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PLANT PATHOLOGY

Efficacy of *Bacillus subtilis* G-1 in suppression of stem rot caused by *Sclerotium rolfsii* and growth promotion of groundnut

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ABSTRACT

A total of seven biocontrol agents with known antifungal activity against other soilborne fungal pathogens were screened for their antagonistic potential against *Sclerotium rolfsii* Sacc, the causal agent of stem rot disease of groundnut (*Arachis hypogaea* L.) by dual culture assay. Among the various biocontrol agents tested *Bacillus subtilis* strain G-1 was the most effective in inhibiting the mycelial growth of *S. rolfsii* and recorded an inhibition of 28%. Groundnut seeds when treated with *B. subtilis* G-1 showed significant increases in root length, shoot length and seedling vigour. A talc-based powder formulation of the highly effective strain, *B. subtilis* G-1, was developed and its efficacy in controlling groundnut stem rot was determined under greenhouse conditions. The results indicated that seed treatment with the powder formulation of *B. subtilis* G-1 alone effectively reduced the incidence of stem rot and increased the pod yield; but combined application through seed and soil increased the efficacy. Seed treatment and soil application with *B. subtilis* G-1 reduced the stem rot incidence from 80 per cent (with non-bacterized seeds) to 5 per cent. When the treated seeds were sown in soil, the antagonist moved to the rhizosphere and multiplied well in it. These results suggest that *B. subtilis* G-1 is an effective bioagent against stem rot of groundnut. Further studies are required to assess its efficacy in controlling stem rot of groundnut under field conditions.

Highlights

- *Bacillus subtilis* strain G-1 has good potential as a microbial agent for biological control of stem rot of groundnut caused by *Sclerotium rolfsii*.

Keywords: *Arachis hypogaea, Sclerotium rolfsii*, stem rot, biological control, *Bacillus subtilis*

Sclerotium rolfsii Sacc, the causal agent of groundnut stem rot, is an important soilborne pathogen in many areas of the world where groundnut (*Arachis hypogaea* L.) is grown. The fungus infects lower stems of groundnut, which are in contact with the soil as well as pegs, pods and roots. Infected plants show wilting of one or few branches initially, but the whole plant may wilt and die within few weeks of infection. Whitish fungal mycelium and light-to-dark brown sclerotia appear on the soil surface and diseased plant tissues (Linderman and Gilbert 1973, Punja and Rahe 1992). High soil moisture, denser plant stands and frequent irrigation favour infection and fungal mycelial spread within and between plants (Coley-Smith and Cooke 1971, Punja 1985, Punja and Rahe 1992, Sconyers *et al*., 2005). Control of this

pathogen is difficult as it produces sclerotia which overwinter in soil and on plant debris and emerge as inoculum and cause disease during the following season (Punja 1988). The fungus once established in the soil is very difficult to eliminate. Presently, there are no commercial groundnut cultivars that are resistant to stem rot. Cultural methods including crop rotation with non-host for *S. rolfsii*, deep ploughing and non-dirting cultivation provides only partial control of stem rot (Garren 1961). Fungicides are widely used for the management of stem rot of groundnut (Hagan *et al.*, 1986, Hagan *et al.*, 1988, Csinos 1989, Grichar 1995). But the ill effects of synthetic fungicides on the environment and their escalating cost, development of resistant mutants of pathogens and frequent breakdown of resistant cultivars strongly demand a sustainable and an alternative management approach to control crop diseases. Biological control of plant diseases has been studied extensively as an alternative to chemical control. Several microorganisms such as *Pseudomonas* spp. (Karthikeyan *et al.*, 2006), *Trichoderma harzianum* (Cilliers *et al.*, 2003), and *Streptomyces* spp. (Adhilakshmi *et al.*, 2014) have been identified as effective biocontrol agents against *S. rolfsii*. The objectives of this research were to examine biocontrol strains with known antifungal activity against other soilborne fungal pathogens for their antifungal activity against *S. rolfsii in vitro* and to test their efficacy in controlling stem rot of groundnut under greenhouse conditions.

Materials and Methods

Microbial cultures

The fungus, *S. rolfsii* was isolated from stem rot infected groundnut plants and maintained on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) medium under laboratory conditions. The antagonistic organisms viz., *Bacillus subtilis* G-1, *Bacillus subtilis* EPCO 8, *Bacillus amyloliquefaciens* B2, *Streptomyces* sp. ANR, *Streptomyces* sp. PDK, *Streptomyces* sp. SA and *Pseudomonas fluorescens* Pf1 were obtained from the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, India. The bioinoculants viz. *Bacillus megaterium* var *phosphaticum* strain PBS, *Rhizobium* strain BMBS, *Azospirillum brasiliense* strain 204 and *Azotobacter chrooccocum* strain AC1 were obtained from the Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India.

