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DISEASE CONTROL

Assessing the prospects of *Streptomyces* sp. RP1A-12 in managing groundnut stem rot disease caused by *Sclerotium rolfsii* Sacc

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Abstract Stem rot of groundnut caused by the soilborne pathogen *Sclerotium rolfsii* can cause significant yield losses. Biological control of stem rot using actinomycetes is a viable alternative to existing fungicidal management. Though actinomycetes are prolific antibiotic producers, reports pertaining to their use in groundnut disease management are limited. Here, actinomycetes were isolated from groundnut rhizospheric soils and screened for antagonism against *S. rolfsii* through a dual culture assay. Culture filtrates and crude extracts of the potential candidates were screened further for extracellular antifungal activity and characterized for biocontrol and plant-growth-promoting traits. A promising candidate was tested under greenhouse conditions as whole organism as well as crude extracts. Isolate RP1A-12 exhibited high antagonism against *S. rolfsii* in dual culture assay (69 % inhibition), culture filtrate assay (78–100 % inhibition at various concentrations) and crude extract assay (100 % inhibition with 1 % crude extracts). Moreover, germination of sclerotia of the test pathogen was inhibited with 1 % crude extracts. Strain RP1A-12 produced hydrogen cyanide, lipase, siderophores and indole acetic acid. Oxalic acid production by *S. rolfsii* was also inhibited by crude extracts of RP1A-12. In greenhouse studies, RP1A-12 reduced stem rot severity. Overall, our results suggest that isolate RP1A-12 has potential biocontrol capabilities against stem rot pathogen. Molecular characterization based on 16S rRNA gene sequencing of RP1A-12 identified it as a species of *Streptomyces*, closely related to *S. flocculus*.

Keywords Groundnut · Stem rot · Biocontrol · *Streptomyces* sp. · Antifungal metabolites

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Introduction

Groundnut (peanut; *Arachis hypogaea* L.) is an important food legume grown in tropical and subtropical environments of more than 100 countries. Apart from several foliar diseases that affect groundnut production, diseases caused by soilborne pathogens can be very serious because they are difficult to control. One such disease is stem rot, caused by *Sclerotium rolfsii* Sacc., that can lead economically significant yield losses in several groundnut-growing countries (Bowen et al. 1992; Le 2011; Pande and Rao 2000). Under ambient conditions of disease development, losses due to stem rot can reach 80 % (Mayee and Datar 1988). Though host-plant resistance is the most economically viable option to control this disease, unfortunately, all improved varieties in cultivation today are more or less susceptible to stem rot. Presently, stem rot is managed using chemical fungicides such as azoxystrobin, chlorothalonil, tebuconazole, carbendazim and mancozeb (Hagan et al. 1991, 2010), but their high cost keeps them from being a viable approach, especially for resource-poor smallholder farmers and where productivity is low (1.0–1.5 t/ha), especially in Asia and sub-Saharan Africa. On the other hand, sustainable and ecologically safe methods to manage soilborne pathogens are gaining popularity as concerns on the harmful effects of chemicals increase.

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Therefore, for devising effective ways to manage these economically significant soilborne diseases, a thorough understanding of alternate and viable disease management options is essential.

Biological control with effective microbes is an important viable alternative. Of the types of bioagents reported, actinomycetes are promising because they are abundant in the soil and produce metabolites that inhibit a wide range of plant pathogens.

Actinomycetes are gram-positive, filamentous bacteria, characterized by high genomic G + C content (Bouizgarne 2013) and are saprophytic in nature (Crawford et al. 1993). Although actinomycetes are known to produce a wide array of antibiotics as secondary metabolites that are specific for several plant pathogens (Stockwell and Duffy 2012), reports on their use for the biological control of stem rot pathogen in groundnut are scarce. Nevertheless, they are well established as promising candidates for biological control of other soilborne pathogens and plant-growth promotion (El-Tarabily et al. 2000; Trejo-Estrada et al. 1998) such as phosphate solubilisation (Jog et al. 2014), production of indole-3-acetic acid (IAA), siderophores, hydrocyanic acid (HCN) and cell-wall-degrading lytic enzymes (Gopalakrishnan et al. 2011, 2013). Understanding these traits and their contribution to controlling pathogens is important for incorporating biocontrol agents into an integrated disease management program. Since the stem rot pathogen survives through sclerotia in soil for longer periods, selection of actinomycete strains with suppressive effects on sclerotial germination is therefore important. Further, these bioagents would be the ideal candidates if they suppress oxalic acid (OA) production of *S. rolfsii*, which is a major virulence factor.

