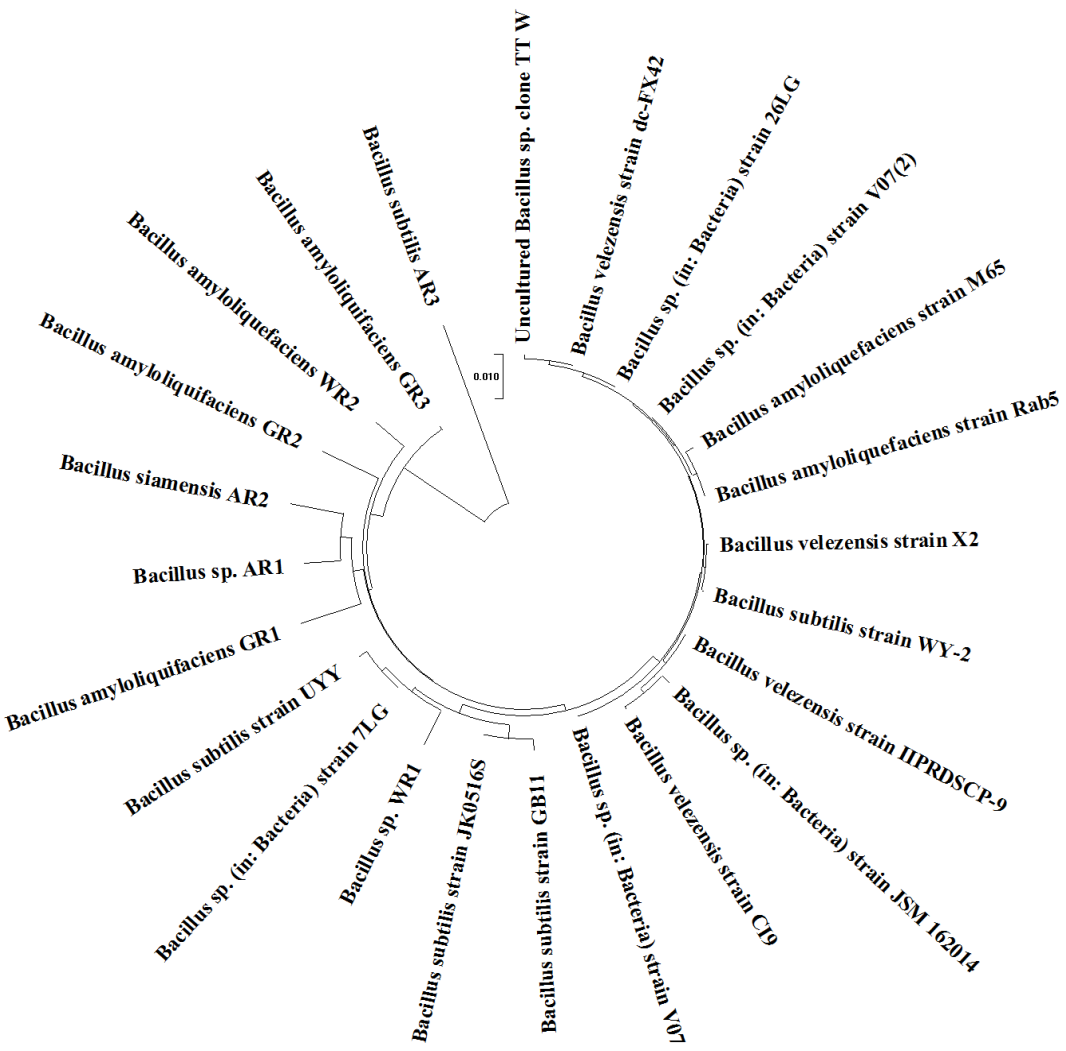


Figure 12. Circular phylogenetic tree of endospore forming endorhizosphere bacterial isolates from *Amaranthus* spp

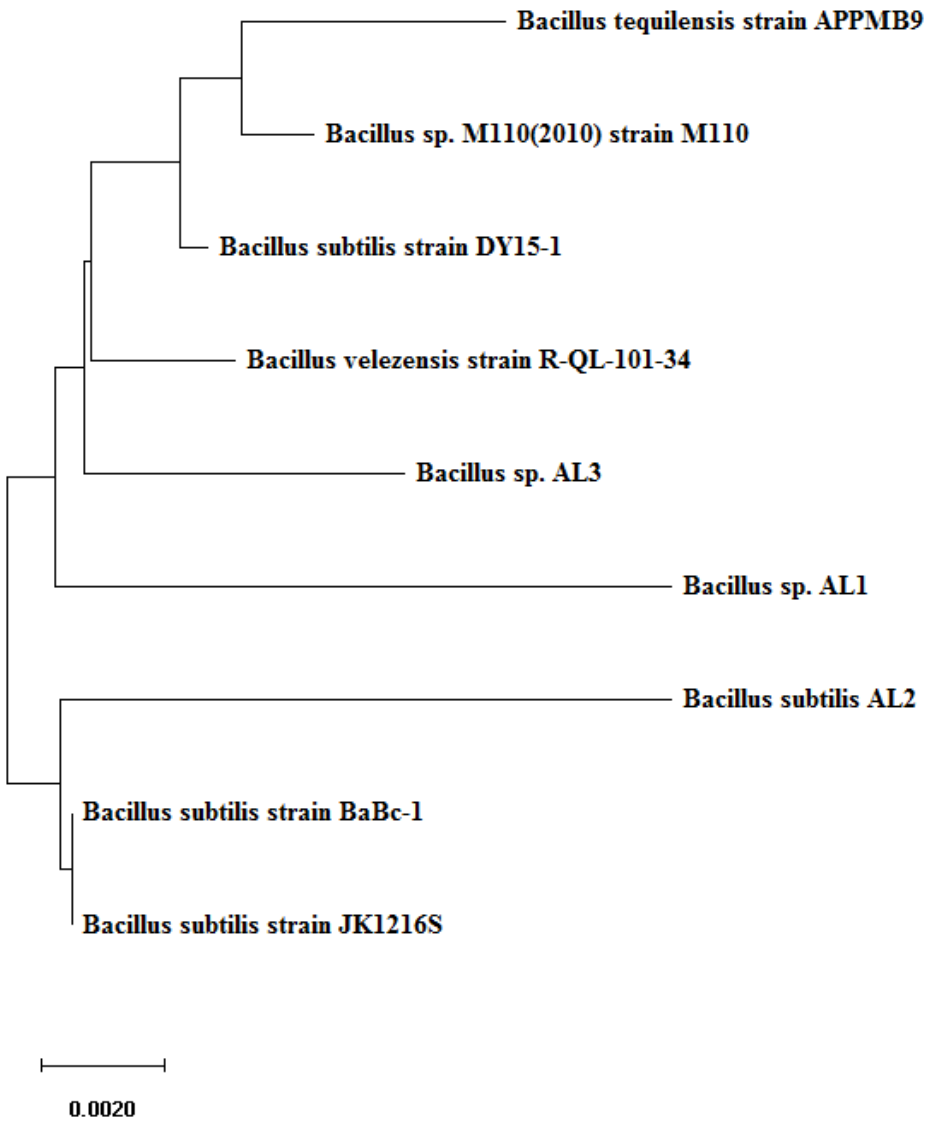


Figure 13. Phylogenetic tree of endospore forming phyllosphere bacterial isolates from red amaranthus variety arun

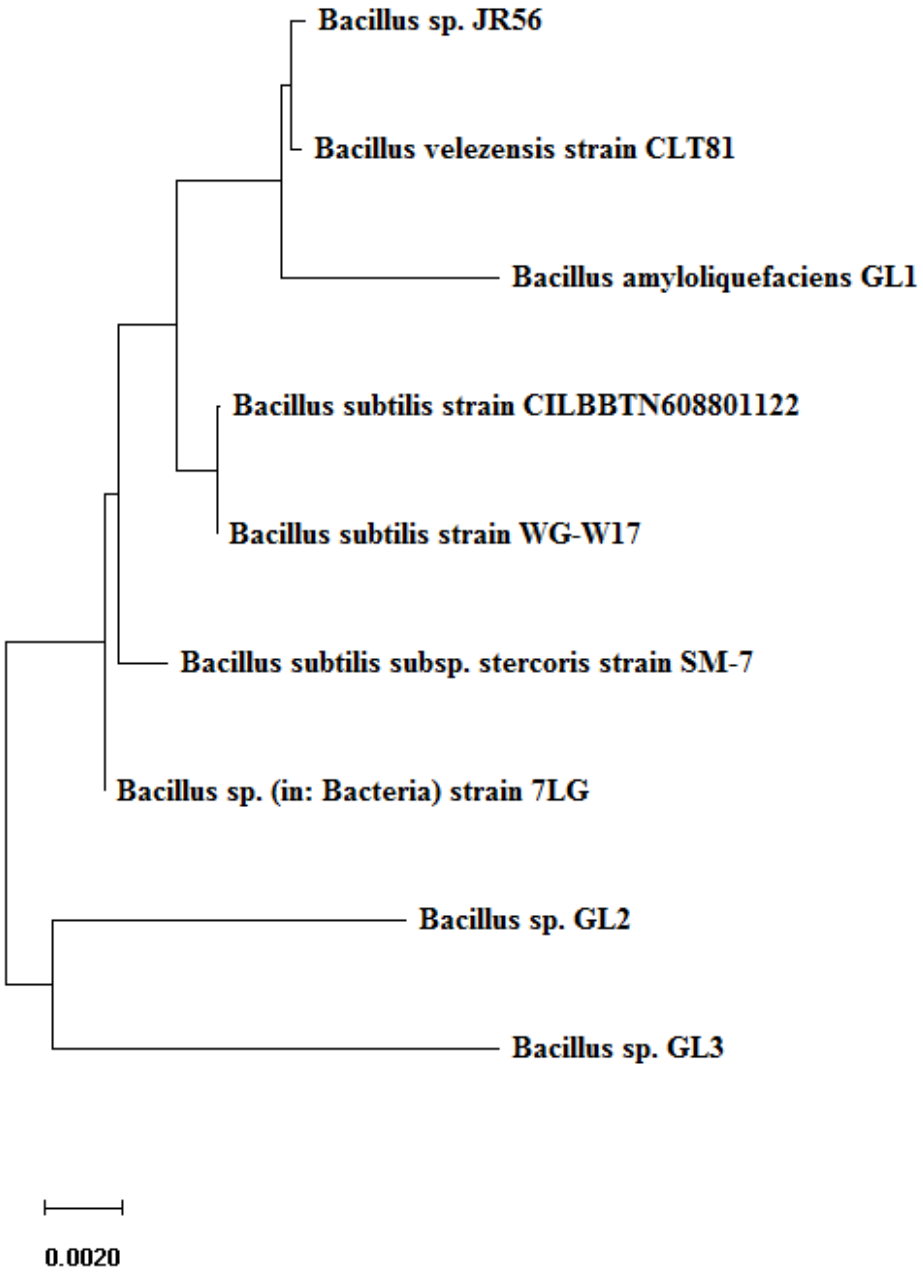


Figure 14. Phylogenetic tree of endospore forming phyllosphere bacterial isolates from green amaranthus variety CO-1

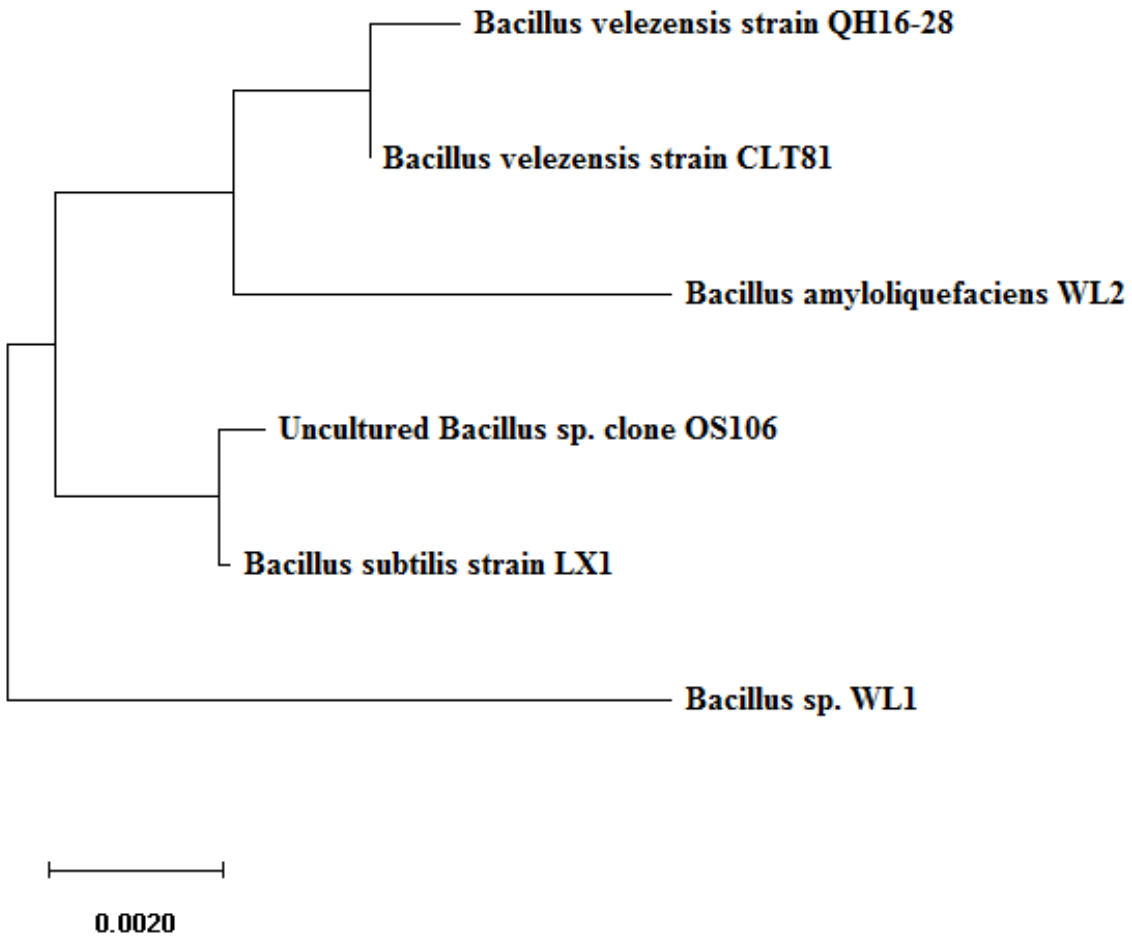


Figure 15. Phylogenetic tree of endospore forming phyllosphere bacterial isolates from wild relative *Amaranthus viridis*