In vitro screening of antagonists against S. rolfsii

The bacterial and actinomycete isolates were tested for their *in-vitro* antagonistic activity against *S. rolfsii* by dual culture technique (Dennis and Webster 1971). The antagonists were streaked on one side of a Petri dish containing PDA medium at 1 cm from the edge of plate. The mycelial disc (8-mm-dia) taken from the margin of 5-day-old cultures of *S. rolfsii* was placed on the opposite side in the Petri dish perpendicular to the antagonist. The plates were incubated at room temperature (28± 2°C) for 6-7 days. The percent inhibition of growth of the test pathogen was calculated using the following formula:

$$I = \frac{C - T}{C} * 100$$

Where; I =percentage of inhibition, C = radial growth of the pathogen in control and T =radial growth of pathogen in treatment.

Efficacy of antagonists on seed germination and seedling vigour

The antagonists viz., *B. subtilis* G-1, *B. subtilis* EPCO8 and *B. amyloliquefaciens* B2 which inhibited the growth of *S. rolfsii* in dual culture assay were evaluated for their effect on seed germination and seedling vigour. The bacterial isolates were grown on nutrient broth with constant shaking at 150 rpm for 48 h at room temperature (28±2°C). The bacterial cells were harvested by centrifugation at 6,000 rpm for 15 min and the pellet was suspended in 0.01 M phosphate buffer (pH 7.0. The final concentration of the suspension was adjusted to approximately 10^8 CFU/ml (OD595 = 0.3) in a spectrophotometer and used as inoculum (Thompson 1996). Groundnut seeds (TMV 7) were soaked in the bacterial suspension for 3 min and dried in shade for 2 h.

The plant growth promoting activity of the bacterial antagonists was assessed based on the seedling vigour following the standard roll towel method (International Seed Testing Association 1996). The treated seeds were placed on coarse blotter paper sheets and covered with a moistened blotter and rolled. The roll was kept on a butter paper sheet and rolled as a bundle, and incubated in a growth chamber at 25°C. Five replications were maintained for each treatment. The root and shoot lengths of seedlings were measured and the germination percentage was calculated after 10 days. The vigour index was calculated by multiplying percent plant stand with the sum of shoot length and root length (Baki and Anderson 1973).

Compatibility tests

The bacterial antagonist viz., *B. subtilis* G-1 which showed the highest mycelial growth inhibition and plant growth promoting activity was used for further studies. *B. subtilis* G-1 was tested *in vitro* for its compatibility with other beneficial soil inoculants by cross-streak assay on nutrient agar medium. *B. subtilis* G-1 was streaked as a strip at one end of the Petri plate and incubated for 24 h at room temperature (28±2°C). The test strains viz. *Bacillus megaterium* var *phosphaticum* strain PBS, *Rhizobium* strain BMBS, *Azospirillum brasiliense* strain 204 and *Azotobacter chrooccocum* strain AC1 were streaked on the Petri plate perpendicular to *B. subtilis*. The plates were incubated further for 48 h at (28±2°C) and observed for the growth inhibition.

Development of formulation of B. subtilis G-1

A loopful of *B. subtilis* G-1 was inoculated into the nutrient broth and incubated in a rotary shaker at 150 rpm for 48 h at room temperature (28±2°C). After 48 h of incubation, the broth containing 9×10^8 cfu/ml was used for the preparation of talc-based formulation. To the 400 ml of bacterial suspension, 1 kg of the sterile talc powder, 15 g of calcium carbonate and 10 g of carboxymethyl cellulose (CMC) were added and mixed under sterile conditions (Vidhyasekaran and Muthuamilan 1995). The product was shade dried to reduce the moisture content to 35% and then packed in white polypropylene bag and sealed. The prepared formulation was tested for its ability to suppress stem rot of groundnut under greenhouse conditions. At the time of application, the population of bacteria in the talc-based powder formulation was 2.5×10^8 cfu/g.