In the present study, we screened actinomycetes against *S. rolfsii* for the efficacy of the cultures and the crude culture metabolite in laboratory and greenhouse (GH) tests to determine candidate antagonists for pilot tests in the field. The long-term goal is to develop a metabolite-based formulation of *Streptomyces* sp. that effectively reduces stem rot disease and enhances groundnut pod yields.

Materials and methods

Isolation of actinomycetes

Actinomycetes were isolated from the groundnut rhizospheric soil samples collected from the Patancheru fields of the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT). The collected samples were subjected to various pre-treatments (1.5 % v/v phenol; dry heat at 100 °C for 1 h; air drying for 24 h; water bath at 70 °C for 15 min) after sampling for high recovery of actinomycetes (Seong et al. 2001). Serial dilutions of the collected soil samples were prepared, and dilutions of 10^{-4}–10^{-6} were spread onto actinomycetes isolation medium (HiMedia), Bennett's agar (composition in g/L: peptone, 10.0; $CaCl_2$, 2.0; Tween 80, 4.7 mL; agar 20.0 g; pH 7.2) and starch casein agar (in g/L: starch, 2.0; casein, 0.3; KNO_3, 2.0; K_2HPO_4, 2.0; NaCl, 2.0; $MgSO_4{\cdot}7H_2O$, 0.05; $CaCO_3$, 0.02; $FeSO_4{\cdot}7H_2O$, 0.01; agar, 20.0 g; pH 7.2) in Petri dishes. The Petri dishes were incubated at 28 ± 2 °C for 15 days. Actinomycete colonies were selected from the Petri dishes and subcultured on Bennett's agar to get pure colonies. These isolates were maintained as 20 % (w/v) glycerol stocks at −30 °C. For each study, a fresh vial of the isolate was used.

Source of *S. rolfsii*

The culture of *S. rolfsii*, from the culture collection of the Groundnut Pathology Laboratory at ICRISAT, was originally isolated from diseased groundnut plants with typical stem rot symptoms at the Patancheru fields and maintained on potato dextrose agar (PDA; HiMedia) plates at 25 °C. The pathogenicity of the fungal culture was confirmed earlier using Koch's postulates. Sclerotia were harvested from media plates cultured for 14 days and stored at 4 °C for further studies.

In vitro antagonistic activities

Dual culture assays

Ability of the isolated actinomycetes (108 isolates) to inhibit the growth of *S. rolfsii* under in vitro conditions was tested using a dual culture assay (Anjaiah et al. 2003). Fresh cultures of actinomycetes were streaked on one end of plates of glucose casamino yeast extract medium (GCY, in g/L: glucose, 15.0; casamino acid, 1.5; yeast extract, 1.0; K_2HPO_4, 1.5; $MgSO_4{\cdot}7H_2O$, 1.0; agar, 20.0; pH 7.2) and incubated for 3 days at 28 ± 2 °C. Later, a mycelial plug (5 mm diameter) from a 3-day-old *S. rolfsii* culture was placed at the other end. Media plates with *S. rolfsii* mycelial discs alone served as controls. Each treatment had three replications. The Petri dishes were incubated for 4 days at 28 ± 2 °C, until the fungus completely covered the control plates. Percentage inhibition of the test pathogen by the actinomycete strain was calculated using the formula $\mathrm{I}\ \ \% = (C - T)/C \times 100$, where C is radial growth of the control and T is radial growth of the treated culture (Dennis and Webster 1971).

Culture filtrate assay

Superior isolates in the dual culture assay were then evaluated for activity of their culture filtrates against *S. rolfsii*.

Cultures were grown in GCY broth for 6 days at room temperature and centrifuged at 10,000 rpm (Sorvall RC-5B, GS-3 rotor, DuPont Instruments, Newton, CT, USA) for 15 min. The obtained supernatants were filtered using 0.45-µm sterile membrane filters (Merck Millipore, Ireland). Mycelial plugs of the test pathogen from actively growing cultures were transferred onto petri dishes with PDA containing 0, 12.5, 25, or 50 % v/v of the filtrates and incubated at 28 ± 2 °C for 5 days. The percentage inhibition of the test pathogen was calculated as described previously. Three replications were maintained for each concentration.

