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Full Length Research Paper

Biocontrol of charcoal-rot of sorghum by actinomycetes isolated from herbal vermicompost

Subramaniam Gopalakrishnan*, Bandru Keerthi Kiran, Pagidi Humayun, Meesala Sree Vidya, Kanala Deepthi, Simi Jacob, Srinivas Vadlamudi, Gottumukkala Alekhya and Om Rupela

Research Program-Grain Legumes, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru 502 324, Andhra Pradesh, India.

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A total of 137 actinomycetes, isolated from 25 different herbal vermicomposts, were characterized for their antagonistic potential against *Macrophomina phaseolina* by dual-culture assay. Of them, eight most promising isolates (CAI-17, CAI-21, CAI-26, CAI-68, CAI-78, KAI-26, KAI-27 and MMA-32) were characterized for the production of siderophore, chitinase, protease, hydrocyanic acid (HCN), indole acetic acid (IAA) and further evaluated for their antagonistic potential against *M. phaseolina* by blotter-paper assay and in greenhouse. All the eight isolates produced HCN and IAA, seven produced siderophore (except CAI-78) and protease (except KAI-27) and four produced chitinase (CAI-26, KAI-26, KAI-27 and MMA-32). In the blotter-paper assay, no charcoal-rot infection was observed in KAI-26 and KAI-27-treated sorghum roots, indicating complete inhibition of the pathogen, while the other isolates showed 47 to 88% lesser charcoal-rot infection compared to the control. In the antifungal activity test against *M. phaseolina* (in greenhouse on sorghum), all the isolates increased in shoot dry mass by 28 to 53% and root dry mass by 5 to 21%, over the control. In order to confirm the plant growth promoting (PGP) traits of the isolates, the green house experiment was repeated, but in the absence of *M. phaseolina*. The results further confirmed the PGP traits of the isolates as evidenced by 15 to 34% increase in shoot dry mass on six isolates (except CAI-26 and KAI-27), 14 to 57% increase in root dry mass on five isolates (except CAI-68, KAI-26 and KAI-27), 17 to 60% increase in root length on five isolates (except CAI-17, CAI-21 and CAI-68) and 10 to 64% increase in root volume on six isolates (except CAI-17 and CAI-68). Culture filtrate of three potential actinomycetes (CAI-21, CAI-26 and MMA-32) at 0.5% inhibited the growth of *M. phaseolina*, indicating that the metabolites of these actinomycetes were responsible for the inhibition. The sequences of 16S rDNA gene of the isolates matched with *Streptomyces* but with different species in BLAST analysis. This study indicates that the selected actinomycetes have the potential for PGP and control of charcoal-rot disease in sorghum.

Key words: Antagonistic actinomycetes, biocontrol, charcoal-rot, *Macrophomina phaseolina*.

INTRODUCTION

Charcoal-rot of sorghum (*Sorghum bicolor*) caused by *Macrophomina phaseolina* (Tassi) Goid. is endemic to tropical and temperate regions of the world (Wyllie,

1998). Significant losses of yield (up to 64%) have been observed in India under conditions favoring the incidence of the disease in post-rainy sorghum, occupying more than 5 million ha in Maharashtra, Karnataka and Andhra Pradesh (Das et al., 2008). In addition to severely damaging the crop, the pathogen also produces a toxin called "phaseolinone" in the diseased stalk that causes anemia in mice (Bhattacharya et al., 1994). Management of charcoal rot of sorghum is difficult, as no single control measure is fully effective. Actinomycetes are a group of Gram-positive bacteria, with high G + C content

*Corresponding author. E-mail: s.gopalakrishnan@cgiar.org.
Tel: +91 40 3071 3610. Fax: +91 40 3071 3074.

Abbreviations: HCN, Hydrocyanic acid; IAA, indole acetic acid; PGP, plant growth promoting.

belonging to the order Actinomycetales, which form branched mycelium and hence have sometimes been classified as fungi imperfecti. These are found most commonly in soil, compost, fresh and marine water and play an important role in the decomposition of organic materials and produce secondary metabolites of commercial interest. The biological degradation and conversion of agricultural wastes or herbals by earthworms and microorganisms, called vermicomposting, is becoming a favored method of recycling wastes (Edwards, 1998).