Greenhouse studies

The stem rot susceptible groundnut cultivar, cv. TMV7 (Bunch type; duration 115–120 days) obtained from the Department of Oilseeds, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India was used. The fungus, *S. rolfsii*, was multiplied in sand-maize medium (Riker and Riker 1936) for 15 days and the sand–maize inoculum was mixed with the sterilised soil in the ratio of 1:19 in polyethylene bags. The polyethylene bags were shaken vigorously to ensure uniform distribution of the inoculum. Earthen pots (30 cm diameter) were filled up with 5 kg of infested soil and arranged on the greenhouse benches. The pots were incubated for three days before planting. Seeds were treated with the powder formulation of *B. subtilis* at the rate of 10 g/kg of seeds and the treated seeds were sown in the infested soil (Ruark and Shew 2010). Five groundnut seeds were planted in each pot. In another set of pots, seed treatment was followed by soil application of talc-based powder formulation at the rate of 5g/ pot at the time of sowing. Seeds mock-treated with the talc powder formulation without *B. subtilis* G-1 were kept as control. Carbendazim (0.2%) was used as a check. Each pot served as a replicate and each treatment was replicated five times. The initial population of *B. subtilis* G-1 in the soil was determined by dilution plating immediately following the treatment. The percentage of stem rot incidence was recorded 25 days after sowing. The experiment was repeated three times.

Population density assays of B. subtilis G-1 in the rhizosphere

The rhizosphere population of *B. subtilis* G-1 was assessed at different time intervals. Groundnut plants from each treatment were pulled out gently with roots intact and root portions were cut in to

small bits. All root bits with adhering soil particles were thoroughly mixed, weighed and transferred to 100 ml of sterile distilled water and shaken for 30 min on a rotary shaker. After thorough shaking the population of *B. subtilis* G-1 in the suspension was estimated by dilution plate method.

Statistical analysis

The completely randomized design was used for the laboratory and greenhouse experiments. Arc sine transformation of data on percentage of stem rot incidence was done and Duncan's multiple range test (DMRT) was first applied to the transformed values and then transferred to the original means (Gomez and Gomez 1984). The data were analyzed using SAS statistical software version 9.2 (SAS Institute, Inc., Cary, NC).

Table 1. *In vitro* evaluation of biocontrol agents against S. *rolfsii* by dual culture technique

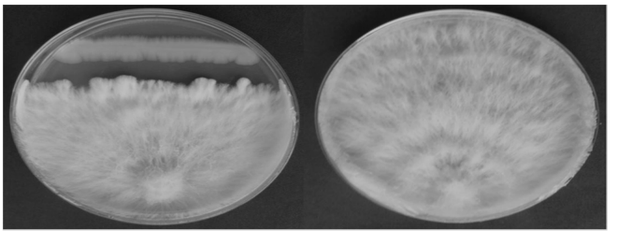
Biocontrol agents	Mycelium growth (cm)	Mean inhibition (%)
Bacillus subtilis G-1	6.50	28.0 (31.95)[a]
Bacillus subtilis EPCO8	6.65	26.0 (30.66)[a]
Bacillus amyloliquefaciens B2	6.55	27.0 (31.31)[a]
Streptomyces sp. ANR	8.85	1.7 (5.16)[b]
Streptomyces sp. PDK	9.00	0 (1.28)[b]
Streptomyces sp. SA	9.00	0 (1.28)[b]
Pseudomonas fluorescens Pf1	9.00	0 (1.28)[b]
Control	9.00	0 (1.28)[b]

The data are mean of three replications.
Values in the parenthesis are arcsine transformed values
Means within a column followed by a common letter are not significantly different (p=0.05) by DMRT.

Results

In vitro antagonistic activity

A total of seven bio-control agents were tested for their efficacy in suppressing mycelial growth of *S. rolfsii in vitro* in dual culture assay. Among the various bio-control agents tested, *B. subtilis* G-1, *B. amyloliquefaciens* B2 and *B. subtilis* EPCO 8 were found effective in inhibiting the mycelial growth of *S. rolfsii* with mean percentage inhibition of 28, 27 and 26 respectively (Table 1). The *in vitro* antifungal activity of *B. subtilis* G-1 against *S. rolfsii* is shown in Figure 1.



B. subtilis G-1 Control

Figure 1. *In vitro* antifungal activity of *B. subtilis* G-1 against *Sclerotium rolfsii*

Plant growth promoting activity

Groundnut seeds when treated with different bio-control agents showed significant increases in seed germination, root length, shoot length and seedling vigour (Table 2). Among the five bio-control agents tested, *B. subtilis* G-1 was the most effective in promoting plant growth followed by *B. amyloliquefaciens* B2 and *B. subtilis* EPCO 8. The *B. subtilis* G-1 recorded a vigour index of 3735; whereas *B. amyloliquefaciens* B2 and *B. subtilis* EPCO 8 recorded vigour index of 3420 and 3241 respectively. Untreated control seeds recorded seedling vigour of 2650.