Crude culture extract assay

Crude extracts of the superior actinomycete isolates based on culture filtrate studies were obtained by treating the culture supernatants (approximately 1 L) with an adsorbent, Diaion HP-20 (SUPELCO Sigma Aldrich, Bellefonte, PA, USA), and desorbing them with methanol. Before treatment, the adsorbent resins were prepared according to Sterner (2012). The desorbed supernatants were concentrated over Rotavapor R-215 (BUCHI, Bern, Switzerland) and collected in 5 mL of methanol. The inhibitory effects of the crude metabolites at 1 % v/v on mycelial growth and sclerotial germination were studied using a poisoned food technique (Nene and Thapliyal 1993). Control plates were prepared by using the same quantity of methanol in the PDA. Three replications were maintained for the study. The percentage inhibition of the test pathogen was calculated as described already.

Characterization for plant-growth promotion and biocontrol

IAA production

For IAA production, the actinomycete isolates were grown in starch casein broth supplemented with L-tryptophan (1 mg/mL) for 72 h at 28 ± 2 °C. Later, the cultures were centrifuged at 13,000 rpm (Eppendorf 5415 D GE-009) for 5 min, and the supernatants were collected. This culture filtrate (2 mL) was allowed to react with 4 mL of Salkowski reagent (1 mL of 0.5 M $FeCl_3$ in 50 mL of 35 % v/v $HClO_4$) at 28 ± 2 °C for 30 min in the dark. The development of a pink colour indicated the production of IAA. IAA was then quantified by measuring the absorbance of the solution at 530 nm in a UV-1800 spectrophotometer (Shimadzu, Maryland, USA). A standard curve was plotted to quantify the IAA (µg/mL) present in the culture filtrate (Patten and Glick 1996).

Siderophore production

Actinomycete isolates were inoculated in King's B broth (in g/L: peptone, 10.0; K_2HPO_4, 1.5; glycerol, 15.0; $MgSO_4$, 5 mL) incubated for 4 days at 28 ± 2 °C. Culture broths were centrifuged at 10,000 rpm (Eppendorf 5415 D GE-009) for 10 min, supernatants were collected, and an equal volume of chrome azurol S solution (Schwyn and Neilands 1987) was added. This mixture was incubated for 30 min in dark. A change from blue to orange indicated a positive reaction (Macagnan et al. 2008).

Phosphate solubilization

Rock-phosphate-buffered medium was used to evaluate the phosphate solubilization capacity of the actinomycete strains according to Gyaneshwar et al. (1998). The culture was grown in Bennett's broth for 1 week, then 15 µL of the culture was placed onto a sterile filter paper disc placed on a fresh plate of Bennett's agar. After 5 days at 28 ± 2 °C, isolates positive for phosphate solubilization were identified by a pink zone around the colonies.

Production of lytic enzymes

Selected actinomycete isolates were tested for the production of major cell-wall-degrading enzymes such as lipase, protease, chitinase, and glucanase. Production of protease and lipase were tested using the methods of Bhattacharya et al. (2009). Chitinase production was determined using the standardized protocols of Hirano and Nagao (1988). Drops (15 µL) of the actinomycete isolates cultured in Bennett's broth were placed onto sterile filter paper discs on casein agar (protease), Tween 20 agar medium (lipase), cellulose chitin agar medium (chitinase). After 5 days at 28 ± 2 °C, plates were examined for halo zones around the paper discs as an indication of protease, lipase and chitinase activity.

For assessing β-1,3-glucanase activity, an actinomycetes isolate was added to tryptic soy broth (TSB; Sigma-Aldrich, Munich, Germany) amended with 1 % (w/v) colloidal chitin and incubated for 4 days at 28 ± 2 °C (Valois et al. 1996). A change from yellow to red indicates the presence of glucose produced by activity of β-1,3-glucanase.

HCN production

HCN was estimated qualitatively by the sulfocyanate colorimetric method (Lorck 1948). The actinomycete cultures were streaked onto Bennett's agar amended with glycine (4.4 g/L). A Whatman no. 1 filter paper (8 cm diameter; GE Healthcare, Little Chalfont, UK) was soaked in 1 % (v/

v) picric acid and sprayed with 1 mL of 10 % (w/v) sodium carbonate and stuck underneath the Petri dish lids. The Petri dishes were sealed with parafilm and incubated at 28 ± 2 °C for 7–10 days. A reddish brown colour on the filter paper indicated HCN production.