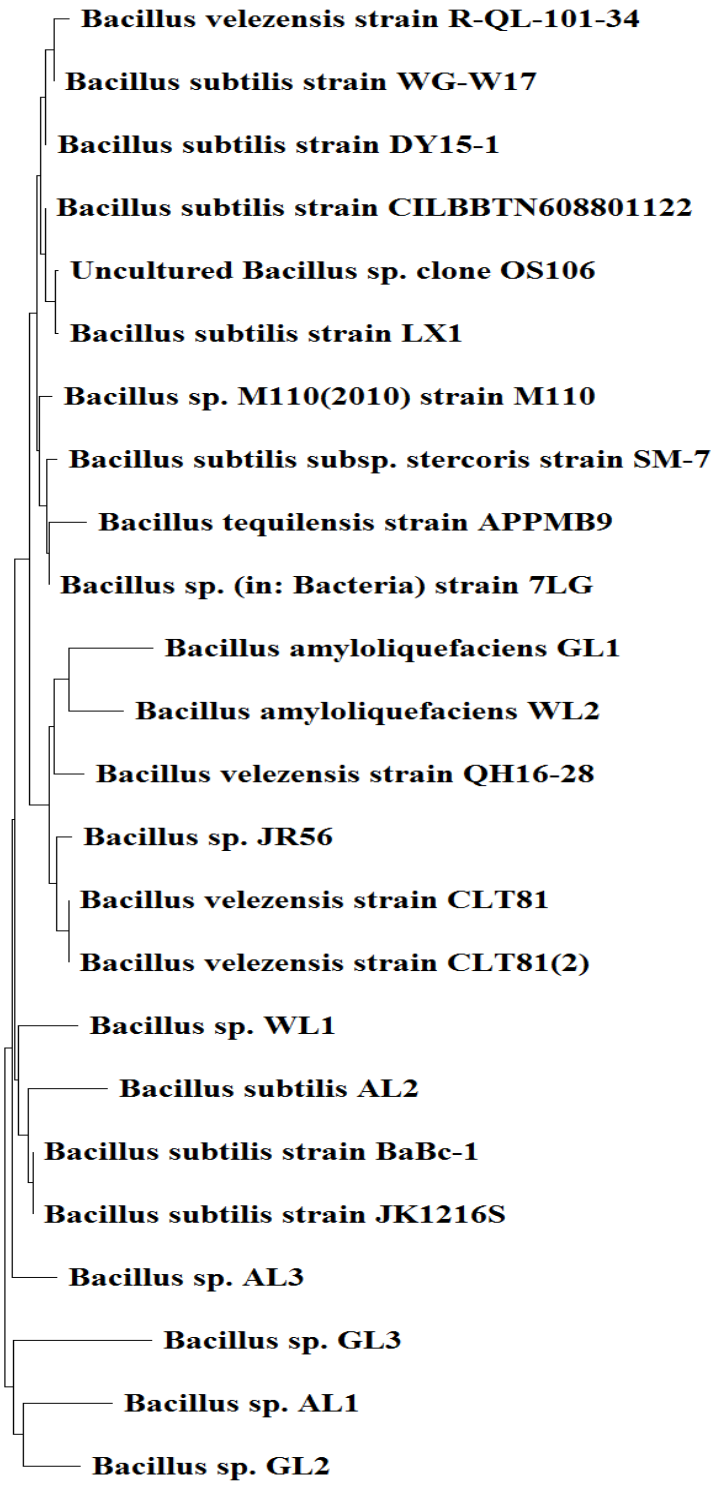


Figure 16. Phylogenetic tree of endospore forming phyllosphere bacterial isolates from *Amaranthus* spp.

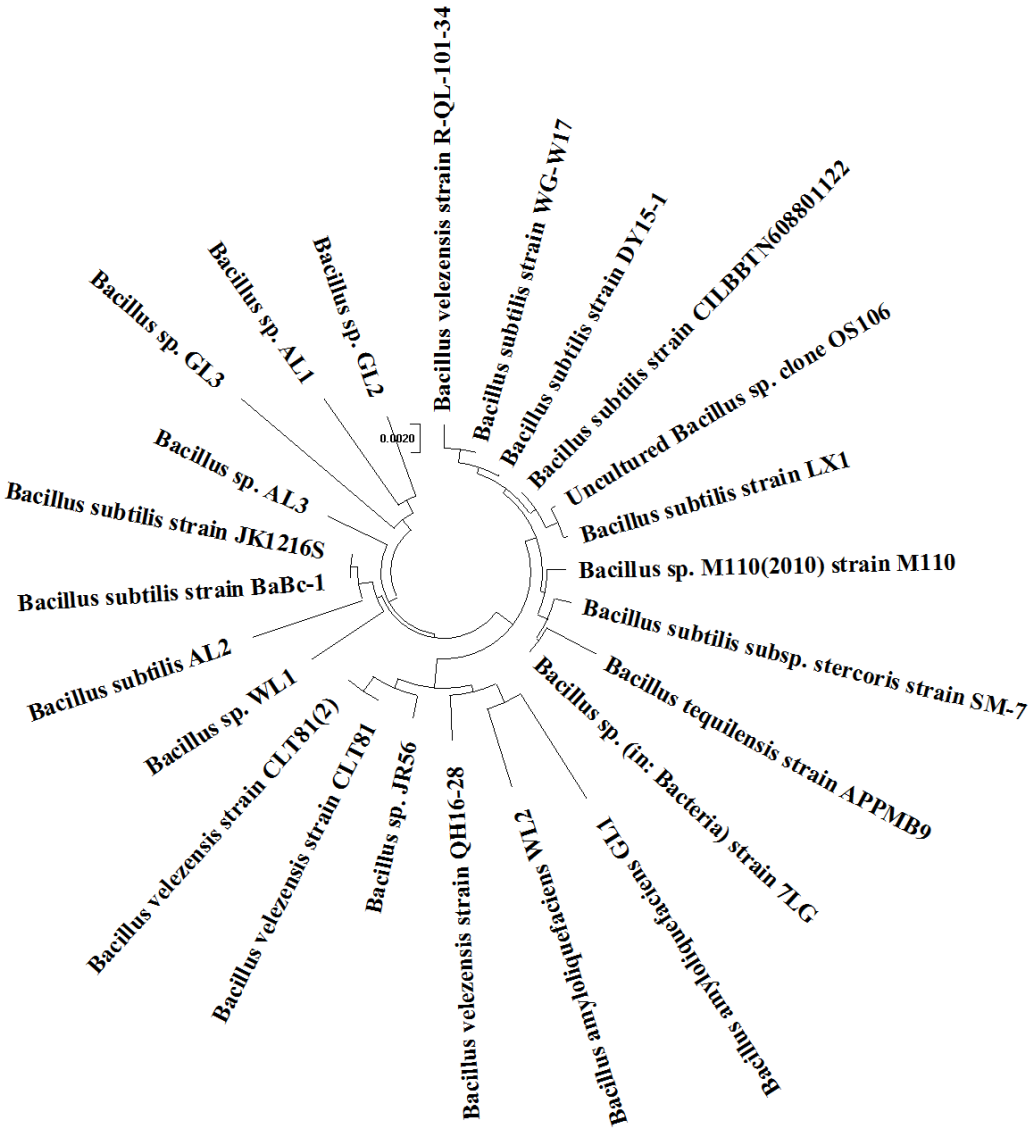


Figure 17. Circular phylogenetic tree of endospore forming phyllosphere bacterial isolates from *Amaranthus* spp.

Based on *in vitro* screening, endospore forming endorhizosphere bacterial isolates *Bacillus* sp AR1, *Bacillus siamensis* AR2, *Bacillus amyloliquefaciens* GR1, *Bacillus amyloliquefaciens* GR2, *Bacillus* sp WR1. and *Bacillus amyloliquefaciens* WR2 were selected as best and were further taken for *in vivo* screening, which helps to confirm the result gained from preliminary studies.

Bacterization of red amaranthus variety Arun seeds with selected isolates was done and seedlings were raised. For efficiency use of water, light, and soil nutrients, seeds must germinate and seedlings emerge, quickly and uniformly. Slow growth and germination of seeds leads to weak and stunted growth of plant and may be more vulnerable to pathogens results in low production (Bisen *et al*., 2015). *In vitro* studies of the present investigation showed the improved seed germination on inoculation with endospore forming endorhizosphere bacterial isolates. Moreover, root dipping of seedlings with bacterial suspension was carried out at the time of transplantation. Introducing microbial inoculants to targeted niche include the seed treatment, soil drenches and root dipping at the time of transplant which improves the survival and colonization ability of the bacteria in the seed, soil and in the roots (Bashan, 1998). Bacteria attached to the root resulted in enhanced growth and productivity in several crops (Paul and Lade, 2014).

Observations on biometric parameters showed significant difference between the treatments. Observations at 25 DAT, plants treated with *Bacillus* sp AR1 showed the maximum number of leaves. Least number of leaves was found in plants treated with *Bacillus amyloliquefaciens* WR2 (Figure 18). Observation on number of branches and plant height was taken at 15 DAT and 25 DAT, showed significant difference. At 25 DAT, highest number of branches was noticed in plants treated with *Bacillus* sp WR1, followed by plants treated with *Bacillus amyloliquefaciens* GR1, found to be on par with each other. Least number of branches per plant was recorded in control plants (Figure 19). Observations on plant height at 25 DAT showed that treatment with *Bacillus siamensis* AR2 was pointedly superior to all other treatments (Figure 20), whereas least

was observed in control plants. Numerous endospore forming *Bacillus* strains have already been reported for their plant growth promotion activities and are being investigated because of their widespread presence in soil, rapid and aggressive colonization on host plants rhizospheres and for the production of a range of plant growth promoting traits (Borriss *et al*., 2011; Kumar *et al*., 2011). Seed treatment or root dip with *Bacillus* spp. has been shown to bring drastic positive change in the composition of rhizosphere leading to increase in growth and yield of different crops. In parallel to our work several other authors have reported the plant growth promoting activities by the *Bacillus* species. Endophytic microorganisms provide beneficial compounds for the plant host and act as potential as plant growth promoters in agriculture (Strobel, 2002; Kuklinsky-Sobral *et al*., 2004). Biological fixation of nitrogen, phosphate solubilization, production of phytohormones and siderophores, synthesize of aminocyclopropane-1-carboxylic acid deaminase responsible for ethylene regulation are different mechanism involved by the endophytic bacteria in plant growth promotion (Berg and Hallmann, 2006). Rice plants inoculated with *Bacillus cereus* TS1 showed significant difference in plant height compared to control with a representing of increase of 15.46 % (Sivakumar *et al*., 2012). Qureshi *et al*. (2012) reported *Bacillus* spp. as an effective inoculant for the growth and yield enhancement of cotton.

Impact of the bacterial isolates on biomass production was assessed. Biometric parameters showed substantial difference between the treated plants and control plants. Maximum mean root fresh weight was observed in plants treated with *Bacillus amyloliquefaciens* GR1. Lowest was observed in plants treated with *Bacillus siamensis* AR2. Plants treated with *Bacillus amyloliquefaciens* GR1 was found to be significantly superior in root dry matter production compared to other treatments. Lowest root dry weight was observed in control plants (Figure 21). Highest shoot fresh weight was observed in plants treated with *Bacillus amyloliquefaciens* GR1, whereas minimum was observed in control plants. *Bacillus siamensis* AR2 treated plants showed expressively highest shoot dry weight and the lowest shoot dry weight was noticed in plants treated with *Bacillus amyloliquefaciens* WR2 (Figure 22). There exist several reports where

Bacillus spp. involved in improving plant biomass. Biopriming of amaranth (*Amaranthus hypochondriacus*) seeds with *Bacillus* sp. showed an average maximum enhancement of 85% in root dry weight, of which two isolates were identified as *Bacillus* pumilus and *Bacillus subtilis* (Pandey *et al*., 2018). Lettuce (*Lactuca sativa*) plants inoculated with *Bacillus subtilis* showed an increase in weight of shoots (64%) and roots (68%) (Galelli *et al*., 2015). *Bacillus altitudinis*, an endophytic spore forming bacteria isolated from yerba mate plant increased shoot dry weight compared to control (Laczeski *et al*., 2020). All these studies were in tune with the results of present study.

Bacillus spp. quickly and aggressively colonize the root system, augment the plant growth and yield directly and indirectly via. Plant Growth Promoting (PGP) activities and control a wide range of phytopathogens including *Erwinia corotovora, Fusarium* species, *Fusarium oxysporum, Macrophomina phaseolina, Phytophthora, Pythium* species and *Rhizoctonia solani* (Kumar *et al*., 2012). Apart from plant growth promoting traits, these beneficial microorganisms are known to antagonize phytopathogens which indirectly contribute to the plant growth promotion. Hence, after checking the plant growth promoting potential of endospore forming endorhizosphere bacterial isolates, their antagonistic activity against Rhizoctonia leaf blight was evaluated in red amaranthus variety Arun.