One of the advantages of using earthworms in composting is that it creates aerobic conditions and release coelomic fluids in the decaying biomass, which contains factor(s) that kills pathogens such as *Salmonella*, *Serratia marcescens* and *Escherichia coli* (Prabha, 2009). Application of vermicompost prepared from the herbals not only benefits crop plants as it contains beneficial microorganisms, that help the plants to mobilize and acquire nutrients, but also promotes plant growth and inhibits many plant pathogenic microorganisms (Postma et al., 2003; Suthar et al., 2005; Perner et al., 2006; Nath and Singh, 2009). Efficacy of *Jatropha*, *Annona* and *Parthenium* vermivash was shown to inhibit *M. phaseolina*, *Sclerotium rolfsii* and FOC (Gopalakrishnan et al., 2010). Hence, in the present investigation, several herbal vermicomposts were screened for actinomycetes that contain antagonistic potential against charcoal-rot in sorghum. The objective of this study was to isolate, characterize and evaluate the actinomycetes isolated from vermicomposts for their ability to suppress *M. phaseolina*.

MATERIALS AND METHODS

Preparation of herbal vermicompost samples

Foliages of 25 different botanicals (*Jatropha curcas*, *Annona squamosa*, *Parthenium hysterophorus*, *Oryza sativa*, *Gliricidia sepium*, *Adhatoda vasica*, *Azadirachta indica*, *Capsicum annuum*, *Calotropis gigantea*, *Calotropis procera*, *Datura metal*, *Allium sativum*, *Zingiber officinale*, *Ipomoea batatas*, *Momordica charantia*, *Moringa oleifera*, *Argyranthemum frutescens*, *Nerium indicum*, *Allium cepa*, *Curcuma aromatica*, *Pongamia pinnata*, *Abacopteris multilineata*, *Nicotiana tabacum*, *Tridax procumbens* and *Vitex negundo*) were collected from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) farm and air-dried at room temperature ($30\pm 2^\circ\text{C}$). The container for vermicomposting was constructed by cutting a 200 L plastic barrel into two halves. A metal grill was placed at the bottom of the barrel (10 cm clearance from the base [floor] of the barrel), and the air-dried foliages of the herbals (bedding material) were composted on top of the grill with earthworms (*Eisenia foetida*). The bedding material was moistened with water before being added to the barrel. The barrel was covered with a lid to keep the moisture intact and to avoid light as earthworms grow well in moist environment and darkness. A layer of foliage was added once a week as feed for the earthworms. The whole set-up was left for 2 months until all the foliages of the herbals were digested. When the herbal compost was ready, about 100 g of the sample was collected and stored in a refrigerator at 4°C for further studies.

Actinomycetes, bacterial and fungal population in the herbal vermicompost

Ten grams of herbal vermicompost from each sample were separately suspended in 90 ml of sterilized physiological saline (0.85% of NaCl) in a flask and placed on an orbital shaker (at 100 rpm) at room temperature ($28\pm 2^\circ\text{C}$) for 1 h. At the end of shaking, the vermicompost samples were serially diluted up to 10^6 dilutions with physiological saline. Dilutions 10^4 to 10^6 were plated for enumerating actinomycetes (on starch casein agar; SCA) and bacteria (on Luria agar), while 10^2 to 10^4 were plated for fungal colonies (on potato dextrose agar) by spread plate technique and incubated at $28\pm 2^\circ\text{C}$ for four days. Bacterial colonies were enumerated at the end of 24 h incubation, while fungal and actinomycetes were enumerated at the end of 72 and 96 h, respectively. The most prominent actinomycete colonies (the ones which were found abundantly in the plate, produced pigments and inhibited the adjacent colonies) were isolated and maintained on SCA slants at 4°C for further studies.