Inhibitory effect on OA production and sclerotial germination of *S. rolfsii* by crude culture extracts of actinomycete strain RP1A-12

Crude extracts of a culture of potential actinomycete strain RP1A-12 were tested for the in vitro inhibition of OA production by *S. rolfsii* using a modification of the method of Gawande et al. (2013). Richard's broth containing 0.5 or 1 % crude extracts were each inoculated with a mycelial plug (5 mm diameter) from a 5-day-old culture of *S. rolfsii*. Flasks containing Richard's broth and only *S. rolfsii* served as the control. The flasks were incubated for 15 days at 28 °C. OA production was quantified as described by Mahadevan and Sridhar (1986), and the percentage reduction in relation to the control was calculated.

The effect of the crude extracts of RP1A-12 on sclerotial germination in vitro was tested by dipping the sclerotia of test pathogen in 1 % concentration of crude extract for 5, 10, 15, 20, 25 or 30 min. Sclerotia dipped in sterile water served as controls. Individual sclerotia were then transferred onto PDA and incubated at 28 ± 2 °C for 7 days, then examined for germination.

Evaluation of strain RP1A-12 for controlling stem rot disease in the greenhouse

RP1A-12, the superior strain in the in vitro antagonistic studies against *S. rolfsii* was further tested in the GH with the culture in a talc-based formulation or as a crude extract in a water-based formulation. Talc formulations were prepared using actively growing cultures (after 5 days in GCY broth) at 10^8 CFU/mL mixed with sterilized talc powder at the rate of 400 mL/kg (Vidhyasekaran and Muthamilan 1995). The formulation was later shade-dried and stored in polyethylene bags at 4 °C until further use, then prepared as described in the previous section and diluted with distilled water at a ratio of 1:100 for use.

Groundnut variety JL-24, susceptible to stem rot, was used to evaluate RP1A-12 for disease control in the GH. Groundnut seeds were sown in plastic pots (8 inch diameter and 7.5 inch height) filled with sterilized soil, mixture of sand and soil at a ratio of 1:2 (v/v). Five seeds per pot were sown equidistantly at a depth of 4 cm. The stem rot pathogen, *S. rolfsii*, multiplied on autoclaved sorghum grains was added to the pots at 15 days after sowing (DAS). For mass multiplication of the pathogen, 600 g of sorghum grains were sterilized in 1 L flasks and 7–10 mycelial discs of actively grown *S. rolfsii* added, then incubated at 28 ± 2 °C for 10 days. For each pot of plants, 15 g of the multiplied inoculum was added 10 cm below the surface layer around the base of the plants and covered with soil.

The treatments with the biocontrol agent or crude culture extract were applied as either a seed treatment or a soil application included (1) seed treatment with talc formulations (10 g/kg seed; ST T_f), (2) seed treatment with crude extracts (10 mL/kg seed; ST C_f), (3) soil application with talc formulations (15 g/pot; SA T_f), (4) soil application with crude extracts (50 mL/pot; SA C_f); (5) (ST + SA) T_f; (6) (ST + SA) C_f. Soil application (15 g of the RP1A-12 formulation was added to the pot and covered with soil) and crude extracts (each pot was drenched with 50 mL crude extract) was carried out at 50 DAS. The fungicide azoxystrobin (Amistar, Syngenta, Switzerland) [250 g/L (23.1 % w/w) azoxystrobin] was used as a chemical check by drenching the soil in each pot with about 50 mL of the fungicide solution (1 mL/L) at 20 DAS. A commercially available *Trichoderma viride* strain procured from Sri Biotech Laboratories (Hyderabad, India) was also evaluated and applied to soil in pots at 10 g/kg soil before sowing. Pathogen-inoculated and uninoculated controls were maintained separately. The experiment was carried out in a randomized complete block design with three replications. Each replication had three pots. The pots were maintained on a greenhouse bench at 26 ± 2 °C and 90 % relative humidity up to 110 days (until harvest). Stem rot disease severity was assessed every 15 days starting from 30 days after inoculation on a 1–5 scale according to Shokes et al. (1996): 1 = apparently healthy plant; 2 = lesions on stem only; 3 = ≤25 % of plants symptomatic (wilted, dead or decaying); 4 = 26–50 % of plants symptomatic; and 5 = >50 % of plants symptomatic. Pod mass was recorded at harvest.

Experimental procedures and statistical analysis

The laboratory and GH experimental data were subjected to a one-way analysis of variance (GenStat 14.0 version 2013, Lawes Agricultural Trust, Rothamsted Experimental Station). Differences between the treatment means were tested for significance at $P = 0.05$. Data on in vitro culture filtrate assays and GH evaluations were analysed with Tukey's test of multiple comparisons.