Results showed a substantial difference in percent disease index between the plants treated with bacterial endophytes and the control plants at three days after inoculation and five days after inoculation. At 3 DAI, no symptoms were developed on the absolute control plants. The plants treated with *Bacillus amyloliquefaciens* GR1 showed the least PDI 12.96 which accounts to 54.35% disease suppression over control plants. Maximum biocontrol activity than mancozeb application was shown by *Bacillus amyloliquefaciens* GR1 treated plants against *R. solani* on 3rd DAI, whereas on 5th DAI by *Bacillus* sp WR1 treated plants (Figure 23). Green amaranthus variety CO-1 and wild relative (*Amaranthus viridis*) are basically resistant to Rhizoctonia leaf blight, compared to red amaranthus variety Arun. The endophytes obtained from green amaranthus and

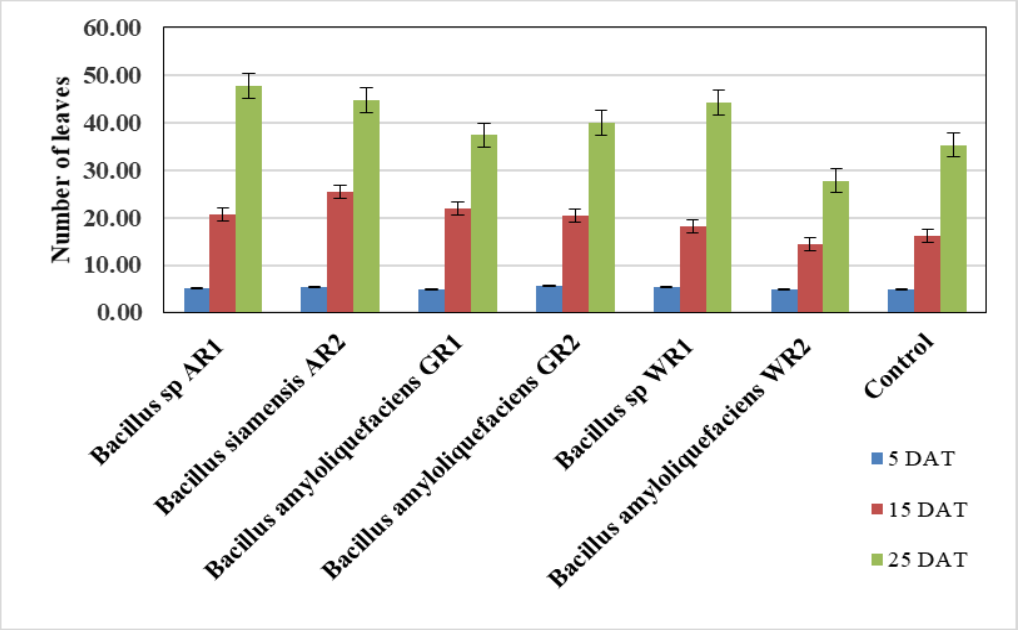


Figure 18. Effect of endospore forming endorhizosphere bacterial isolates on number of leaves of red amaranthus var. Arun

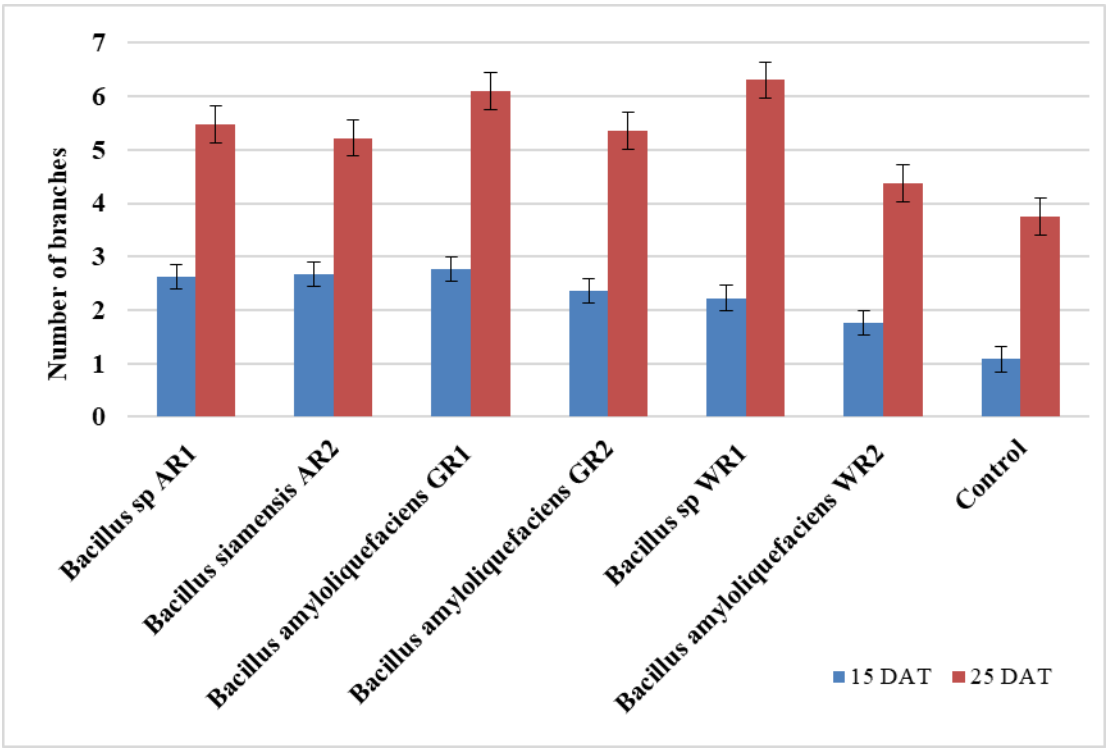


Figure 19. Effect of endospore forming endorhizosphere bacterial isolates on number of branches of red amaranthus var. Arun

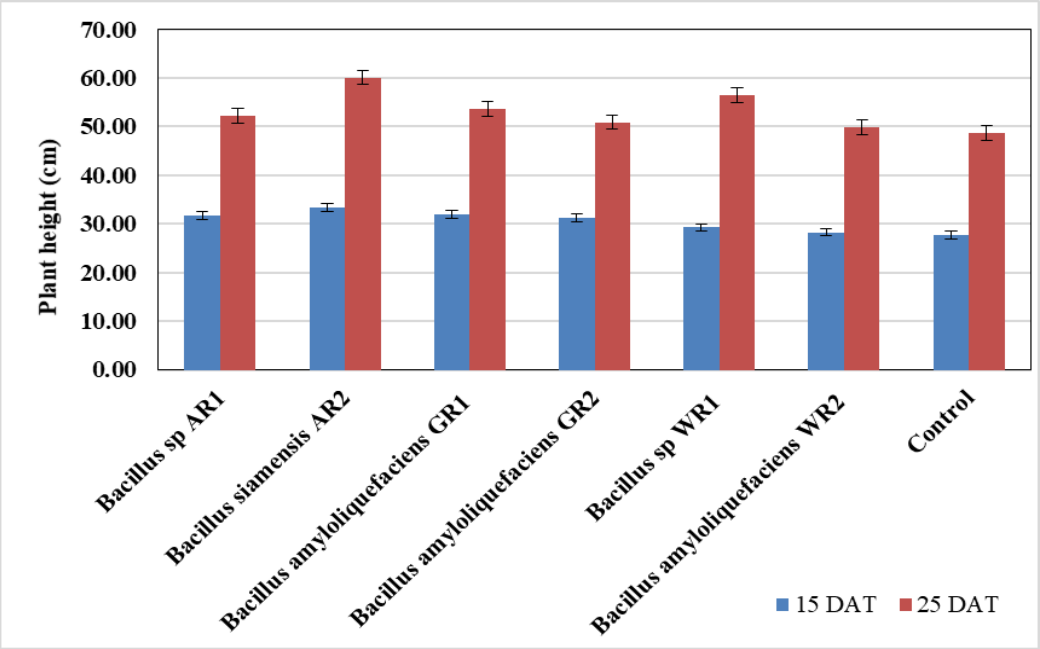


Figure 20. Effect of endospore forming endorhizosphere bacterial isolates on plant height of red amaranthus var. Arun

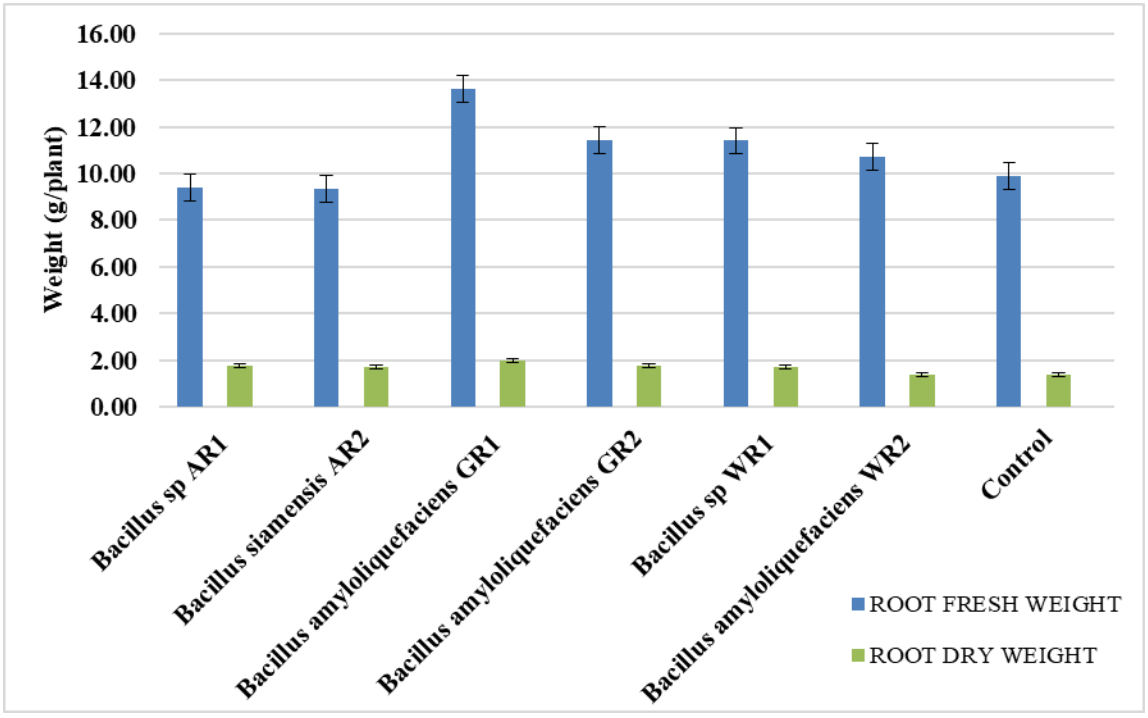


Figure 21. Effect of endospore forming endorhizosphere bacterial isolates on fresh and dry weight of root of red amaranthus var. Arun

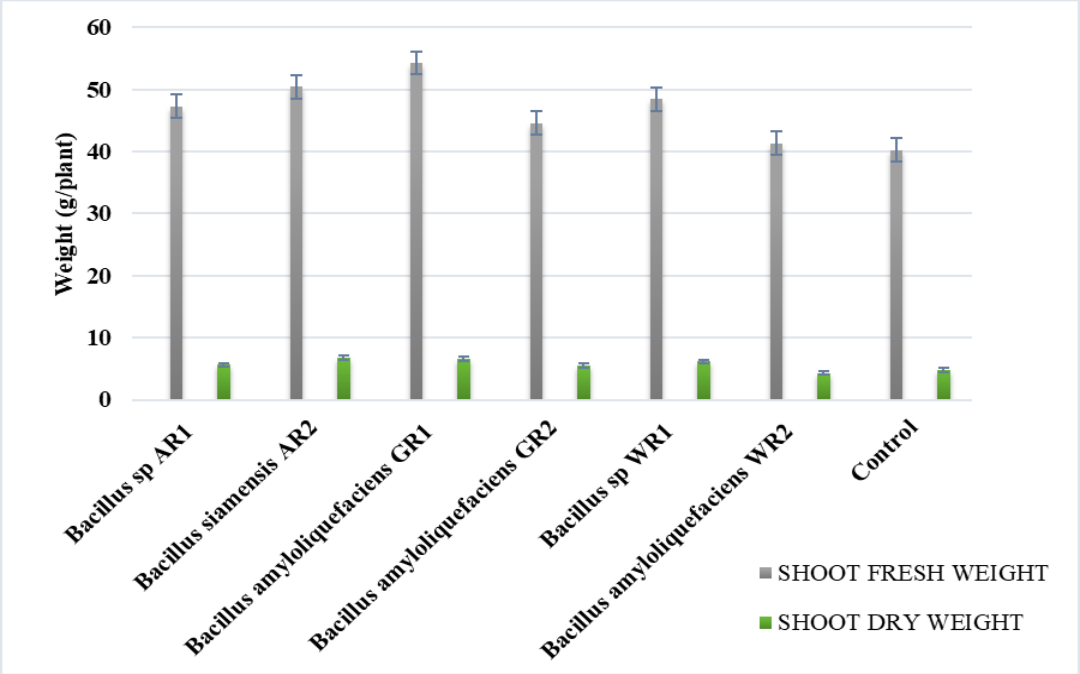


Figure 22. Effect of endospore forming endorhizosphere bacterial isolates on fresh and dry weight of shoot of red amaranthus var. Arun

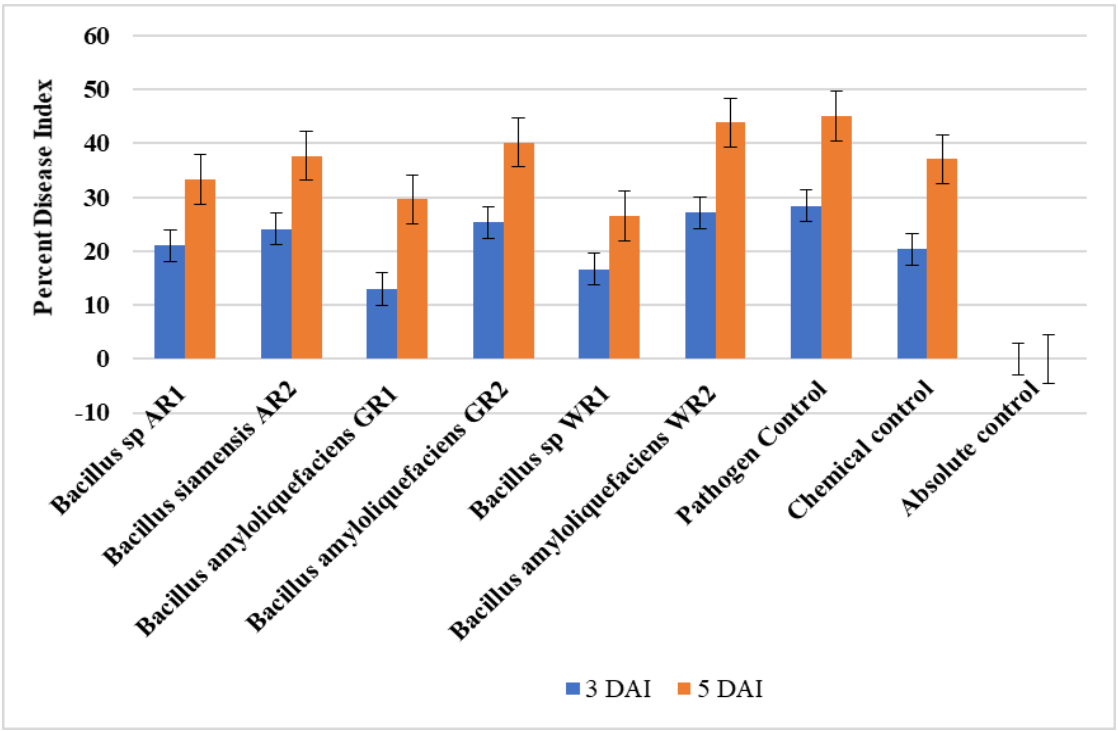


Figure 23. Disease suppression in terms of percent disease index of lesions produced on leaves treated with endospore forming endorhizosphere bacteria

wild relative might have successfully established themselves in treated plants exhibiting their antagonism against *R. solani*. The production of any antifungal compounds, competition, and induced systemic resistance might be the reasons for their antagonistic activities.

Pertaining to our work there are several reports that emphasize the effectiveness of endophytic bacteria in reducing the disease incidence and disease severity. Inhibition of sharp eye spot of wheat caused by *Rhizoctonia* spp. was reported by endophytic *Bacillus cereus* strain B946 (Mei *et al*., 2005). Raaijmakers and Mazzola (2012) reported the use of *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus pumilus* as bio-fertilizer and bio-control agents for against phytopathogens. In a study conducted by Uppala *et al*., (2010) bacterial endophytes identified as *Bacillus* spp. showed growth promotion as well as antagonistic activity against Rhizoctonia leaf blight in amaranthus. Recently, endophytes isolated from *Piper* sp. showed growth promotion as well as antagonistic activity against foot rot disease caused by *Phytopthora capsici* (Kollakkodan *et al*., 2017).