In vitro antifungal activity

Actinomycete isolates were evaluated for their antifungal activity against *M. phaseolina* by dual-culture assay. *M. phaseolina* was acquired from the Directorate of Sorghum Research, Hyderabad, India. A fungal disk (*M. phaseolina*) of 6 mm diameter was placed on one edge (1 cm from the corner) of the glucose casamino acid yeast extract (GCY) agar plate and the actinomycete isolate was streaked on the other edge of the plate (1 cm from the corner), followed by incubation at $28\pm 2^\circ\text{C}$ for four days or until the pathogen covered the entire plate in the control plate. Inhibition of fungal mycelium (halo zone) around the actinomycete colony was noted as positive and the inhibition zone measured.

In vitro plant growth-promoting attributes of the actinomycetes isolates

Siderophore production

Siderophore production was determined according to the methodology described by Schwyn and Neilands (1987). Actinomycetes were streaked on Chrome Azurol S (CAS) agar media and incubated at $28\pm 2^\circ\text{C}$ for four days. When the actinomycetes consume iron present in the blue-colored CAS media, orange halos are produced around the colonies, which indicate the presence of siderophores. Observations were recorded on a 0 to 4 rating scale as follows: 0 = no change; 1 = positive (slight halo); 2 = halo zone of 1 to 3 mm; 3 = halo zone of 4 to 6 mm and 4 = halo zone of 7 mm and above.

Chitinase production

Colloidal chitin was prepared freshly and used in the Chitin agar as per the standard protocols of Hirano and Nagao (1988). Actinomycetes were streaked on Chitin agar and incubated at $28\pm 2^\circ\text{C}$ for four days. At the end of the incubation, the plates were observed for halo zones around the colonies, which indicate the production of chitinase. Observations were recorded on a 0 to 4 rating scale as follows: 0 = no change; 1 = positive (slight halo); 2 = halo zone of 1 to 3 mm; 3 = halo zone of 4 to 6 mm and 4 = halo zone of 7 mm and above.

Protease production

It was done as per the protocols of Bhattacharya et al. (2009).

Actinomycetes were streaked on Casein agar and incubated at $28\pm 2^\circ\text{C}$ for four days. At the end of the incubation, the plates were observed for halo zones around the colonies, which indicate the production of protease. Observations were recorded on a 0 to 4 rating scale as follows: 0 = no change; 1 = positive (slight halo); 2 = halo zone of 1 to 3 mm; 3 = halo zone of 4 to 6 mm and 4 = halo zone of 7 mm and above.

Hydrocyanic acid (HCN) production

HCN was estimated qualitatively by sulfocyanate colorimetric method (Lorck, 1948). The actinomycetes were grown on Bennett agar amended with glycine (4.4 g l^{-1}). One sheet of Whatman filter paper no. 1 (8 cm diameter) was soaked in 1% picric acid (in 10% sodium carbonate; filter paper and picric acid were sterilized separately) for a minute and stuck underneath the Petri dish lids. The plates were sealed with parafilm and incubated at $28\pm 2^\circ\text{C}$ for four days. Development of reddish brown color on the filter paper indicated positive for HCN production. Observations were recorded on a 0 to 3 rating scale (they were rated based on the intensity of the reddish brown color) as follows: 0 = no color change; 1 = light reddish brown; 2 = medium reddish brown and 3 = dark reddish brown.

Indole acetic acid (IAA) production

It was done as per the protocols of Patten and Glick (1996). The actinomycetes were grown in Starch casein broth supplemented with L-tryptophan ($1\text{ }\mu\text{g ml}^{-1}$) for four days. At the end of the incubation, the cultures were centrifuged at $10,000 \times g$ for 10 min, and the supernatants collected. One ml of this culture filtrate was allowed to react with 2 ml of Salkowsky reagent (1 ml of 0.5 M FeCl_3 in 50 ml of 35% HClO_4) at $28\pm 2^\circ\text{C}$ for 30 min. At the end of the incubation, development of pink color indicated the presence of IAA. Quantification of IAA was done by measuring the absorbance in a spectrophotometer at 530 nm. A standard curve was plotted to quantify the IAA ($\mu\text{g ml}^{-1}$) present in the culture filtrate.