Molecular characterization of strain RP1A-12

Pure culture of the isolate (RP1A-12) was grown on ISP-7 (Tyrosine Agar, HiMedia) agar slants at 28 ± 2 °C until log phase (4 days). The culture was sent to Macrogen (Seoul, South Korea) for 16S rRNA gene sequencing. The

sequences obtained were compared with those from the GenBank databases using BLASTn (Altschul et al. 1990), aligned using the CLUSTAL_X software (Thompson et al. 1997), and phylogenetic trees were constructed using the neighbour-joining method (Saitou and Nei 1987). Bootstrap analysis was performed to estimate statistical stability of the branches in the cluster with 1000 replicates using the MEGA version 6 program (Tamura et al. 2013).

Results

In vitro antagonistic activities

Dual culture assays

Of the 108 actinomycete isolates screened, 26 isolates inhibited growth of *S. rolfsii* ranging from 21 to 69 %. Of these, 10 strains exhibiting >45 % inhibition of test pathogen were selected for further antagonistic studies. Maximum inhibition was achieved by isolate RP1A-12 (69 %; Fig. 1), which caused a clear growth inhibition zone with the pathogen that prevailed for up to 10 days.

Culture filtrate assays

Of the culture filtrates of the 10 isolates evaluated, those from RCE-10, RP1A-10 and RP1A-12 at 25 and 50 % concentrations completely inhibited mycelial growth of *S. rolfsii* (Table 1). The culture filtrates of these three strains were also effective (47, 74 and 78 % inhibition, respectively) at 12.5 %. Strains RCE-22 and RP9A-16 did not inhibit fungal growth at any of the concentrations used. For the rest of the strains, the inhibition of test pathogen ranged from 28.6 % (RCE-20) to 79.6 % (RP9A-8) at 50 % concentration. In general, for the effective strains, inhibition of the fungus increased with increasing concentrations of the culture filtrate.

When the culture filtrates were tested on sclerotial body germination, the filtrate from RP1A-12 completely inhibited germination at the 50 % concentration. Sclerotial growth was inhibited from 10 to 36 % by the 50 % concentrations of the culture filtrates from the other promising strains.

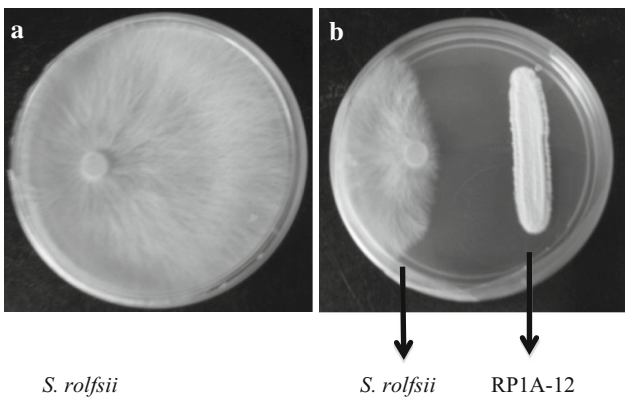

Fig. 1 Antagonist assay of actinomycete isolate RP1A-12 against *Sclerotium rolfsii*. **a** Control plate with *S. rolfsii* alone. **b** Assay plate with *S. rolfsii* on one side and potential antagonist on the other side

Crude extract assays

Studies on the antagonistic activity of crude extracts (1 % concentration) of five elite isolates (RCE-10, RP1A-10, RP1A-12, RP1A-15 and RP9A-8) indicated that all the isolates inhibited mycelial growth of *S. rolfsii* to some degree (Fig. 2). Complete inhibition of *S. rolfsii* was obtained with crude metabolites of RP1A-12, followed by RP1A-15, RCE-10, RP1A-10 and RP9A-8. Similar results were observed against sclerotial bodies.

Characterization of plant-growth promotion and biocontrol

Characterization studies of five elite isolates revealed that all the strains, RCE-10, RP1A-10, RP1A-12, RP1A-15 and RP9A-8 produced lipase (Table 2). However, none produced protease, chitinase, or β-1,3 glucanase. RCE-10, RP1A-10 and RP1A-12 produced HCN, and three isolates (RP1A-10, RP1A-12 and RP1A-15) produced siderophores. All strains produced IAA to various degrees. Strain RP1A-15 produced the most IAA, followed by RP1A-12. None of the *Streptomyces* strains were able to solubilize phosphate.

Inhibitory effect on OA production and sclerotial germination of *S. rolfsii* by crude culture extracts of an actinomycete strain RP1A-12

RP1A-12 at the two studied concentrations inhibited the fungus by 96.1 (at 0.5 % concentration) to 97.2 % (at 1 % concentration) compared with the control. Germination of sclerotia was inhibited after a soak in 1 % crude extract for 20 min or more.