Genus *Bacillus* encompasses various species which include several strains having biocontrol activity till date (Jacobsen *et al*., 2004). Rajasekar *et al*. (2019) reported that phylloplane bacterial isolates act as antagonistic to some foliar diseases. Considering this, further an *in vivo* study was conducted to test for their disease suppressive activity of endospore forming phyllosphere bacterial isolates *Bacillus* sp AL1, *Bacillus* sp AL3, *Bacillus subtilis* GL2, *Bacillus* sp GL3, *Bacillus subtilis* WL1 and *Bacillus amyloliquefaciens* WL2 selected based on the *in vitro* studies against Rhizoctonia leaf blight in red amaranthus variety Arun.

Endospore forming phyllosphere bacterial isolates were sprayed onto the leaves of amaranthus plants. Since the isolates were obtained from phyllosphere, spraying onto the leaves would help them for rapid, aggressive and effective colonization on phyllosphere, thereby preventing the attack of pathogens. Least PDI represent more antagonistic activity against the pathogen which indicates the efficient disease suppressiveness of the

bacteria against the pathogen. Here, even though *Bacillus* sp GL3 and *Bacillus* sp AL3 showed maximum biocontrol activity on 3 DAI, only *Bacillus* sp AL3 showed significant disease suppressing activity on 5 DAI. Less lesion size on inoculated leaves and maximum percent disease index recorded by *Bacillus* sp AL3 indicated the potential antagonistic activity against Rhizoctonia leaf blight (Figure 24). Over all, *Bacillus* sp GL3 was the superior one in antagonistic activity against *R. solani* under *in vitro* conditions. However, during the initial stages of pathogen inoculation under *in vivo* conditions *Bacillus* sp GL3 showed remarkable antagonism, later *Bacillus* sp AL3 showed maximum antagonism. Present study reports the possibility of using endospore forming phyllosphere bacteria in suppressing the Rhizoctonia leaf blight for the first time. There are numerous reports which conclusively support the indirect plant growth promotion by different strains of *Bacillus* spp. through antagonism. *Bacillus* BCAs rely on different mechanisms which directly restricts the pathogen growth by the release of bioactive antifungal compounds that are potentially involved in antibiosis, including proteins, lipopeptides, diffusible antibiotics, volatiles compounds (Alvarez *et al*., 2011; Baysal *et al*., 2013) and siderophores (Jenifer *et al*., 2013). Under iron deficiency conditions, siderophores acts as a competitive factor especially on leaf surface, which are important in the suppression of fungal diseases (Nabti *et al*., 2013). Volatile substances produced by *Bacillus thuringiensis* and *Bacillus pumilis* showed a significant inhibition of *Colletotrichum gloeosporioides* (Zheng *et al*., 2013). Hunter (2016) reported strains of *Bacillus amyloliquefaciens* subsp. plantarum and *Bacillus subtilis* as potential biocontrol agents of *R. solani* under *in vitro* and *in vivo* conditions. Additionally, results from many works on biocontrol activity of *Bacillus* sp against *R. solani* in different crops such as tomato (Szczech and Shoda, 2004) and cucumber (Huang *et al*., 2012), lettuce (Chowdhury *et al*., 2013) strengthen the results of present investigation.

In addition to the evaluation of antagonistic potential, endospore forming phyllosphere bacterial isolates were tested for their influence on biometric parameters such as root fresh and dry weight, shoot fresh and dry weight. Bacterial treated plants showed significant difference in biometric parameters when compared to control plants.

Maximum fresh weight was recorded in plants treated with *Bacillus* sp GL3 whereas the least was recorded in *Bacillus subtilis* WL1. Plants treated with *Bacillus* sp GL3 showed maximum root dry weight (Figure 25). *Bacillus* sp GL3 treated plants recorded highest shoot fresh weight whereas the least was observed in absolute control. Maximum shoot dry weight was recorded in plants inoculated with *Bacillus* sp GL3, least shoot dry weight was observed in absolute control plants (Figure 26). Here, remarkable improvement in biometric observations was noticed in the plants treated with phyllosphere bacteria isolated from green amaranthus. It was very interesting that phyllopshere bacterial isolates plays an important role in enhancing growth attributes of amaranthus plants. The present investigation established that the endospore forming phyllosphere bacterial isolates have the potential to be employed as plant growth promoters as well as can be used as biocontrol agents against Rhizoctonia leaf blight in amaranthus. Giongo *et al*. (2013), in an experiment on plant growth promotion of plyllospheric bacteria inhabiting *Vriesea gigantea* Gaud. and *Tillandsia aeranthos* (Loiseleur) L.B. Smith (Bromeliaceae) concluded that 70% of the evaluated bacteria presented the ability of siderophore production and phosphate solubilization, and possessed the *nif* H gene, there by promote plant growth. Similarly, *B. thuringiensis* strain SNKr10 isolated from phyllosphere of spinach, showed increase in seedling length, root and shoot length compared to control (Sharma and Saharan, 2017). Inoculation of the isolates obtained from the phylloplane of *Jatropha curcas* to maize seed significantly increased shoot and root lengths of maize seedlings (Dubey *et al*., 2017)

Summing up the overall results of this study, it is evident that the endospore forming endorhizosphere bacteria *Bacillus amyloliquefaciens* GR1 had better efficiency in plant growth promotion and the endospore forming phyllosphere bacteria *Bacillus* sp AL3 as a promising biocontrol agent in reducing Rhizoctonia leaf blight of amaranthus. Therefore, present study concludes the significant role of endospore forming endorhizosphere and phyllosphere bacterial isolates in the enhancement of plant growth either directly or indirectly

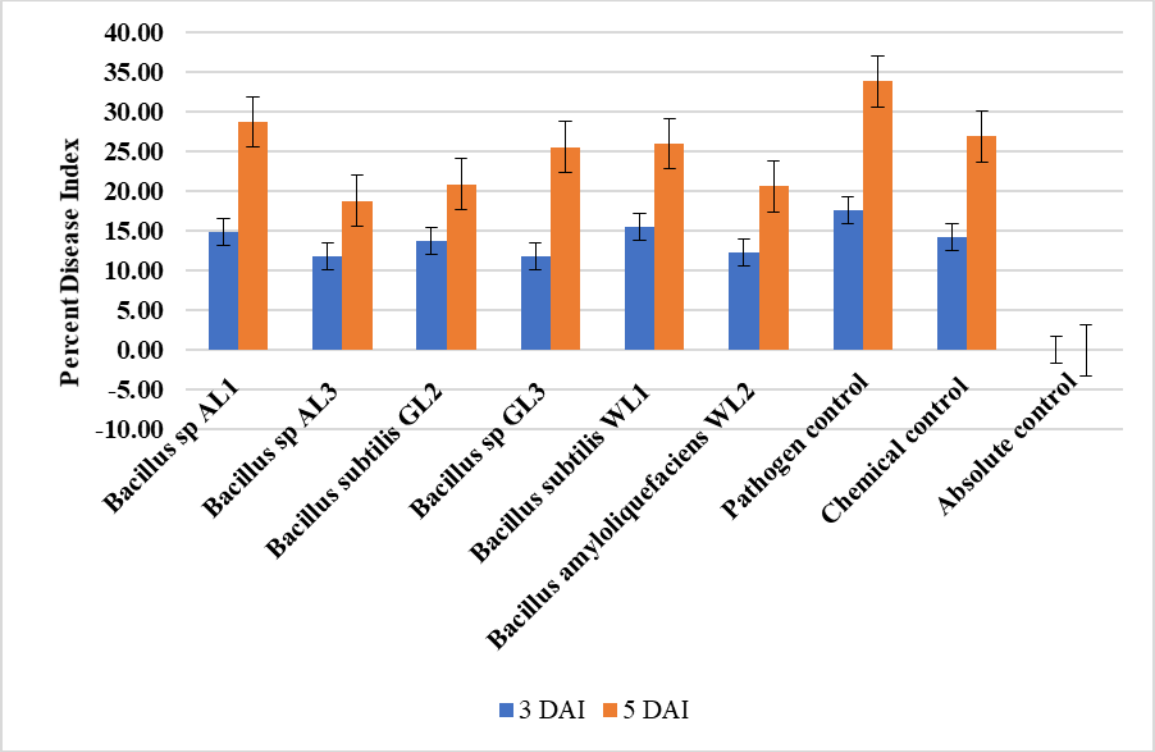


Figure 24. Disease suppression in terms of percent disease index of lesions produced on leaves treated with endospore forming phyllosphere bacteria

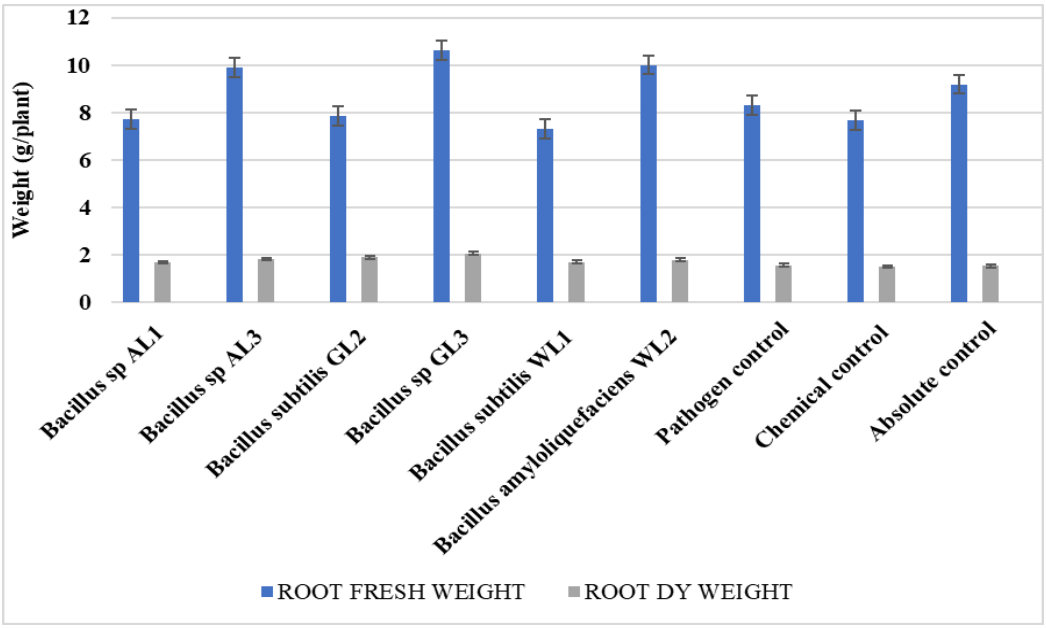


Figure 25. Effect of endospore forming phyllosphere bacterial isolates on fresh and dry weight of root of red amaranthus var. Arun

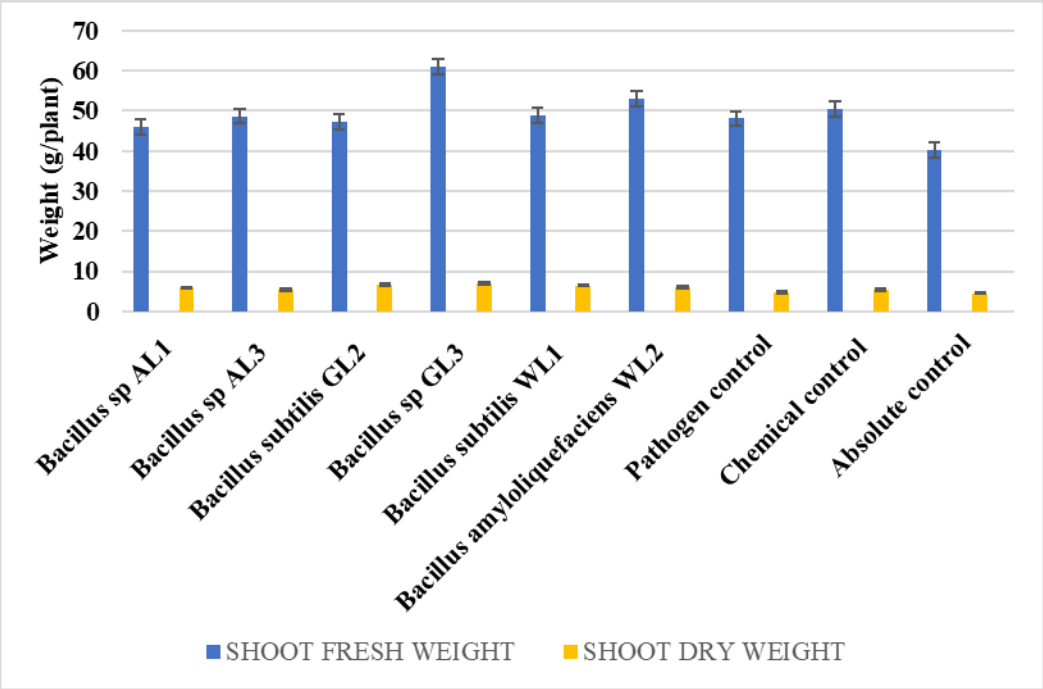


Figure 26. Effect of endospore forming phyllosphere bacterial isolates on fresh and dry weight of shoot of red amaranthus var. Arun

Summary

6. SUMMARY

Leaves are the primary determinant of economic yield in Amaranthus. However, Rhizoctonia leaf blight, is a major constraint in cultivation of amaranthus, especially during the monsoon period. Among the biocontrol agents, bacterial endophytes are important as they bring about disease suppression as a well as plant growth promotion. Recently, endospore forming bacteria have been reported as promising biocontrol agents. Endospore forming bacteria possess the characteristics that enable them to thrive under unfavourable conditions. They are known to play an important role in increasing plant growth and disease suppression. Hence, developing endospore forming bacteria as biocontrol agents and plant growth promoters is a promising strategy.

In this context the programme entitled "Plant associated endospore forming bacteria from amaranthus as growth promoters and biocontrol agents against Rhizoctonia leaf blight" was undertaken in the Department of Agricultural Microbiology, College of Agriculture, Vellayani, Thiruvananthapuram.

The main objectives of the present study were isolation and characterization of endospore forming bacteria from endorhizosphere and phyllosphere of Amaranthus and evaluate their effect on growth promotion and leaf blight disease suppression. The salient findings from the present study are briefed below.