In vivo antifungal activity by blotter paper assay

Determination of *in vivo* antifungal activity of the eight most potential actinomycetes against *M. phaseolina* was done by blotter paper assay technique (Nene et al., 1981). Inoculum of *M. phaseolina* was prepared by homogenizing (with a tissue mizer; Techmar type T 25, Japan) a 5-day-old *M. phaseolina* culture grown on potato dextrose broth (PDB) at $28\pm 2^\circ\text{C}$. Two-week-old seedlings of sorghum (raised in sterilized vermiculite in 12 cm pots; variety R16- susceptible to charcoal-rot) were dipped in the inoculum of *M. phaseolina* for 30 min and placed side by side on a blotter paper (45×25 cm with one fold), so that only the roots were covered. Actinomycete isolates (CAI-17, CAI-21, CAI-26, CAI-68, CAI-78, KAI-26, KAI-27 and MMA-32) were inoculated (5 ml plant^{-1}) separately into plants.

Fifteen plants per replicate and three replications were made for each actinomycete isolate. Positive and negative controls were made by inoculating the plants only with *M. phaseolina* and sterile water, respectively. The blotter paper was kept moist all the time with sterilized water and incubated at $28 \pm 2^\circ\text{C}$ for 8 days with a 12-h day length provided by fluorescent lights ($120\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$). At the end of the incubation, the disease symptoms of the charcoal-rot (black-colored microsclerotia infection on the root surface) were recorded on a 0 to 4 rating scale (0 represents no visible charcoal-rot symptom, while 4 represents maximum disease symptoms), and the percentage of infected roots in actinomycete inoculated

treatments compared with the control was calculated.

In vivo antifungal activity in a greenhouse

Eight potential antagonistic actinomycete isolates (CAI-17, CAI-21, CAI-26, CAI-68, CAI-78, KAI-26, KAI-27 and MMA-32) were evaluated in greenhouse for their antagonistic potential against *M. phaseolina*. A total of 9 treatments (eight actinomycetes + *M. phaseolina* inoculated- positive control) were made with six replications. *M. phaseolina* inoculum was mass multiplied on sorghum grains (variety R16) as per the protocols of Gupta et al. (2002). Pot mixture (800 g) was prepared by mixing red soil, sand and farm yard manure at 3:2:2 and filled in 8" plastic pots followed by inoculation with *M. phaseolina* inoculum (20% of pot weight, 200 g pot^{-1}). Inoculum was mixed thoroughly with the pot mixture. Water (100 ml) was added to each pot to wet the potting mixture and the pots were covered with polythene sheets. The whole set-up was incubated at $32\pm 2^\circ\text{C}$ in green house for 15 days for charcoal-rot symptoms to develop.

Two weeks later, sorghum seeds (variety R16) were surface-sterilized with sodium hypochlorite (2.5%) for 5 min and rinsed eight times with sterilized water before allowing it to sprout in a Petri plate overnight. The sprouted seeds were transferred into test actinomycete isolates (grown in Starch casein broth separately) for an hour before being sown in the pots (6 seeds/pot but thinned to 3 after 1 week). Booster doses of actinomycete isolates (5 ml per seedling , 10^8 CFU ml^{-1}) were applied twice (at 15 and 30 days after sowing) by soil drench method. Growth parameters including root length, root dry weight, shoot dry weight, shoot root ratio, percentage of root and shoot dry weight increase over the control and the disease incidence were determined at day 60 after sowing.

In vivo PGP activity in a greenhouse

For evaluating the PGP potential of the eight actinomycete isolates as potential biocontrol agents against *M. phaseolina* actinomycete isolates, the previously explained greenhouse experiment was repeated without applying *M. phaseolina*. However, one new treatment was included in which only water was added and the positive control (only *M. phaseolina* inoculated) was removed. A total of nine treatments (eight antagonistic actinomycetes + negative [water] control) were made with six replications. Growth parameters including root length, root volume, root dry weight, shoot dry weight, shoot root ratio and % root and shoot dry weight and length increase over the control were determined at day 60, after sowing.