GH evaluation of strain RP1A-12 in reducing stem rot disease

Of the different treatments evaluated, T_f and C_f of RP1A-12 reduced disease severity only when used as ST but not as SA (Table 3). For ST T_f, disease control was significantly higher than SA T_f, which did not differ significantly from the pathogen-inoculated control. C_f was only effective when used as ST but proved ineffective when used

Table 1 In vitro effect of culture filtrates of selected actinomycetes in inhibiting the radial growth of *Sclerotium rolfsii*

Actinomycete isolates (identity no.)	Inhibition of *S. rolfsii*[1] at different concentrations of culture filtrate of antagonists (%)		
	12.5 %	25 %	50 %
RCE-10	47.1[c]	100.0[f]	100.0[e]
RCE-20	8.7[a]	20.0[b]	28.6[b]
RCE-22	0.0[a]	0.0[a]	0.0[a]
RP1A-10	74.5[d]	92.2[e]	100.0[e]
RP1A-12	78.0[d]	100.0[f]	100.0[e]
RP1A-15	29.4[b]	47.5[d]	52.9[c]
RP9A-8	38.0[bc]	45.9[cd]	79.6[d]
RP9A-15	12.5[a]	23.5[b]	32.9[b]
RP9A-16	0.0[a]	0.0[a]	0.0[a]
RP9A-23	11.8[a]	39.2[c]	47.1[e]

Percentage inhibition of fungus was calculated relative to growth on control plates. PDA was amended with diluted culture filtrates (12.5, 25, and 50 % of stock). Values are means of three replications and were compared across a column (at the same concentration); treatments with similar letters did not differ significantly in Tukey's test, $P = 0.05$

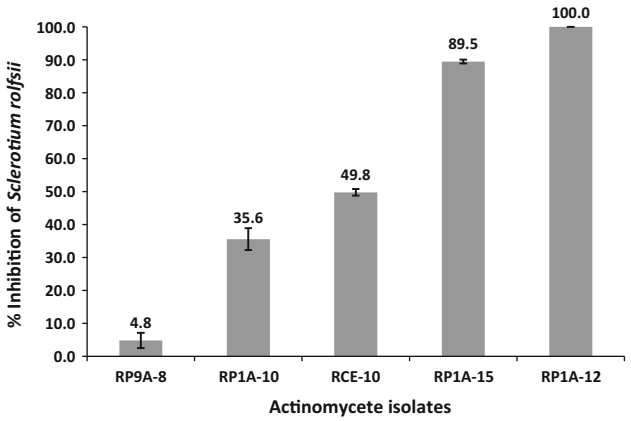

Fig. 2 Percentage inhibition of radial growth of *Sclerotium rolfsii* after 5 days incubation at 28 ± 2 °C on PDA amended with a crude culture extract of the potent actinomycete isolates. The extracts were diluted to 1 % of original stock. *Error bars* are standard errors of the mean

either as SA or as combination use (ST + SA). Maximum disease control was obtained with RP1A-12 used as ST T_f, followed by (ST + SA) T_f. Azoxystrobin and *T. viride* were not effective; stem rot severities were comparable to that of the pathogen-inoculated control.

Pod yields after (ST + SA) T_f and ST C_f treatments were significantly higher than in the pathogen-inoculated control, and yields after the two treatments did not differ significantly (Table 3) and were comparable to that of the untreated negative control. For the remaining treatments, pod yields did not differ significantly from the pathogen-inoculated control. Overall, T_f of strain RP1A-12 proved most effective as biocontrol agent and was more effective than the water-based C_f.

Molecular characterization of candidate strain RP1A-12

The BLAST analysis using 1357 bp of the16S rRNA gene sequence indicated that the RP1A-12 isolate is related (99 %) to *Streptomyces* isolates such as *S. flocculus*, *S. albus*, *S. gibsonii*, *S. rangoonensis*, and *S. almquistii.* Phylogenetic analysis using the neighbour joining dendrogram indicated that RP1A-12 is closely related to *S.*

Table 2 Characterization of elite actinomycete isolates for biocontrol and plant-growth-promoting indicators