A total of eight endospore forming endorhizosphere bacteria were isolated from healthy roots of red amaranthus variety Arun, green amaranthus variety CO-1 and wild relative (*Amaranthus viridis*) by enrichment method on NA medium. The isolates were designated as AR1, AR2, AR3, GR1, GR2, GR3, WR1 and WR2. Similarly, using the same method a total of eight endospore forming phyllosphere bacteria were isolated form healthy leaves of red amaranthus variety Arun, green amaranthus variety CO-1 and wild relative and were designated as, AL1, AL2, AL3, GL1, GL2, GL3, WL1 and WL2. Both endospore forming endorhizosphere and phyllosphere bacteria were identified as *Bacillus* sp. based on morphological, biochemical and molecular characterization. These isolates

designated as AR1, AR2, AR3, GR1, GR2, GR3, WR1, WR2, AL1, AL2, AL3, GL1, GL2, GL3, WL1 and WL2 were identified as *Bacillus* sp., *Bacillus siamensis, Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus amyloliquefaciens, Bacillus amyloliquefaciens, Bacillus* sp., *Bacillus amyloliquefaciens, Bacillus* sp., *Bacillus subtilis, Bacillus* sp., *Bacillus amyloliquefaciens, Bacillus* sp., *Bacillus* sp., *Bacillus subtilis* and *Bacillus amyloliquefaciens* respectively based on 16S rRNA sequence.

Further, endospore forming endorhizosphere bacterial isolates were studied for their plant growth promoting activity, and the endospore forming phyllosphere bacterial isolates were tested for their disease suppressiveness against *Rhizoctonia solani* leaf blight, in red amaranthus variety Arun.

Indole-Acetic Acid (IAA) production by the different isolates of endospore forming endorhizosphere bacteria showed wide variations ranging from 4.09 µg mL^{-1} to 9.73 µg mL^{-1} of culture filtrate and the isolate AR3 produced the maximum IAA of 9.73 µg mL^{-1} of culture filtrate.

Studies on the effect of endospore forming endorhizosphere bacteria on seed germination and seedling growth using roll towel assay recorded a significant increase in germination percentage, seedling shoot length, root length and seedling vigour index compared to control. The isolate WR1 showed the maximum germination (43.33%) whereas control recorded a germination of 30%. The isolate WR1 showed maximum seedling vigour index (223.66) compared to the control (136.53).

Protray experiment conducted to study the influence of endospore forming endorhizosphere bacterial isolates on plant growth parameters of amaranthus in the nursery stage revealed the growth promotion efficacy of the isolates. Maximum germination percentage of 100 was observed in seeds treated with the isolate WR2. Significant influence of the bacterial isolates on seedling vigour index was observed wherein the isolate WR2 recorded maximum of 884.50 and the control recorded 665.35. Highest root fresh weight was recorded in the plants treated with isolate GR1 (8.12

mg/plant) and the lowest root fresh weight of 3.56 mg/plant was recorded in control plants. It was found that the highest root dry weight was found in plants treated with isolate WR1 (2.99 mg/plant) as against the control (2.46 mg/plant).

The antagonistic activities of all endospore forming phyllopshere bacterial isolates were assessed against *Rhizoctonia solani* which causes leaf blight disease in amaranthus using dual culture plate assay on Potato Dextrose Agar medium. Even though, all the isolates inhibited the mycelial growth of *Rhizoctonia solani*, GL3 and GL1 exhibited the maximum and minimum zone of inhibition (ZOI) of 8.73 and 1.00 mm respectively.

Apart from direct antagonistic activity, all the endospore forming phyllopshere bacterial isolates were tested for their indirect antagonism against *Rhizoctonia solani* mycelial growth, in which isolate GL3 showed the maximum ZOI of 2.88 mm and the isolate GL1 showed the minimum ZOI of 0.50 mm.

A detached leaf assay was carried out to study the effect of endospore forming phyllosphere bacterial isolates to test the antagonistic activity against *R. solani*. Treatments exerted significant effect in the development of symptoms in the leaves. A significant difference in the lesion size observed on the detached leaves treated with bacterial isolates and water (control) on 3^{rd} and 4^{th} day after pathogen inoculation. Maximum lesion area of 3.49 and 12.23 cm^2 was observed in control plants and the minimum lesion area of 0.42 and 3.11 cm^2 was developed on the leaves treated with isolate WL2 on 3^{rd} and 4^{th} day after pathogen inoculation respectively.

After the preliminary assessment of plant growth promotion and antagonistic activity against *R. solani* under *in vitro* conditions, the endospore forming endorhizosphere bacterial isolates AR1, AR2, GR1, GR2, WR1 and WR2 and endospore forming phyllosphere bacterial isolates AL1, AL3, GL2, GL3, WL1 and WL2 were selected as superior isolates and were used for further pot culture study.

A pot culture experiment was conducted in completely randomized design to evaluate the efficacy of selected endospore forming endorhizosphere bacteria for their plant growth promoting activity in amaranthus (var. Arun). Significantly higher values with respect to biometric parameters such as number of leaves, number of branches, plant height and dry matter production were observed in plants treated with bacteria compared to the control plants. Maximum shoot fresh weight of 54.30 g/plant was observed in *Bacillus amyloliquefaciens* GR1 treated plants, whereas the control plants were with the minimum of 40.24 g/plant., Maximum shoot dry matter production was recorded in plants treated *Bacillus siamensis* AR2 (6.74 g/plant) and the control plants recorded 4.76 g/plant. Maximum root fresh weight of 13.64 g/plant was recorded in plants inoculated with *Bacillus amyloliquefaciens* GR1 whereas the control plants had root fresh weight of 9.88 g/plant. Maximum root dry weight was exhibited by plants treated with *Bacillus amyloliquefaciens* GR1 (1.98 g/plant) and the lowest was produced by control plants with 1.37 g/plant.

Endospore forming endorhizosphere bacterial isolates were tested for their biocontrol activity against Rhizoctonia leaf blight. Percent disease index was calculated based on scoring the lesion size on inoculated leaves at three days after inoculation and five days after inoculation, treatments showed significant results. At 3 DAI, no symptoms were developed on the absolute control plants. The plants treated with *Bacillus amyloliquefaciens* GR1 showed the least PDI 12.96 which accounts to 54.35% disease suppression over control plants. Maximum PDI was observed in control plants (28.39).

Plants treated with *Bacillus* sp WR1 showed the minimum PDI of 26.54 with 41.10% disease suppression over the pathogen control, which was followed by *Bacillus amyloliquefaciens* GR1 with PDI of 29.62 and percentage disease suppression of 34.25 over the pathogen control. Pathogen control plants showed maximum percent disease index of 45.06. *Bacillus amyloliquefaciens* GR1 treated plants showed more biocontrol activity against *R. solani* on 3rd DAI, whereas on 5th DAI *Bacillus* sp WR1 treated plants showed the maximum biocontrol activity against *R. solani.*

Evaluation of disease suppressiveness of endospore forming phyllosphere bacterial isolates against *Rhizoctonia* foliar blight was carried out using red amaranthus variety Arun as test crop by challenge inoculation with the pathogen on intact leaves. Plants treated with isolate *Bacillus* sp. GL3 showed the minimum percent disease index of 11.80 with 32.92% disease suppression over the pathogen control on 3rd day after pathogen inoculation, which was on par with the isolate *Bacillus* sp. AL3 with 11.81 percent disease index. Interestingly, on 5th day after pathogen inoculation, isolate *Bacillus* sp. AL3 treated plants showed the minimum percent disease index of 18.75 with 44.51% disease suppression over the pathogen inoculated control.

In the present investigation, *Bacillus amyloliquefaciens* GR1 and *Bacillus* sp. AL3 were selected as the best isolates for plant growth promotion and disease suppression respectively. Seed bacterization of amaranthus plants with endospore forming endorhizosphere and foliar spray of phyllosphere bacterial isolates improves plant growth and suppresses Rhizoctonia leaf blight incidence respectively and helps in better establishment of plants.

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Appendices

APPENDIX - I

COMPOSITION OF MEDIA USED

1. Nutrient Agar

Peptone	- 5g
NaCl	- 5g
Beef extract	- 3g
Agar	- 20g
Distilled water	- 1000 ml

Peptone, NaCl and beef extract were dissolved in 500 ml distilled water and volume made up to 1000 ml. 20 g agar-agar was added into this mixture and autoclaved at 15 lbs pressure and 121^0C for 15 min.

2. Potato Dextrose Agar

Peeled and sliced potatoes	- 200g
Dextrose ($C_6H_{12}O_6$)	- 20g
Agar-agar	- 20g
Distilled water	- 1000 ml

Potatoes were boiled in 500 ml of distilled water and the extract was collected by filtering through a muslin cloth. Agar-agar was dissolved separately in 500 ml of distilled water. The potato extract was mixed in the molten agar and 20 g of dextrose was dissolved in to the mixture. The volume was made up to 1000 ml with distilled water and medium was sterilized at 15 lbs pressure and 121^0C for 15 min.

APPENDIX - II

COMPOSITION OF STAIN USED

1. Crystal violet

One volume saturated alcohol solution of crystal violet in four volumes of one per cent aqueous ammonium oxalate.

2. Gram's iodine

Iodine crystals	- 1.0g
Potassium iodide	- 2.0g
Distilled water	- 300ml

3. Safranin

Ten ml saturated solution of safranin in 100 ml distilled water.

4. Malachite Green

Malachite green - 1.0 gm

Distilled water - 100 ml

APPENDIX - III
SEQUENCE PRODUCING SIGNIFICANT ALIGNMENTS

a. *Bacillus* sp AR1

Sequences producing significant alignments Download Manage Columns Show 10

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Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Bacillus sp. (in: Bacteria) strain 26LG 16S ribosomal RNA gene, partial sequence	2218	2218	98%	0.0	96.63%	MT762874.1
Bacillus velezensis strain X2 16S ribosomal RNA gene, partial sequence	2211	2211	98%	0.0	96.55%	KY052002.1
Uncultured Bacillus sp. clone TT W 16S ribosomal RNA gene, partial sequence	2211	2211	98%	0.0	96.55%	KY393358.1
Bacillus amyloliquefaciens strain 2M5-10 16S ribosomal RNA gene, partial sequence	2211	2211	98%	0.0	96.55%	KX267880.1
Bacillus velezensis NJN-6, complete genome	2211	17524	98%	0.0	96.55%	CP007165.1
Bacillus amyloliquefaciens strain zy2 16S ribosomal RNA gene, partial sequence	2211	2211	98%	0.0	96.55%	JN160740.1
Bacillus amyloliquefaciens subsp. plantarum strain Nt10-11 16S ribosomal RNA gene, partial sequence	2211	2211	98%	0.0	96.55%	HQ831429.1
Bacillus subtilis strain HDYM-23 16S ribosomal RNA gene, partial sequence	2211	2211	98%	0.0	96.55%	EF428247.2
Bacillus sp. (in: Bacteria) strain V13 16S ribosomal RNA gene, partial sequence	2209	2209	98%	0.0	96.68%	MH820047.1
Bacillus sp. (in: Bacteria) strain HC19 16S ribosomal RNA gene, partial sequence	2209	2209	98%	0.0	96.55%	MH398510.1

b. *Bacillus siamensis* AR2

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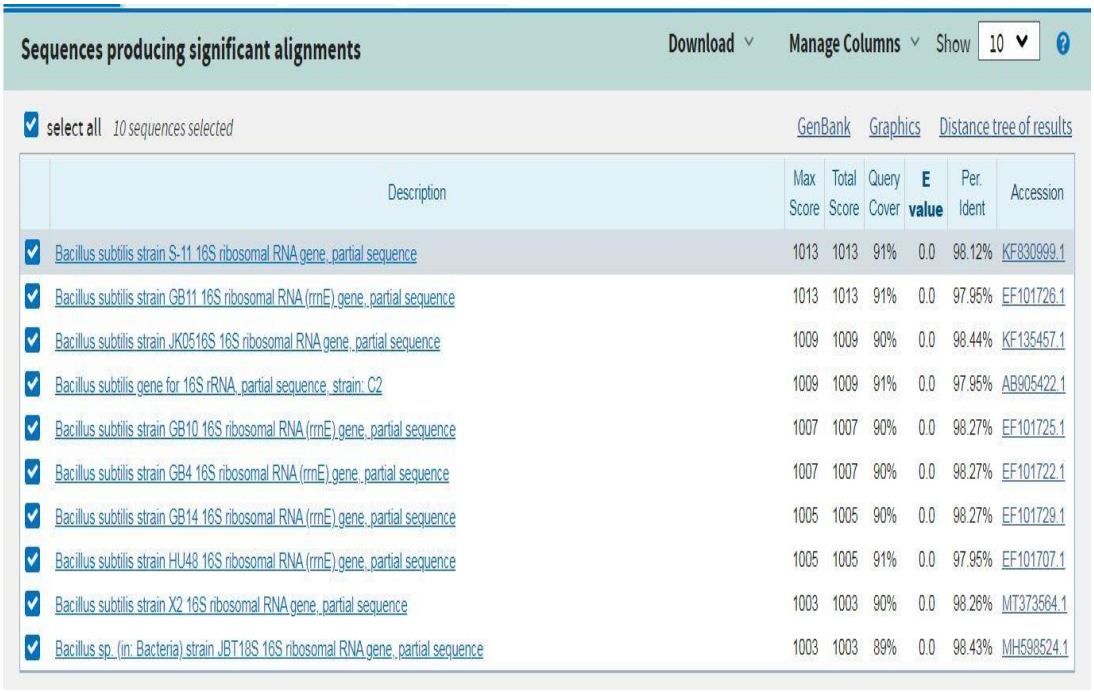
Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Bacillus siamensis strain SZ004 16S ribosomal RNA gene, partial sequence	2209	2209	98%	0.0	96.69%	MK788212.1
Bacillus sp. (in: Bacteria) strain JSM 162014 16S ribosomal RNA gene, partial sequence	2200	2200	98%	0.0	96.54%	MG893194.1
Bacillus velezensis strain CI9 16S ribosomal RNA gene, partial sequence	2198	2198	98%	0.0	96.54%	KU681039.1
Bacillus amyloliquefaciens strain SSH100-3 16S ribosomal RNA gene, partial sequence	2198	2198	98%	0.0	96.54%	KU321525.1
Bacillus amyloliquefaciens gene for 16S rRNA, partial sequence, strain: NCCP-49	2198	2198	98%	0.0	96.54%	AB547229.1
Bacillus velezensis strain QH16-28 16S ribosomal RNA gene, partial sequence	2196	2196	98%	0.0	96.54%	MT078625.1
Bacillus subtilis strain DJ1 16S ribosomal RNA gene, partial sequence	2196	2196	98%	0.0	96.54%	KU317795.1
Bacillus amyloliquefaciens strain JA9 16S ribosomal RNA gene, partial sequence	2196	2196	98%	0.0	96.54%	KR072656.1
Bacillus methylotrophicus strain BHR3P2B1S 16S ribosomal RNA gene, partial sequence	2196	2196	98%	0.0	96.54%	KJ567098.2
Bacillus methylotrophicus strain IHB B 7249 16S ribosomal RNA gene, partial sequence	2196	2196	98%	0.0	96.54%	KJ767354.1