Effect of the culture filtrates of actinomycetes on fungal pathogens

Culture filtrates (1 L) of three actinomycetes (CAI-21, CAI-26 and MMA-32) were prepared from 5-day-old cultures grown in Starch casein broth by collecting the supernatants after centrifuging the cultures for 20 min at $10,000 \times g$. After adjusting the pH to 3.0, the supernatants were partitioned three times against ethyl acetate (EtOAc) resulting in organic and aqueous fractions. The organic fraction was combined, dried over anhydrous sodium sulphate and the EtOAc removed by film evaporation on a rotary evaporator (BUCHI V-850, Switzerland) at 35°C . The residues were dissolved in 10 ml of methanol and stored in a freezer at -20°C . The aqueous fraction was combined, evaporated on a rotary evaporator at 35°C and dissolved in minimal volumes of methanol (10 ml). Both organic as well as aqueous fractions were evaluated for their antagonistic potential against *M. phaseolina*.

Table 1. Microbial population* (actinomycetes, bacteria and fungal), diversity and pH of 25 different herbal vermicomposts used in this study.

| Common name | Scientific name | Actinomycetes | | Bacteria | | Fungi | | pH |
|-------------------------|---------------------------------|---------------|-----------|----------|----------|-------|----------|------|
| | | Count | Diversity | Count | Division | Count | Division | |
| Rice straw | <i>Oryza sativa</i> | 7.41 | 6 | 8.67 | 14 | 6.63 | 3 | 7.01 |
| Gliricidia foliage | <i>Gliricidia sepium</i> | 7.53 | 6 | 8.92 | 13 | 6.93 | 7 | 8.13 |
| Neem foliage | <i>Azadirachta indica</i> | 7.51 | 8 | 9.26 | 17 | 6.97 | 5 | 7.76 |
| Adhatoda foliage | <i>Adhatoda vasica</i> | 7.12 | 4 | 8.89 | 12 | 6.52 | 6 | 8.27 |
| Annona foliage | <i>Annona squamosa</i> | 7.74 | 7 | 9.31 | 8 | 7.05 | 5 | 7.94 |
| Chilli foliage | <i>Capsicum annuum</i> | 6.83 | 2 | 8.88 | 11 | 6.57 | 7 | 8.14 |
| Calotrophis foliage | <i>Calotrophis gigantean</i> | 7.04 | 3 | 8.99 | 10 | 6.64 | 6 | 8.06 |
| Chrysanthemum foliage | <i>Chrysanthemum morifolium</i> | 7.4 | 12 | 7.4 | 12 | 6.55 | 6 | 7.63 |
| Datura foliage | <i>Datura metal</i> | 0 | 0 | 8.78 | 13 | 6.84 | 3 | 7.7 |
| Garlic foliage | <i>Allium sativum</i> | 7.12 | 4 | 8.59 | 10 | 7.05 | 8 | 8.18 |
| Ginger foliage | <i>Zingiber officinale</i> | 7.46 | 7 | 8.82 | 11 | 7.13 | 6 | 8.79 |
| Sweet potato foliage | <i>Ipomoea batatas</i> | 7.27 | 6 | 8.73 | 10 | 6.27 | 4 | 7.8 |
| Jatropha foliage | <i>Jatropha curcas</i> | 6.82 | 2 | 8.76 | 10 | 6.95 | 7 | 8.05 |
| Jatropha seed | <i>Jatropha curcas</i> | 6.88 | 1 | 8.7 | 10 | 6.99 | 6 | 7.42 |
| Bitter gueard foliage | <i>Momordica charantia</i> | 7.29 | 3 | 8.74 | 11 | 6.74 | 6 | 8.14 |
| Drum stick foliage | <i>Moringa oleifera</i> | 6.68 | 2 | 8.73 | 9 | 6.59 | 7 | 7.78 |
| Oleander foliage | <i>Nerium indicum</i> | 7.25 | 5 | 8.8 | 15 | 6.7 | 4 | 8.01 |
| Onion foliage | <i>Allium cepa</i> | 6.33 | 1 | 8.59 | 11 | 6.65 | 5 | 8.73 |
| Parthenium foliage | <i>Parthenium hysterophorus</i> | 7.51 | 8 | 8.8 | 10 | 6.81 | 8 | 8.03 |
| Wild turmeric foliage | <i>Curcuma aromatica</i> | 7.2 | 5 | 8.77 | 13 | 7.06 | 6 | 9.07 |
| Pongamia foliage | <i>Pongamia pinnata</i> | 7.4 | 6 | 8.73 | 7 | 7.33 | 9 | 7.32 |
| Yellow oleander foliage | <i>Thevetia peruviana</i> | 7.17 | 6 | 8.65 | 11 | 6.86 | 3 | 8.02 |
| Tobacco foliage | <i>Nicotiana tabacum</i> | 2 | 2 | 8.74 | 8 | 7 | 7 | 8.37 |
| Tridax foliage | <i>Tridax procumbens</i> | 6.83 | 3 | 8.57 | 7 | 6.83 | 7 | 8.32 |
| Vitex foliage | <i>Vitex negundo</i> | 7.12 | 2 | 8.91 | 6 | 6.99 | 4 | 7.49 |