Isolate	Biocontrol traits						Plant growth-promoting traits	
	Lipase	Protease	Chitinase	β-1,3 glucanase	HCN	Siderophore	Phosphate solublization	IAA ± SE (μg/mL)
RCE-10	+	−	−	−	+	−	−	7.29 ± 1.66
RP1A-10	+	−	−	−	+	+	−	8.83 ± 2.76
RP1A-12	+	−	−	−	+	+	−	14.42 ± 2.26
RP1A-15	+	−	−	−	−	+	−	16.77 ± 0.57
RP9A-8	+	−	−	−	−	−	−	10.74 ± 0.46

+ positive; – negative for trait; IAA values are means of three replicates with standard error of mean

Table 3 Greenhouse evaluation of formulations of *Streptomyces* sp. RP1A-12 on severity of groundnut stem rot and pod yield

Treatment	Severity	Yield (g/pot)
ST T_f	1.8^{a}	13.1^{ab}
SA T_f	4.8^{d}	4.6^{ab}
(ST + SA) T_f	2.2^{ab}	17.5^{b}
ST C_f	2.4^{abc}	18.8^{b}
SA C_f	3.9^{bcd}	9.7^{ab}
(ST + SA) C_f	3.9^{bcd}	10.2^{ab}
Azoxystrobin	4.3^{cd}	4.7^{ab}
Trichoderma viride	4.3^{cd}	13.6^{ab}
Pathogen inoculated control	5.0^{d}	0.0^{a}
Uninoculated negative control	1.0^{a}	17.0^{b}

Azoxystrobin was applied as a soil drench at 20 DAS; 10 g/kg soil *Trichoderma viride* (commercial formulation) was applied to soil before sowing. Disease severity was estimated on a 1–5 scale described in methods. Values are mean of three replicates. Both variables were analyzed using a one-way ANOVA; means separated at $p = 0.05$. Values for a particular treatment followed by the same letter did not differ significantly

T_f talc powder formulation, C_f crude extract, *ST* seed treatment, *SA* soil application

flocculus (Fig. 3). This gene sequence of RP1A-12 was deposited at GenBank (accession no. KR049226).

Discussion

Actinomycete strains are important biocontrol agents and have been reported against a wide range of plant pathogens (Doumbou et al. 2001; Yuan and Crawford 1995). Important mechanisms of action by actinomycetes in plant disease suppression include antibiosis (Trejo-Estrada et al. 1998) and competition for nutrients and space (Tokala et al. 2002). Actinomycetes are important antagonists in plant rhizospheres and can colonize plant root surfaces (Kortemaa et al. 1994; Tokala et al. 2002). In our studies, we identified *Streptomyces* sp. RP1A-12 with significant activity against *S. rolfsii* in various in vitro dual culture assays using the organism (Fig. 1), culture filtrates (Table 1) and crude extracts (Fig. 2). That germination of sclerotia was inhibited by crude extract is important because the pathogen survives for long periods in the soil as sclerotia.

In our GH studies, the talc formulations of the organism and the water formulation of the crude extract were effective individually in reducing disease severity only when used as ST (Table 3). Stem rot severities after treatments ST T_f, ST C_f and (ST + SA) T_f were on par with that of uninoculated healthy control. However, the SA T_f and SA C_f treatments were not effective in controlling the disease, and the severity did not differ significantly from that of the pathogen-inoculated control, perhaps due to the difference in the times of their application. SA T_f was applied at 50 DAS, when the pathogen, applied at 15 DAS, should have been well established in the soil. The ST T_f, though effective against stem rot, did not contribute to pod yields significantly; for plant-growth promotion and pod formation, population density might not have been sufficient in the ST T_f treatment compared with (ST + SA) T_f, which had adequate colony forming units of RP1A-12 in the crop rhizosphere.

Reduction in pathogen growth and stem rot severity by RP1A-12 in this study can be attributed to the production of HCN, siderophores, cell-wall-degrading enzyme such as lipase (Table 2) and reduction of OA production by *S. rolfsii*. Rhizobacteria that produce siderophores are known to be strong antagonists against several plant pathogenic fungi (Chaiharn et al. 2009). These siderophores deprive native rhizosphere microflora of plants of iron, thereby contributing to plant-growth promotion through pathogen control (Duijff et al. 1993); iron is essential for microbial growth, so microbes have adopted siderophore production as a strategy for growth under iron-limiting conditions.