c. *Bacillus subtilis* AR3



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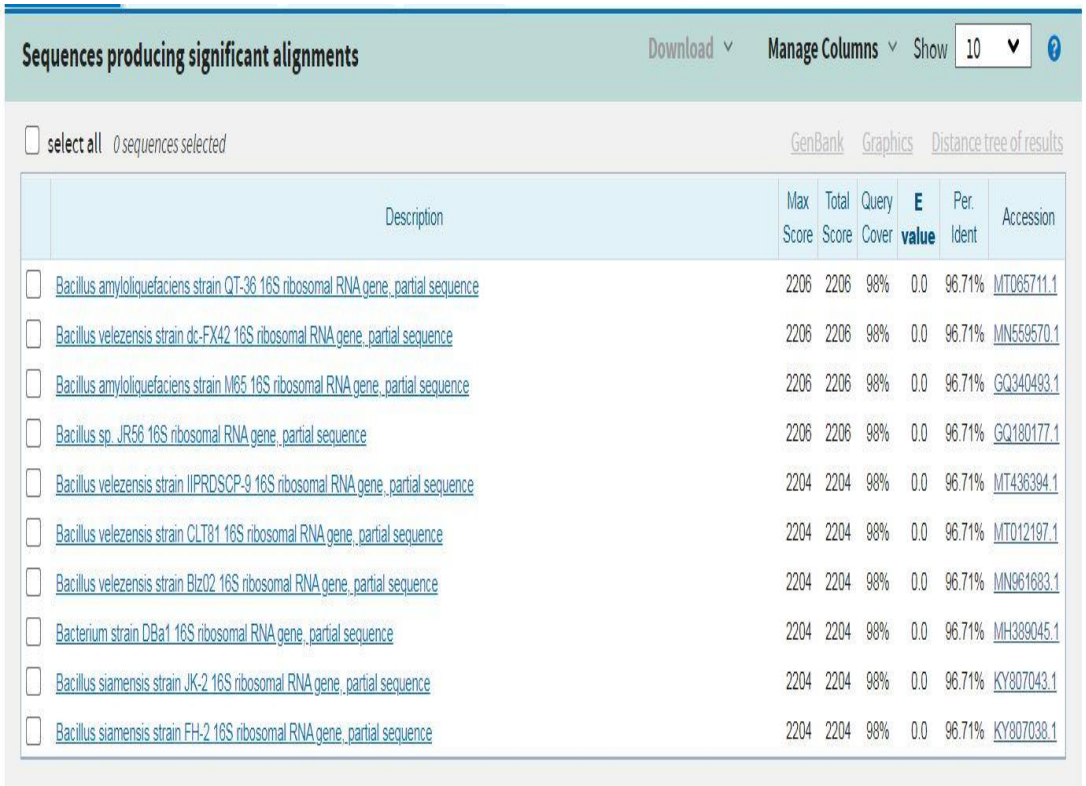
Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Bacillus subtilis strain S-11 16S ribosomal RNA gene, partial sequence	1013	1013	91%	0.0	98.12%	KF830999.1
Bacillus subtilis strain GB11 16S ribosomal RNA (rrnE) gene, partial sequence	1013	1013	91%	0.0	97.95%	EF101726.1
Bacillus subtilis strain JK0516S 16S ribosomal RNA gene, partial sequence	1009	1009	90%	0.0	98.44%	KF135457.1
Bacillus subtilis gene for 16S rRNA, partial sequence, strain: C2	1009	1009	91%	0.0	97.95%	AB905422.1
Bacillus subtilis strain GB10 16S ribosomal RNA (rrnE) gene, partial sequence	1007	1007	90%	0.0	98.27%	EF101725.1
Bacillus subtilis strain GB4 16S ribosomal RNA (rrnE) gene, partial sequence	1007	1007	90%	0.0	98.27%	EF101722.1
Bacillus subtilis strain GB14 16S ribosomal RNA (rrnE) gene, partial sequence	1005	1005	90%	0.0	98.27%	EF101729.1
Bacillus subtilis strain HU48 16S ribosomal RNA (rrnE) gene, partial sequence	1005	1005	91%	0.0	97.95%	EF101707.1
Bacillus subtilis strain X2 16S ribosomal RNA gene, partial sequence	1003	1003	90%	0.0	98.26%	MT373564.1
Bacillus sp. (in: Bacteria) strain JBT18S 16S ribosomal RNA gene, partial sequence	1003	1003	89%	0.0	98.43%	MH598524.1

d. *Bacillus amyloliquefaciens* GR1



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Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Bacillus amyloliquefaciens strain QT-36 16S ribosomal RNA gene, partial sequence	2206	2206	98%	0.0	96.71%	MT065711.1
Bacillus velezensis strain dc-FX42 16S ribosomal RNA gene, partial sequence	2206	2206	98%	0.0	96.71%	MN559570.1
Bacillus amyloliquefaciens strain M65 16S ribosomal RNA gene, partial sequence	2206	2206	98%	0.0	96.71%	GQ340493.1
Bacillus sp. JR56 16S ribosomal RNA gene, partial sequence	2206	2206	98%	0.0	96.71%	GQ180177.1
Bacillus velezensis strain IIPRDSCP-9 16S ribosomal RNA gene, partial sequence	2204	2204	98%	0.0	96.71%	MT436394.1
Bacillus velezensis strain CLT81 16S ribosomal RNA gene, partial sequence	2204	2204	98%	0.0	96.71%	MT012197.1
Bacillus velezensis strain Blz02 16S ribosomal RNA gene, partial sequence	2204	2204	98%	0.0	96.71%	MN961683.1
Bacterium strain DBa1 16S ribosomal RNA gene, partial sequence	2204	2204	98%	0.0	96.71%	MH389045.1
Bacillus siamensis strain JK-2 16S ribosomal RNA gene, partial sequence	2204	2204	98%	0.0	96.71%	KY807043.1
Bacillus siamensis strain FH-2 16S ribosomal RNA gene, partial sequence	2204	2204	98%	0.0	96.71%	KY807038.1

e. *Bacillus amyloliquefaciens* GR2

Sequences producing significant alignments Download Manage Columns Show 10

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Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Bacillus amyloliquefaciens strain I2ScCTM4 16S ribosomal RNA gene, partial sequence	2189	2189	97%	0.0	96.61%	MH985211.1
Bacillus sp. (in: Bacteria) strain V07 16S ribosomal RNA gene, partial sequence	2189	2189	97%	0.0	96.61%	MH820054.1
Bacillus subtilis strain WY-2 16S ribosomal RNA gene, partial sequence	2189	2189	97%	0.0	96.61%	KM884947.1
Bacillus amyloliquefaciens strain GC25 16S ribosomal RNA gene, partial sequence	2189	2189	97%	0.0	96.61%	KF158226.1
Bacillus amyloliquefaciens strain M34 16S ribosomal RNA gene, partial sequence	2189	2189	97%	0.0	96.61%	GQ340497.1
Bacillus sp. (in: Bacteria) strain V03 16S ribosomal RNA gene, partial sequence	2187	2187	97%	0.0	96.53%	MH820051.1
Bacillus siamensis strain JK-1 16S ribosomal RNA gene, partial sequence	2187	2187	97%	0.0	96.53%	KY807042.1
Bacillus amyloliquefaciens strain SSH100-3 16S ribosomal RNA gene, partial sequence	2187	2187	97%	0.0	96.61%	KU321525.1
Bacillus velezensis strain B268 chromosome, complete genome	2185	19621	97%	0.0	96.46%	CP053764.1
Bacillus velezensis strain UB2017 chromosome, complete genome	2185	19627	97%	0.0	96.46%	CP049741.1

f. *Bacillus amyloliquefaciens* GR3

Sequences producing significant alignments Download Manage Columns Show 10

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Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Bacillus amyloliquefaciens strain GC25 16S ribosomal RNA gene, partial sequence	2270	2270	98%	0.0	97.48%	KF158226.1
Bacillus sp. (in: Bacteria) strain 26LG 16S ribosomal RNA gene, partial sequence	2270	2270	98%	0.0	97.48%	MT762874.1
Bacillus velezensis strain IIPRDSCP-9 16S ribosomal RNA gene, partial sequence	2268	2268	98%	0.0	97.48%	MT436394.1
Bacillus velezensis strain QH16-28 16S ribosomal RNA gene, partial sequence	2268	2268	98%	0.0	97.48%	MT078625.1
Bacillus velezensis strain CLT81 16S ribosomal RNA gene, partial sequence	2268	2268	98%	0.0	97.48%	MT012197.1
Bacillus sp. (in: Bacteria) strain V07 16S ribosomal RNA gene, partial sequence	2268	2268	98%	0.0	97.48%	MH820054.1
Bacterium strain DBa1 16S ribosomal RNA gene, partial sequence	2268	2268	98%	0.0	97.48%	MH389045.1
Bacillus velezensis strain FME1 16S ribosomal RNA gene, partial sequence	2268	2268	98%	0.0	97.48%	KX821761.1
Bacillus velezensis strain FMP5 16S ribosomal RNA gene, partial sequence	2268	2268	98%	0.0	97.48%	KX821759.1
Bacillus siamensis strain FL23 16S ribosomal RNA gene, partial sequence	2268	2268	98%	0.0	97.48%	KY818941.1

g. *Bacillus* sp WR1

Sequences producing significant alignments Download Manage Columns Show 10

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Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Bacillus sp. strain 4N 16S ribosomal RNA gene, partial sequence	2237	2237	98%	0.0	97.13%	MF765317.1
Bacillus sp. (in: Bacteria) strain 7LG 16S ribosomal RNA gene, partial sequence	2237	2237	98%	0.0	97.13%	MT762864.1
Bacillus subtilis strain UYY 16S ribosomal RNA gene, partial sequence	2231	2231	98%	0.0	97.06%	MT613862.1
Bacillus subtilis subsp. stercoris strain SM-7 16S ribosomal RNA gene, partial sequence	2231	2231	98%	0.0	96.99%	MT377873.1
Bacillus subtilis strain CILBBTN607105190 16S ribosomal RNA gene, partial sequence	2231	2231	98%	0.0	97.06%	MT254858.1
Bacillus subtilis strain NBRINN3.2 16S ribosomal RNA gene, partial sequence	2231	2231	98%	0.0	97.06%	MK168627.1
Bacillus subtilis subsp. spizizenii strain EB7 16S ribosomal RNA gene, partial sequence	2231	2231	98%	0.0	97.06%	MK184212.1
Bacillus licheniformis KR7-12 gene for 16S rRNA, partial sequence	2231	2231	98%	0.0	97.06%	LC512758.1
Bacillus subtilis subsp. stercoris strain JCM 30051 16S ribosomal RNA gene, partial sequence	2231	2231	98%	0.0	97.06%	MN536904.1
Bacillus subtilis strain VT-V-4 16S ribosomal RNA gene, partial sequence	2231	2231	98%	0.0	97.06%	MN524177.1

h. *Bacillus amyloliquefaciens* WR2

Sequences producing significant alignments Download Manage Columns Show 10

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Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Bacillus sp. (in: Bacteria) strain MK08 16S ribosomal RNA gene, partial sequence	2268	2268	97%	0.0	97.87%	MN007100.1
Bacillus amyloliquefaciens strain Rab5 16S ribosomal RNA gene, partial sequence	2268	2268	97%	0.0	97.87%	MK782759.1
Bacillus sp. (in: Bacteria) strain V07 16S ribosomal RNA gene, partial sequence	2268	2268	97%	0.0	97.87%	MH820054.1
Bacillus amyloliquefaciens strain S29 16S ribosomal RNA gene, partial sequence	2268	2268	97%	0.0	97.87%	KX855957.1
Bacillus methylotrophicus strain EHF5 16S ribosomal RNA gene, partial sequence	2268	2268	97%	0.0	97.87%	KP851951.1
Bacillus subtilis strain WY-2 16S ribosomal RNA gene, partial sequence	2268	2268	97%	0.0	97.87%	KM884947.1
Bacillus vallismortis strain A7-8 16S ribosomal RNA gene, partial sequence	2268	2268	97%	0.0	97.87%	JF496368.1
Bacillus amyloliquefaciens strain HR62 16S ribosomal RNA gene, partial sequence	2268	2268	97%	0.0	97.87%	JF700435.1
Bacillus amyloliquefaciens strain SSH100-3 16S ribosomal RNA gene, partial sequence	2266	2266	97%	0.0	97.87%	KU321525.1
Bacillus sp. NE2 16S ribosomal RNA gene, partial sequence	2265	2265	97%	0.0	97.73%	KR868765.1

i. *Bacillus* sp AL1

Sequences producing significant alignments Download Manage Columns Show 10

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Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Bacillus sp. T4a 16S ribosomal RNA gene, partial sequence	2239	2239	98%	0.0	97.05%	JX155393.1
Bacillus tequilensis strain APPMB9 16S ribosomal RNA gene, partial sequence	2231	2231	98%	0.0	96.98%	MN640098.1
Bacillus sp. M110(2010) strain M110 16S ribosomal RNA gene, partial sequence	2231	2231	99%	0.0	96.84%	GQ340509.1
Bacillus subtilis strain SASCBT01 16S ribosomal RNA gene, partial sequence	2230	2230	98%	0.0	96.97%	KY921596.1
Bacillus subtilis strain MML5327 16S ribosomal RNA gene, partial sequence	2230	2230	98%	0.0	96.97%	MF687954.1
Bacillus sp. strain NAB37 16S ribosomal RNA gene, partial sequence	2230	2230	98%	0.0	96.97%	MF663661.1
Bacillus sp. K2DN334 16S ribosomal RNA gene, partial sequence	2230	2230	98%	0.0	96.97%	KT308214.1
Bacillus sp. RC29 16S ribosomal RNA gene, complete sequence	2230	2230	98%	0.0	96.97%	KJ669214.1
Bacillus licheniformis strain B5 16S ribosomal RNA gene, partial sequence	2228	2228	99%	0.0	96.77%	JF338810.1
Bacillus subtilis strain BS40-4 16S ribosomal RNA gene, partial sequence	2226	2226	98%	0.0	96.90%	MT348512.1

j. *Bacillus subtilis* AL2

Sequences producing significant alignments Download Manage Columns Show 10

select all *0 sequences selected* GenBank Graphics Distance tree of results

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Bacillus subtilis strain MES146 16S ribosomal RNA gene, partial sequence	2206	2206	98%	0.0	96.59%	MN960406.1
Bacillus subtilis strain BaBc-1 16S ribosomal RNA gene, partial sequence	2206	2206	98%	0.0	96.59%	MK254686.1
Bacillus subtilis strain JK1216S 16S ribosomal RNA gene, partial sequence	2206	2206	98%	0.0	96.59%	KF135464.1
Bacillus subtilis strain M10 16S ribosomal RNA gene, partial sequence	2202	2202	98%	0.0	96.59%	KY090785.1
Bacillus subtilis strain HU48 16S ribosomal RNA (rrnE) gene, partial sequence	2202	2202	98%	0.0	96.52%	EF101707.1
Bacillus subtilis strain AB7 16S ribosomal RNA gene, partial sequence	2200	2200	98%	0.0	96.52%	KP326364.1
Bacillus subtilis strain MSEB 34 16S ribosomal RNA gene, partial sequence	2200	2200	98%	0.0	96.39%	KP261061.1
Bacillus tequilensis strain RPB6 16S ribosomal RNA gene, partial sequence	2200	2200	98%	0.0	96.52%	MT772214.1
Bacillus sp. M101(2010) strain M101 16S ribosomal RNA gene, partial sequence	2200	2200	98%	0.0	96.52%	GQ340483.1
Bacillus subtilis strain BS40-4 16S ribosomal RNA gene, partial sequence	2198	2198	98%	0.0	96.52%	MT348512.1