The compatibility tests with other bio-agents revealed that *B. subtilis* G-1 was compatible with other beneficial rhizobacteria including *Bacillus megaterium* var *phosphaticum* strain PBS, *Rhizobium* strain BMBS, *Azospirillum brasiliense* strain 204 and *Azotobacter chrooccocum* strain AC1 (Data not shown).

Pot experiment

B. subtilis G-1 was selected based on its *in vitro* antagonism on dual plate technique and plant growth promoting activity for further studies. A talc-based formulation of *B. subtilis* G-1 was prepared and tested for its efficacy in controlling stem rot of groundnut under greenhouse conditions. The results of the greenhouse experiments showed that

inoculation of *S. rolfsii* in groundnut caused 80% stem rot disease incidence. Seed treatment or soil application of powder formulation of *B. subtilis* G-1 significantly reduced the incidence of stem rot and increased the plant height (Table 3). Seed treatment with the powder formulation of *B. subtilis* G-1 alone was effective in controlling stem rot disease compared to control; but combined application through seed and soil increased the efficacy. Maximum reduction in the disease incidence and enhancement of the plant height were noticed in pots treated with B. *subtilis* G-1 through seed and soil. Seed treatment and soil application with *B. subtilis* G-1 recorded the stem rot incidence of 5 percent whereas in control, it was 80 percent. Control of stem rot with application of *B. subtilis* G-1 by seed treatment and soil application was not statistically different from that obtained with seed treatment and soil application with carbendazim (Table 3). Seed treatment and soil application with the powder formulation of *B. subtilis* G-1 significantly increased the pod yield besides controlling stem rot disease.

Table 2 Effect of seed treatment with biocontrol agents on seed germination and seedling vigour of groundnut

Biocontrol agents	Germination (%)	Shoot length (cm)	Root length (cm)	Vigour index
B. *subtilis* G-1	99(86.16)a	20.3a	17.4a	3735a
B. amyloliquefaciens B2	99(86.16)a	18.6b	16.0ab	3420ab
B. subtilis EPCO 8	98 (83.6)a	18.4b	14.7b	3241b
Streptomyces sp. ANR	96(83.15)a	18.3b	12c	2859c
Control	95(80.6)a	15.7c	11.9c	2650c

The data are mean of five replications.
Values in the parenthesis are arcsine transformed values
Data followed by the same letter in a column are not significantly different (p = 0.05) from each other according to DMRT.

The population of *B. subtilis* G-1 in the rhizosphere of groundnut was assessed at different time intervals. When the groundnut seeds were treated with the powder formulation of *B. subtilis* G-1 and sown, the bacteria multiplied well in the rhizosphere and the rhizosphere population increased with increase in the age of the crop (Table 4).

Discussion

Several strains of *Bacillus subtilis* are known to suppress plant pathogens and improve plant health

Table 3 Efficacy of *Bacillus subtilis* strain G-1 in stem rot management and yield enhancement of groundnut under greenhouse conditions

Treatments	Disease incidence (%)	% reduction over control	Plant height (cm)	% increase over control	Pod yield (g/pot)	% increase over control
Soil application (SA) with *B. subtilis* (5g/pot)	30 (32.9)^{b}	62.50	67.5^{c}	7.1	101.25^{cd}	278.5
Seed treatment (ST) with *B. subtilis* (10g/kg)	7.5 (11.3)^{bc}	90.63	71.75^{b}	13.88	104.5^{b}	290.65
ST + SA with *B. subtilis*	5 (6.6)^{c}	93.75	74.5^{a}	18.25	114.5^{a}	328.03
Soil application with Carbendazim (0.2%)	20 (23.1)^{bc}	75.00	68^{c}	7.9	100^{d}	273.83
Seed treatment Carbendazim (2g/kg)	30 (32.9)^{b}	62.50	68.25^{c}	8.3	102.5^{c}	283.17
SA + ST with Carbendazim	13.8 (19)^{bc}	83.13	72.5^{b}	15.1	113.5^{a}	324.29
Control	80 (66.6)^{a}		63^{d}		26.75^{e}	

The data are mean of five replications.
Stem rot incidence was recorded 25 days after sowing.
Values in the parenthesis are arcsine transformed values

Means within a column followed by a common letter are not significantly different (p=0.05) by DMRT.