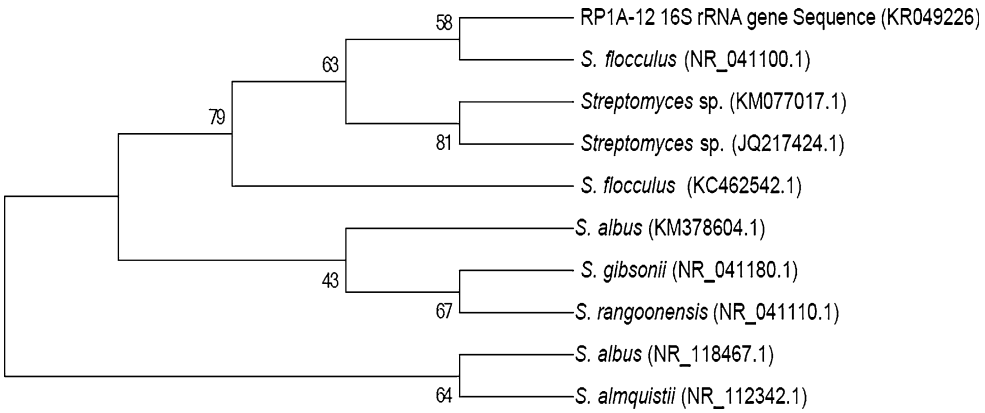

Fig. 3 Neighbour joining dendrogram of actinomycetes isolate RP1A-12, which was antagonistic to *Sclerotium rolfsii*, and representative actinomycetes species constructed using the 16S rRNA gene sequences obtained from a BLAST analysis. The number at each branch is the percentage of time the group of strains in that branch occurred, based on 1000 cycles in bootstrap analysis

Similarly, HCN is a volatile antibiotic produced by antagonists for protecting plants from phytopathogenic fungi (Ahmad et al. 2008; Voisard et al. 1989). Enzymes such as lipases that are produced by antagonists can lead to leakage and lysis of hyphae of pathogenic fungi (Gopalakrishnan et al. 2013).

Actinomycetes are extensively used in plant protection as they are potential sources of agro-active compounds (Doumbou et al. 2001). Some of these metabolites interfere with fungal cell wall synthesis by inhibiting chitin synthase (Endo et al. 1970). Besides, the efficacy of crude extracts of RP1A-12 in inhibiting the growth of *S. rolfsii* (Fig. 2) can be attributed to the production of antifungal metabolites. Further studies are required to isolate and characterize the specific metabolite associated with RP1A-12 using analytical techniques such as liquid chromatography-mass spectrometry (LCMS), fourier transformation infrared spectroscopy (FTIR) and nuclear magnetic resonance spectroscopy (NMR). Targeting the pathogenicity factors of plant pathogens is another approach for biocontrol agents in disease management. OA is considered as one of the major determinants of virulence and pathogenicity of *S. rolfsii* in groundnut (Cessna et al. 2000); mutant fungal strains deficient in OA production are unable to cause disease (Godoy et al. 1990). In our studies, OA production by *S. rolfsii* was significantly reduced by the 0.5 and 1 % concentrations of the crude extracts. Organic biocides can also reduce OA production by *S. rolfsii* (Paramasivan et al. 2013).

Earlier studies have also established the efficacy of culture filtrates of *Streptomyces* sp. in promoting plant growth. In a study by Aldesuquy et al. (1998), grain priming with culture filtrates of *S. olivaceoviridis*, *S. rimosus* and *S. rochei* resulted in increased growth, vigour and yields of wheat. In our studies, though ST with C_f of RP1A-12 strain did not contributed significantly to more pod yields, yields were higher than in control pots in the GH (Table 3). Dey et al. (2004) reported that *Streptomyces* sp. produced different categories of growth-promoting compounds such as auxins, gibberellins, and cytokinins that directly influence plant growth. Keeping that in mind, we also tried to quantify IAA, an important hormone in promoting biometric characteristics including yield by beneficial microbes. Potent strain RP1A-12 in our present study produced up to 14.4 µg/mL IAA (Table 2), that might have played a direct role in pod yield enhancement in groundnut in the GH.

Overall, our results suggest that *Streptomyces* sp. RP1A-12 is a promising bioagent for managing stem rot disease in groundnut and, to a certain extent, enhancing pod yields. To the best of our knowledge, this report is the first on the antifungal activity of *Streptomyces* sp. against the stem rot pathogen and on its plant growth-promoting abilities in groundnut. Field studies using the whole organism and crude extract of RP1A-12 to assess the field efficacy against stem rot are mandatory before recommending the strain as a candidate biocontrol agent.

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Compliance with ethical standards

Conflict of interest The authors confirm that there are no conflicts of interest regarding any experimental data.

Ethical standards No laws have been violated while carrying out any of the experiments for this study.

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