k. *Bacillus* sp AL3

Sequences producing significant alignments Download Manage Columns Show 10

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Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Bacillus sp. RC29 16S ribosomal RNA gene, complete sequence	2239	2239	99%	0.0	96.84%	KJ669214.1
Bacillus subtilis strain DY15-1 16S ribosomal RNA gene, partial sequence	2237	2237	99%	0.0	96.77%	KU862331.1
Bacillus velezensis strain R-QL-101-34 16S ribosomal RNA gene, partial sequence	2235	2235	99%	0.0	96.76%	MT078641.1
Bacillus sp. M112(2010) strain M112 16S ribosomal RNA gene, partial sequence	2235	2235	99%	0.0	96.77%	GQ340478.1
Bacillus subtilis strain 2014-3557 chromosome, complete genome	2233	22265	99%	0.0	96.76%	CP045672.1
Bacillus subtilis subsp. stercoris strain EGI299 16S ribosomal RNA gene, partial sequence	2233	2233	99%	0.0	96.77%	MN704508.1
Bacillus subtilis strain P9_B1 chromosome, complete genome	2233	22281	99%	0.0	96.76%	CP045811.1
Bacillus subtilis strain P8_B3 chromosome, complete genome	2233	22281	99%	0.0	96.76%	CP045812.1
Bacillus subtilis strain P8_B1 chromosome, complete genome	2233	22281	99%	0.0	96.76%	CP045922.1
Bacillus subtilis strain MB8_B7 chromosome, complete genome	2233	22281	99%	0.0	96.76%	CP045821.1

l. *Bacillus amyloliquefaciens* GL1

Sequences producing significant alignments Download Manage Columns Show 10

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Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Bacillus amyloliquefaciens gene for 16S rRNA, partial sequence, strain: NCCP-49	2248	2248	99%	0.0	96.99%	AB547229.1
Bacillus sp. JR56 16S ribosomal RNA gene, partial sequence	2248	2248	99%	0.0	96.99%	GQ180177.1
Bacillus velezensis strain CLT81 16S ribosomal RNA gene, partial sequence	2246	2246	99%	0.0	96.99%	MT012197.1
Bacterium strain DBa1 16S ribosomal RNA gene, partial sequence	2246	2246	99%	0.0	96.99%	MH389045.1
Bacillus siamensis strain JK-2 16S ribosomal RNA gene, partial sequence	2246	2246	99%	0.0	96.99%	KY807043.1
Bacillus siamensis strain FH-2 16S ribosomal RNA gene, partial sequence	2246	2246	99%	0.0	96.99%	KY807038.1
Bacillus velezensis strain FME1 16S ribosomal RNA gene, partial sequence	2246	2246	99%	0.0	96.99%	KX821761.1
Bacillus velezensis strain FMP5 16S ribosomal RNA gene, partial sequence	2246	2246	99%	0.0	96.99%	KX821759.1
Bacillus siamensis strain FL23 16S ribosomal RNA gene, partial sequence	2246	2246	99%	0.0	96.99%	KY818941.1
Bacillus amyloliquefaciens strain ML456 16S ribosomal RNA gene, partial sequence	2246	2246	99%	0.0	96.99%	KC692174.1

m. *Bacillus* sp GL2

Sequences producing significant alignments — Download — Manage Columns — Show 10

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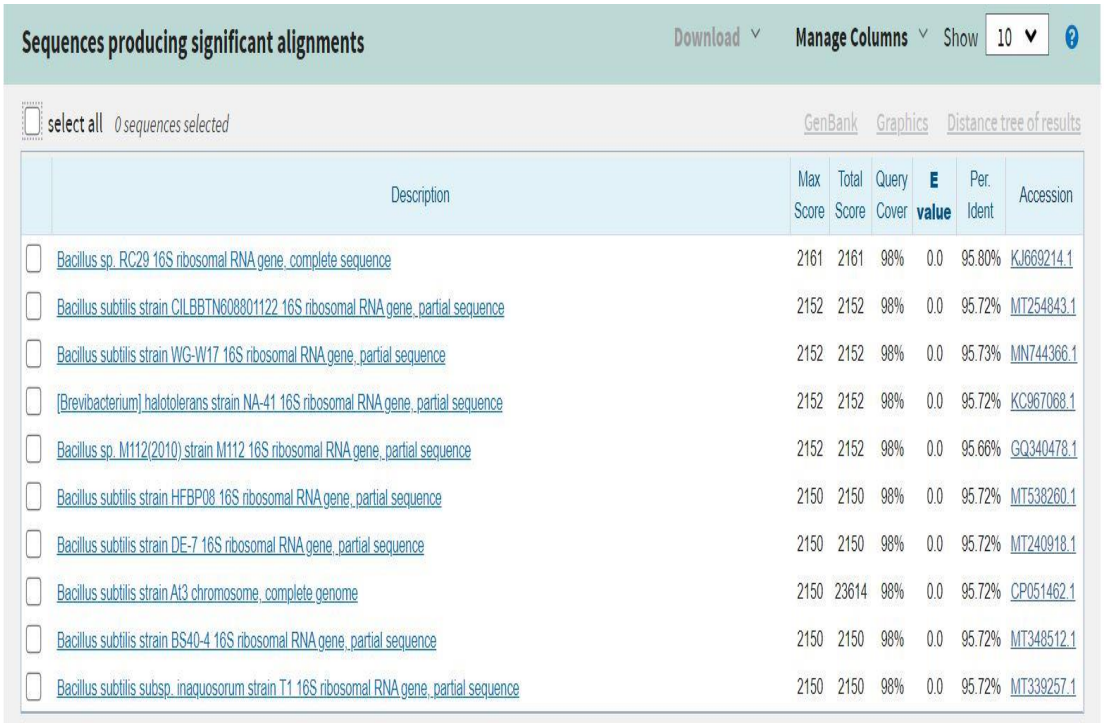
Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Bacillus sp. strain 4N 16S ribosomal RNA gene, partial sequence	2268	2268	98%	0.0	97.64%	MF765317.1
Bacillus sp. (in: Bacteria) strain 7LG 16S ribosomal RNA gene, partial sequence	2268	2268	98%	0.0	97.64%	MT762864.1
Bacillus subtilis subsp. stercoris strain SM-7 16S ribosomal RNA gene, partial sequence	2265	2265	98%	0.0	97.50%	MT377873.1
Bacillus subtilis strain RKMVC 79 16S ribosomal RNA gene, partial sequence	2263	2263	98%	0.0	97.56%	MF939144.1
Uncultured bacterium clone HAKV2 16S ribosomal RNA gene, partial sequence	2263	2263	99%	0.0	97.42%	KY924876.1
Uncultured bacterium clone HAKD3 16S ribosomal RNA gene, partial sequence	2263	2263	99%	0.0	97.42%	KY924875.1
Bacillus sonorensis strain T1Ni 16S ribosomal RNA gene, partial sequence	2263	2263	98%	0.0	97.56%	KY419149.1
Bacillus sonorensis strain N3 16S ribosomal RNA gene, partial sequence	2263	2263	99%	0.0	97.42%	KX890098.1
Bacillus tequilensis strain N1 16S ribosomal RNA gene, partial sequence	2263	2263	99%	0.0	97.42%	KX890096.1
Bacillus subtilis gene for 16S ribosomal RNA, partial sequence, strain: AECSB04	2263	2263	98%	0.0	97.56%	AB748943.1

n. *Bacillus* sp GL3



Sequences producing significant alignments — Download — Manage Columns — Show 10

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Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Bacillus sp. RC29 16S ribosomal RNA gene, complete sequence	2161	2161	98%	0.0	95.80%	KJ669214.1
Bacillus subtilis strain CILBBTN608801122 16S ribosomal RNA gene, partial sequence	2152	2152	98%	0.0	95.72%	MT254843.1
Bacillus subtilis strain WG-W17 16S ribosomal RNA gene, partial sequence	2152	2152	98%	0.0	95.73%	MN744366.1
[Brevibacterium] halotolerans strain NA-41 16S ribosomal RNA gene, partial sequence	2152	2152	98%	0.0	95.72%	KC967068.1
Bacillus sp. M112(2010) strain M112 16S ribosomal RNA gene, partial sequence	2152	2152	98%	0.0	95.66%	GQ340478.1
Bacillus subtilis strain HFBP08 16S ribosomal RNA gene, partial sequence	2150	2150	98%	0.0	95.72%	MT538260.1
Bacillus subtilis strain DE-7 16S ribosomal RNA gene, partial sequence	2150	2150	98%	0.0	95.72%	MT240918.1
Bacillus subtilis strain At3 chromosome, complete genome	2150	23614	98%	0.0	95.72%	CP051462.1
Bacillus subtilis strain BS40-4 16S ribosomal RNA gene, partial sequence	2150	2150	98%	0.0	95.72%	MT348512.1
Bacillus subtilis subsp. inaquosorum strain T1 16S ribosomal RNA gene, partial sequence	2150	2150	98%	0.0	95.72%	MT339257.1

o. *Bacillus subtilis* WL1

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Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Bacillus sp. (in: Bacteria) strain LB5 16S ribosomal RNA gene, partial sequence	2268	2268	98%	0.0	97.57%	MK774786.1
Uncultured Bacillus sp. clone OS106 16S ribosomal RNA gene, partial sequence	2268	2268	98%	0.0	97.57%	MF360159.1
Bacillus subtilis partial 16S rRNA gene, strain LX1	2268	2268	98%	0.0	97.57%	HE985180.1
Bacillus subtilis strain B15 16S ribosomal RNA gene, partial sequence	2266	2266	98%	0.0	97.50%	KJ870195.1
Bacillus subtilis strain 30P3-2 16S ribosomal RNA gene, partial sequence	2265	2265	98%	0.0	97.50%	JN366775.1
Bacillus tequilensis strain K74 16S ribosomal RNA gene, partial sequence	2263	2263	98%	0.0	97.50%	MN905086.1
Bacillus subtilis strain SSR17 16S ribosomal RNA gene, partial sequence	2263	2263	98%	0.0	97.49%	MH889071.1
Bacillus subtilis strain NIBSM OsG3 16S ribosomal RNA gene, partial sequence	2263	2263	98%	0.0	97.49%	KY930334.1
Bacillus subtilis strain NIBSM_OsS2 16S ribosomal RNA gene, partial sequence	2263	2263	98%	0.0	97.49%	KY927394.1
Bacillus subtilis strain NIBSM_OsS1 16S ribosomal RNA gene, partial sequence	2263	2263	98%	0.0	97.49%	KY927393.1

n. *Bacillus amyloliquefaciens* WL2

Sequences producing significant alignments Download Manage Columns Show 10

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Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Bacillus amyloliquefaciens gene for 16S rRNA, partial sequence, strain: NCCP-49	2261	2261	98%	0.0	97.48%	AB547229.1
Bacillus velezensis strain QH16-28 16S ribosomal RNA gene, partial sequence	2257	2257	98%	0.0	97.41%	MT078625.1
Bacillus velezensis strain CLT81 16S ribosomal RNA gene, partial sequence	2257	2257	98%	0.0	97.41%	MT012197.1
Bacterium strain DBa1 16S ribosomal RNA gene, partial sequence	2257	2257	98%	0.0	97.41%	MH389045.1
Bacillus velezensis strain FME1 16S ribosomal RNA gene, partial sequence	2257	2257	98%	0.0	97.41%	KX821761.1
Bacillus velezensis strain FMP5 16S ribosomal RNA gene, partial sequence	2257	2257	98%	0.0	97.41%	KX821759.1
Bacillus siamensis strain FL23 16S ribosomal RNA gene, partial sequence	2257	2257	98%	0.0	97.41%	KY818941.1
Bacillus methylotrophicus strain BHR3P2B1S 16S ribosomal RNA gene, partial sequence	2257	2257	98%	0.0	97.41%	KJ567098.2
Bacillus methylotrophicus strain IHB B 7249 16S ribosomal RNA gene, partial sequence	2257	2257	98%	0.0	97.41%	KJ767354.1
Bacillus amyloliquefaciens strain ML456 16S ribosomal RNA gene, partial sequence	2257	2257	98%	0.0	97.41%	KC692174.1

PLANT ASSOCIATED ENDOSPORE FORMING BACTERIA FROM AMARANTHUS AS GROWTH PROMOTERS AND BIOCONTROL AGENTS AGAINST RHIZOCTONIA LEAF BLIGHT

by

YASHASWINI M. S.

(2018-11-131)

ABSTRACT

Submitted in partial fulfilment of the

requirements for the degree of

MASTER OF SCIENCE IN AGRICULTURE

Faculty of Agriculture

Kerala Agricultural University

DEPARTMENT OF AGRICULTURAL MICROBIOLOGY

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM- 695 522

KERALA, INDIA

2020

ABSTRACT

The study entitled “Plant associated endospore forming bacteria from amaranthus as growth promoters and biocontrol agents against Rhizoctonia leaf blight”, was conducted during 2018-2020, in the Department of Agricultural Microbiology, College of Agriculture, Vellayani, Thiruvananthapuram, with the objective of isolation and characterization of endospore forming bacteria from endorhizosphere and phyllosphere of Amaranthus and evaluate their effect on growth promotion and disease suppression.

Endospore forming bacteria from endorhizosphere and phyllosphere were isolated from healthy roots and leaves of red amaranthus variety Arun, green amaranthus var. CO1 and wild relative (*Amaranthus viridis*) respectively by enrichment method on NA medium. A total of eight endospore forming bacterial isolates were obtained each from endorhizosphere and phyllosphere of the variety Arun, variety CO-1 and wild relative (*Amaranthus viridis*). All the isolates were identified as *Bacillus* spp. based on morphological, biochemical and molecular characterization. These isolates designated as AR1, AR2, AR3, GR1, GR2, GR3, WR1, WR2, AL1, AL2, AL3, GL1, GL2, GL3, WL1 and WL2 were identified as *Bacillus* sp., *Bacillus siamensis, Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus amyloliquefaciens, Bacillus amyloliquefaciens, Bacillus* sp., *Bacillus amyloliquefaciens, Bacillus* sp., *Bacillus subtilis, Bacillus* sp., *Bacillus amyloliquefaciens, Bacillus* sp., *Bacillus* sp., *Bacillus subtilis* and *Bacillus amyloliquefaciens* respectively based on 16S rRNA sequence.