*Microbial populations were expressed in Log₁₀ values; Div = diversity.

A loop full of *M. phaseolina* were inoculated on the center of a PDA plate and incubated at 28°C for 4 days. Upon its growing, one disc of 6 mm diameter was perforated from the growing mycelia using a sterile borer and inoculated into a flask containing 20 ml of PDB. Both organic and aqueous fraction samples (100 µl) were inoculated separately into the aforementioned flask, whereas in the control 100 µl of methanol was added. Six replications were made for each treatment and the flasks were incubated at room temperature (26 to 29°C) on a shaker (G10 Gyrotary shaker, USA) at 160 rpm for 5 days. At the end of incubation, the fungal dry weight was compared with the control.

Molecular identification of the isolates

Pure cultures of potential *M. phaseolina* antagonistic actinomycetes were grown in Starch casein broth until log phase (four days) and genomic DNA were isolated according to Bazzicalupo and Fani (1995). The amplification of 16S rDNA gene was done by using universal primer 1492R (5'-TACGGYTACCTTGTTACGACTT-3') and 27F (5'-AGAGTTTGATCMTGGCTCAG-3') as per the conditions described by Pandey et al. (2005). The PCR product was sequenced at Macrogen Inc. Seoul, Korea. The sequences obtained were compared with those from the GenBank using the BLAST program (Altschul et al., 1990); aligned using the Clustal W software (Thompson et al., 1997) and phylogenetic trees inferred using the neighbor-joining method (Saitou and Nei, 1987).

Bootstrap analysis was performed to estimate statistical stability of the branches in the cluster with 1000 replicates using MEGA version 4 program (Tamura et al., 2007)

Statistical analysis

All the greenhouse experiments were arranged in completely randomized block design with six replications. The data were subjected to analysis of variance (ANOVA) (Genstat 10.1 version 2007, Lawes Agricultural Trust, Rothamsted Experimental Station) to evaluate the efficiency of the biocontrol agent's application in the greenhouse studies. Significance of differences between the treatment means was tested at P = 0.01 and 0.05.

RESULTS

Actinomycetes, bacterial and fungal population in the herbal vermicomposts

No actinomycete was found in the compost prepared from *Datura* foliage, while all the other vermicomposts contained actinomycetes in the range of 6.3 to 7.7 Log₁₀ (CFU) populations with an exception of tobacco compost, which contained only 2.0 Log₁₀ populations (Table 1).

Table 2. The eight most potential antagonistic actinomycetes against *M. phaseolina*, based on their different *in vitro* biocontrol and PGP traits.

| Isolate | % Inhibition against MP | Production scale for | | | | IAA ($\mu\text{g ml}^{-1}$) |
|---------|-------------------------|----------------------|-------|-------|------|-------------------------------|
| | | Sid* | Chit* | Prot* | HCN* | |
| CAI-17 | 70 | 2 | 0 | 3 | 3 | 0.34 |
| CAI-21 | 63 | 1 | 0 | 3 | 3 | 1.13 |
| CAI-26 | 60 | 2 | 1 | 4 | 3 | 1.17 |
| CAI-68 | 73 | 3 | 0 | 4 | 2 | 0.22 |
| CAI-78 | 50 | 0 | 0 | 2 | 2 | 0.95 |
| KAI-26 | 63 | 3 | 2 | 3 | 1 | 0.4 |
| KAI-27 | 63 | 1 | 2 | 0 | 2 | 0.47 |
| MMA-32 | 55 | 3 | 1 | 2 | 2 | 4.66 |