(Leifert *et al.*, 1995, Collins *et al.*, 2003, McSpadden Gardener 2004, Toure *et al.*, 2004, Jayaraj *et al.*, 2005, Hu *et al.*, 2014, Khabbaz and Abbasi 2014, Zhao *et al.*, 2014). *Bacillus* spp. are capable of growing in diverse

Table 4 Survival of B. subtilis G-1 in groundnut rhizosphere after application through seed and soil

Treatment	Rhizosphere population (10^5 cfu/g)		
	30 DAS	60 DAS	90 DAS
Soil application (SA) with *B. subtilis*	18. 0b	28.0c	34.3c
Seed treatment (ST) with *B. subtilis*	18.3b	31.7b	37.3b
SA + ST	23.3a	41.7a	45.0a
Control	0.0c	0.0d	0.0d

Data are mean of five replications.
Data followed by the same letter in a column are not significantly different (p = 0.05) by DMRT.

environments due to the production of endospores that can tolerate extreme pH, temperature, and osmotic conditions; therefore, they offer several advantages over other antagonistic microorganisms (Earl *et al.*, 2008). A number of *B. subtilis* strains have been integrated successfully into several pest management programs (Jacobsen *et al.*, 2004). A number of commercial products based on *B. subtilis* including Kodiak (Gufstafson Biologicals, Plano, TX), Serenade (Agraquest Inc., Davis, CA), Subtilex (Becker Underwood, Ames, IA) have been developed for the control of various plant diseases (Schisler *et al.*, 2004). *B. subtilis* is known to rapidly colonize plant roots and has the capacity to multiply on the roots (Dijkstra *et al.*, 1987). It remains close to the root tip by passive displacement on the elongating cells. *B. subtilis* produce more than two dozen structurally diverse antifungal and antibacterial compounds (Stein 2005). Furthermore several strains of *Bacillus* sp. are known to induce systemic resistance by producing volatile organic compounds (Ryu *et al.*, 2004) and to promote plant and root growth through the production of phytohormones and extracellular enzymes (Yao *et al.*, 2006, Forchetti *et al.*, 2007, Lee *et al.*, 2008, Swain and Ray 2009, Lahlali *et al.*, 2013). In the present study, it was observed that among the various antagonists tested in vitro, *B. subtilis* G-1 was the most effective in inhibiting the growth of *S. rolfsii in vitro*. Groundnut seeds when treated with *B. subtilis* G-1 showed significant increases in per cent germination, root length, shoot length and seedling vigour.

Seed treatment or soil application of talc-based powder formulations of *B. subtilis* G-1 significantly increased the plant height and reduced the incidence of stem rot. The antagonist when applied through seed and soil reduced the stem rot incidence up to 93% under greenhouse conditions and its effects were equal to or greater than those achieved with the commercial fungicide. It is possible that the physiological alterations induced in groundnut due to the plant growth promoting substances like auxins (Cameco *et al.*, 2001) produced by the *B. subtilis* might have resulted in increased plant height. These results suggest that *B. subtilis* strain G-1 is an effective biocontrol agent against *S.rolfsii*.

When the groundnut seeds were treated with the powder formulation of *B. subtilis* G-1 and sown, the bacteria multiplied well in the rhizosphere and the rhizosphere population increased with increase in the age of the crop. The increase in population of *B. subtilis* G-1 indicates the potential of the antagonist to provide effective and long-lasting protection against stem rot of groundnut.

The compatibility tests with other bio-agents revealed that *B. subtilis* G-1 was compatible with other beneficial rhizobacteria including *Bacillus megaterium* var *phosphaticum* strain PBS, *Rhizobium* strain BMBS, *Azospirillum brasiliense* strain 204 and *Azotobacter chrooccocum* strain AC1. This *B. subtilis* strain G-1 may have potential use in the integrated management of *S. rolfsii* in groundnut. It has been reported that the biocontrol agents that are effective in greenhouse bioassays do not perform similarly under field conditions (Lewis *et al.*, 1993; Jones and Samac 1996). Various factors including chemical and physical properties of the soil, weather conditions, host plant species, presence of non-target plant pathogens, and interactions with other soil microflora and fauna influence the ability of applied biocontrol agents to colonize, multiply, disperse, produce necessary compounds, or parasitize plant pathogens

(Weller 1988). Hence, rigorous evaluation under field conditions in hot spot areas and extensive studies on its biology will be required.

Conclusion

The bacterial antagonist, *Bacillus subtilis* G-1 significantly controlled stem rot disease of groundnut and increased the plant growth under greenhouse conditions. This strain warrants further investigation for its ability to control stem rot and other soil-borne diseases of groundnut under field conditions. The antagonistic activity of *B. subtilis* G-1 is likely due to volatile and diffusible metabolites. Further research on the field efficacy and mode of action of this strain is in progress.

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