Indole Acetic Acid production by the different endospore forming endorhizosphere bacterial isolates ranged from 4.09 to 9.73 µg mL^{-1} of culture filtrate. The isolate AR3 produced the maximum IAA of 9.73 µg mL^{-1} of culture filtrate. In roll towel assay significant increase in germination percentage, seedling shoot length, root length and seedling vigour index compared to control was observed when seeds were treated with the endospore forming endorhizosphere bacterial isolates. Isolate WR1 showed the maximum germination (43.33%) whereas control recorded a germination of

30%. Isolate WR1 showed maximum seedling vigour index (223.66) compared to the control (136.53). A portray experiment was also conducted to study the influence of endospore forming endorhizosphere bacterial isolates on plant growth parameters of amaranthus in the nursery stage. Highest root length was observed in the seedlings treated with isolate GR1 (4.76 cm) whereas the control recorded 3.91 cm**.** Shoot length was the maximum in seeds treated with WR1 (6.34 cm) whereas control recorded a shoot length of 5.59 cm. Maximum germination percentage of 100 was obtained in seeds treated with the isolate WR2. Significant influence of the bacterial isolates on seedling vigour index was observed wherein the isolate WR2 recorded maximum of 884.50. Highest root fresh weight was recorded in the plants treated with isolate GR1 (8.12 mg/plant) and the lowest root fresh weight of 3.56 mg/plant was recorded in control plants. It was found that the highest root dry weight was found in plants treated with isolate WR1 (2.99 mg/plant) as against the control (2.46 mg/plant).

Dual culture plate assay was carried out on Potato Dextrose Agar medium to check the ability of endospore forming phyllopshere bacterial isolates to inhibit *Rhizoctonia solani* causing foliar blight disease. All the isolates inhibited the mycelial growth of *Rhizoctonia solani*, of which GL3 and GL1 exhibited the maximum and minimum zone of inhibition (ZOI) of 8.73 and 1.00 mm respectively. All the endospore forming phyllopshere bacterial isolates tested for their indirect antagonism inhibited *Rhizoctonia solani* mycelial growth, of which isolate GL3 showed the maximum ZOI of 2.88 mm and the minimum was recorded by the isolate GL1 (0.50 mm). In detached leaf assay, significant difference was noticed in the lesion size appeared on the detached leaves treated with endospore forming phyllosphere bacterial isolates and water (control) on 3rd and 4th day after pathogen inoculation. Control exhibited maximum lesion area of 3.49 and 12.23 cm^2 and the minimum lesion area of 0.42 and 3.11 cm^2 was developed on the leaves treated with isolate WL2 on 3rd and 4th day after pathogen inoculation respectively.

Based on *in vitro* studies on plant growth promotion and antagonistic activity against *R. solani* the endospore forming endorhizosphere bacterial isolates AR1, AR2, GR1, GR2, WR1 and WR2 and endospore forming phyllosphere bacterial isolates - AL1, AL3, GL2, GL3, WL1 and WL2 were selected for further pot culture study.

A pot culture experiment was conducted to evaluate the efficacy of selected endospore forming endorhizosphere bacteria for their plant growth promoting activity in Amaranthus (var. Arun). Endospore forming endorhizosphere bacteria inoculated plants showed significant increase in biometric parameters such as number of leaves, number of branches, plant height and dry matter production compared to control. Plants treated with *Bacillus amyloliquefaciens* GR1 exhibited the maximum shoot fresh weight of 54.30 g/plant, whereas the control plants showed the minimum of 40.24 g/plant. Shoot dry matter production was maximum with plants treated with the isolate AR2 (6.74 g/plant) and the control plants recorded 4.76 g/plant. *Bacillus amyloliquefaciens* GR1 treated plants produced maximum root fresh weight of 13.64 g/plant, whereas the control plants had root fresh weight of 9.88 g/plant. Maximum root dry weight was exhibited by plants treated with *Bacillus amyloliquefaciens* GR1 (1.98 g/plant), lowest was produced by control plants with 1.37 g/plant.

Suppression of *Rhizoctonia* leaf blight in the variety Arun treated with selected endospore forming phyllosphere bacterial isolates was studied by challenge inoculation with the pathogen on intact leaves. The bacterial suspension containing bacterial cells of concentration 10^7 cfu ml^{-1} was sprayed on to the intact leaves of plants one week prior to pathogen inoculation. On 3rd day after pathogen inoculation, plants treated with *Bacillus* sp GL3 showed the minimum percent disease index of 11.80 with 32.92% disease suppression over the pathogen control, which was on par with the *Bacillus* sp AL3 with 11.81 percent disease index. No symptom development was observed in absolute control. On 5th day after pathogen inoculation, *Bacillus* sp AL3 treated plants showed the minimum percent disease index of 18.75 with 44.51% disease suppression over the pathogen inoculated control.

The present study revealed that seed bacterization of amaranthus plants with endospore forming endorhizosphere and foliar spray of phyllosphere bacterial isolates improved plant growth and suppressed *Rhizoctonia solani* leaf blight incidence respectively and helps in better establishment of plants. *Bacillus amyloliquefaciens* GR1 and *Bacillus* sp AL3 were selected as the best isolates for plant growth promotion and disease suppression respectively.

സംഗ്രഹം

റൈസോക്റ്റോണിയ ഇല വരൾച്ചയ്ക്കെതിരായി ചീരയിൽ നിന്ന് സസ്യ അനുബന്ധ എൻഡോസ്പോർ രൂപപ്പെടുന്ന ബാക്ടീരിയകൾ സസ്യ വളർച്ചാ പ്രൊമോട്ടർമാരും ബയോകൺട്രോൾ ഏജന്റുമാരുമായി" എന്ന തലക്കെട്ടിലുള്ള പഠനം 2018-2020 കാലയളവിൽ, വെള്ളായണി കാർഷിക കോളേജിലെ കാർഷിക മൈക്രോബയോളജി വിഭാഗത്തിൽ നടത്തി. വളർച്ചാ പ്രോത്സാഹനത്തിലും രോഗം അടിച്ചമർത്തലിലും ഉള്ള അവയുടെ സ്വാധീനം വിലയിരുത്തുക എന്നതായിരുന്നു പഠനത്തിന്റെ ലക്ഷ്യം.

ആരോഗ്യകരമായ വേരുകളിൽ നിന്നും എൻഡോറിസോസ്ഫിയർ, ഫൈലോസ്ഫിയർ എന്നിവയിൽ നിന്നും (ചുവന്ന ചീര ഇനമായ അരുൺ, പച്ച ചീര ഇനങ്ങളായ CO1, കാട്ടു ചീര (*അമരാന്തസ് വിരിഡിസ്*). എൻഎ) എന്ന മാധ്യമത്തിൽ സമ്പുഷ്ടീകരണ രീതി ഉപയോഗിച്ച് എൻഡോസ്പോർ രൂപപ്പെടുന്ന ബാക്ടീരിയകൾ ലഭിച്ചു. എല്ലാ ബാക്ടീരിയകളും മോർഫോളജിക്കൽ, ബയോകെമിക്കൽ, മോളിക്യുലർ ക്യാരക്ടറൈസേഷൻ അടിസ്ഥാനമാക്കി തിരിച്ചറിഞ്ഞു.

വ്യത്യസ്ത എൻഡോസ്പോർ രൂപീകരിക്കുന്ന ബാക്ടീരിയകളുടെ സംസ്ക്കരണ ഫിൽട്രേറ്റ് ഇൻഡോൾ അസറ്റിക് ആസിഡ് ഉൽപാദനം 4.09 മുതൽ 9.73 g mL-1 വരെ രേഖപ്പെടുത്തി. റോൾ ടവൽ പരിശോധനയിൽ വിത്തുപാകി മുളയ്ക്കുന്ന ശതമാനത്തിൽ ഗണ്യമായ വർദ്ധനവ്, ഷൂട്ട് ദൈർഘ്യം, വേര് നീളം, നിയന്ത്രണവുമായി താരതമ്യപ്പെടുത്തുമ്പോൾ എൻഡോറിസോസ്ഫിയർ ബാക്ടീരിയകൾക്കു രേഖപ്പെടുത്തി. നഴ്സറി ഘട്ടത്തിൽ ചീരയുടെ വളർച്ച പരാമീറ്ററുകളിൽ എൻഡോസ്പോർ രൂപപ്പെടുന്ന എൻഡോറിസോസ്ഫിയർ ബാക്ടീരിയകളുടെ സ്വാധീനം പഠിക്കുന്നതിനായി ഒരു പോർട്രേ പരീക്ഷണവും നടത്തി. തൈകളിൽ ബാക്ടീരിയ ഇൻസുലേറ്റുകളുടെ ഗണ്യമായ സ്വാധീനം കണ്ടെത്തി, അതിൽ ബാക്ടീരിയ ഡബ്ല്യുആർ 2 പരമാവധി രേഖപ്പെടുത്തി.

റൈസോക്റ്റോണിയ തടയുന്നതിനായി എൻഡോസ്പോർ രൂപപ്പെടുന്ന ഫിലോപ്ഷിയർ ബാക്ടീരിയൽ ഇൻസുലേറ്റുകളുടെ കഴിവ് പരിശോധിക്കുന്നതിനായി പിഡിഎ എന്ന മാധ്യമത്തിൽ ഇരട്ട കൾച്ചർ പ്ലേറ്റ് പരിശോധന നടത്തി. എല്ലാ ഇൻസുലേറ്റുകളും റൈസോക്റ്റോണിയ സോളാനിയുടെ വളർച്ചയെ തടഞ്ഞു, അതിൽ ജിഎൽ3 (8.73 മില്ലിമീറ്റർ), ജിഎൽ 1 (1.00 മില്ലിമീറ്റർ) എന്നിവ യഥാക്രമം, പരമാവധി, കുറഞ്ഞ ഇൻഹിബിഷന്റെ മേഖലയെ പ്രദർശിപ്പിച്ചു. വേർതിരിച്ച ഇല പരിശോധനയിൽ, എൻഡോസ്പോർ ഉപയോഗിച്ച് ചികിത്സിച്ച വേർതിരിച്ച ഇലകളിൽ കാര്യമായ വ്യത്യാസം കണ്ടെത്തി, രോഗകാരി കുത്തിവയ്പ്പിനു ശേഷം 3, 4 ദിവസങ്ങളിൽ ഫൈലോസ്ഫിയർ ബാക്ടീരിയൽ ഇൻസുലേറ്റുകളും വെള്ളവും (നിയന്ത്രണം) രൂപം കൊള്ളുന്നു. രോഗകാരി കുത്തിവയ്പ്പിനു ശേഷം യഥാക്രമം 3, 4 ദിവസങ്ങളിൽ ഒറ്റപ്പെട്ട ഡബ്ല്യുഎൽ 2 ഉപയോഗിച്ച് ചികിത്സിച്ച ഇലകളിൽ ഏറ്റവും കുറഞ്ഞ നിഖേദ് പ്രദേശവും വികസിപ്പിച്ചെടുത്തു. പോട്ട് കൾച്ചർ പഠനത്തിനായി ഡബ്ല്യുഎൽ2 തിരഞ്ഞെടുത്തു.

തിരഞ്ഞെടുത്ത എൻഡോസ്പോർ രൂപീകരിക്കുന്ന എൻഡോറിസോസ്ഫിയർ ബാക്ടീരിയയുടെ ഫലപ്രാപ്തി വിലയിരുത്തുന്നതിന് ഒരു പോട്ട് കൾച്ചർ പരീക്ഷണം നടത്തി. എൻഡോസ്പോർ രൂപപ്പെടുന്ന എൻഡോറിസോസ്ഫിയർ ബാക്ടീരിയ കുത്തിവച്ചുള്ള സസ്യങ്ങൾ ബയോമെട്രിക് പാരാമീറ്ററുകളിൽ (ഇലകളുടെ എണ്ണം, ശാഖകളുടെ എണ്ണം, ചെടികളുടെ ഉയരം, വരണ്ട വസ്തുക്കളുടെ ഉൽപാദനം) നിയന്ത്രണവുമായി താരതമ്യപ്പെടുത്തുമ്പോൾ ഗണ്യമായി വർദ്ധിച്ചു.

തിരഞ്ഞെടുത്ത എൻഡോസ്പോർ ഉപയോഗിച്ച് ഫിലോസ്ഫിയർ ബാക്ടീരിയ ഉപയോഗിച്ച് ചികിത്സിച്ച അരുൺ ഇനത്തിലുള്ള റൈസോക്റ്റോണിയ ഇല വരൾച്ചയെ അടിച്ചമർത്തുന്നത് ഇലകളിലെ രോഗകാരിയുമായി ചലഞ്ച് കുത്തിവയ്പ്പിലൂടെയാണ്. ബാക്ടീരിയ കോശങ്ങൾ അടങ്ങിയ ബാക്ടീരിയ സസ്പെൻഷൻ രോഗകാരി കുത്തിവയ്പ്പിന് ഒരാഴ്ച മുമ്പ് സസ്യങ്ങളുടെ കേടുകൂടാത്ത ഇലകളിലേക്ക് തളിച്ചു. ശേഷം മൂന്നാം ദിവസം, *ബാസിലസ്*

എസ്പി ജിഎൽ 3 ഉപയോഗിച്ച് ചികിത്സിച്ച സസ്യങ്ങൾ ഏറ്റവും കുറഞ്ഞ ശതമാനം രോഗ സൂചിക 11.80 കാണിക്കുന്നു, രോഗകാരി നിയന്ത്രണത്തെക്കാൾ 32.92 ശതമാനം രോഗം അടിച്ചമർത്തുന്നു, ഇത് 11.81 ശതമാനം രോഗ സൂചികയുമായി *ബാസിലസ്* എഎൽ 3 ന് തുല്യമാണ്. സമ്പൂർണ്ണ നിയന്ത്രണത്തിൽ രോഗലക്ഷണ വികസനമൊന്നും കണ്ടെത്തിയില്ല.

എന്റോസ്പോർ രൂപപ്പെടുന്ന *ബാസിലസ് അമിലോളിക്വേസിയൻസ്* ജിആർ 1, *ബാസിലസ്* എസ്പി എഎൽ3 ചീരസസ്യങ്ങളുടെ വിത്ത് ബാക്ടീരിയവൽക്കരണവും ഫിലോസ്ഫിയർ ബാക്ടീരിയൽ ഇൻസുലേറ്റുകളുടെ സസ്യജാലങ്ങളുടെ തളിക്കലും സസ്യങ്ങളുടെ വളർച്ച മെച്ചപ്പെടുത്തുകയും *റൈസോക്ടോണിയ സൊളാനി* ഇല വരൾച്ചയെ യഥാക്രമം അടിച്ചമർത്തുകയും സസ്യങ്ങളെ മികച്ച രീതിയിൽ സ്ഥാപിക്കാൻ സഹായിക്കുകയും ചെയ്യുന്നു ഇപ്പോഴത്തെ പഠനം വെളിപ്പെടുത്തുന്നത്.