MP = *Macrophomina phaseolina*; CAI = Compost actinomycete isolate; KAI = Karnataka actinomycete isolate; MMA = Masala mitti actinomycete; Sid = Siderophore; Chit = Chitinase; Prot = Protease; HCN = Hydrocyanic Acid; IAA = Indole acetic acid; * = rating scale for siderophore, chitinase, cellulase, lipase, protease and HCN were as follows: 0 = Negative; 1 = Positive; 2 = 1 to 3 mm; 3 = 4 to 6 mm; 4 = > 6mm

The bacterial and fungal populations were found in the range of 7.4 to 9.3 and 6.3 to 7.3 Log₁₀ values, respectively (Table 1). Maximum diversity of actinomycetes, bacteria and fungal population were found in vermicomposts prepared with Chrysanthemum, Oleander and Pongamia, respectively (Table 1). A total of 137 actinomycetes, the most prominent ones (the ones which were found abundantly, produced pigments and inhibited the adjacent colonies) in the SCA plate, were isolated and further screened for their antagonistic potential against *M. phaseolina* by *in vitro* dual-culture assay.

***In vitro* biocontrol and plant growth promoting attributes of the actinomycetes**

Of the 137 actinomycete isolates studied for their biocontrol trait, only 79 of them were found to have the antagonistic potential against *M. phaseolina*. When the eight most potential antagonistic actinomycetes [compost actinomycete isolate; CAI-17, CAI-21, CAI-26, CAI-68, CAI-78, Karnataka actinomycete isolate; KAI-26, KAI-27 and Masala Matti actinomycete (MMA)-32; all originated from 25 different herbal vermicomposts] were evaluated further for their biocontrol and plant growth promoting traits, all the eight isolates produced HCN and IAA (MMA-32 produced highest IAA; 4.66 $\mu\text{g ml}^{-1}$), seven isolates produced both siderophore (except CAI-78) and protease (except KAI-27) and four (CAI-26, KAI-26, KAI-27 and MMA-32) isolates produced chitinase (Table 2).

***In vivo* antifungal activity by blotter paper assay**

When all the eight actinomycete isolates were evaluated for their *in vivo* antifungal potential against *M. phaseolina*

by blotter paper assay, neither charcoal-rot disease symptoms (rating 0 in 0 to 4 visual rating scale) nor root infections (0%) were observed in the KAI-26 and KAI-27-treated sorghum roots, whereas very little disease symptoms (rating 1 to 2) and lesser root infection (47 to 77% lesser than the control) were observed in the other actinomycete-treated sorghum roots (Figure 1).

Evaluation of actinomycetes in greenhouse conditions

The isolates were further evaluated for their *in vivo* antagonistic potential against *M. phaseolina* in green house on sorghum crop. Root length was found greater in 6/8 isolates (except CAI-68 and CAI-78), the maximum being found in two isolates namely 174 cm in CAI-17 and 171 cm in CAI-26, compared to 150 cm in the control plant roots (Table 3). All the eight isolates increased both sorghum shoot biomass (28 to 53%) and root biomass (5 to 21%) over the control (Table 3). The highest increase of both shoot and root biomass (45 and 21%, respectively) was found in CAI-26 (Table 3). Shoot root ratio was found higher in all the isolates, ranging 3.01 to 3.74 compared to 2.74 in the control (Table 3). No negative effect was found in any of the isolates.

In order to confirm the PGP traits, all the isolates were further evaluated in green house but without inoculating *M. phaseolina* on sorghum crop. All the eight isolates increased sorghum shoot biomass by 10 to 34%; however, only six isolates increased more than 15% biomass (except CAI-26 and KAI-27) (Table 4). Root biomass was also found increased in all eight isolates (up to 57%) but only five isolates (CAI-17, CAI-21, CAI-26, CAI-78 and MMA-32) increased more than 14% (Table 4). Root length (except CAI-68), root volume (except CAI-

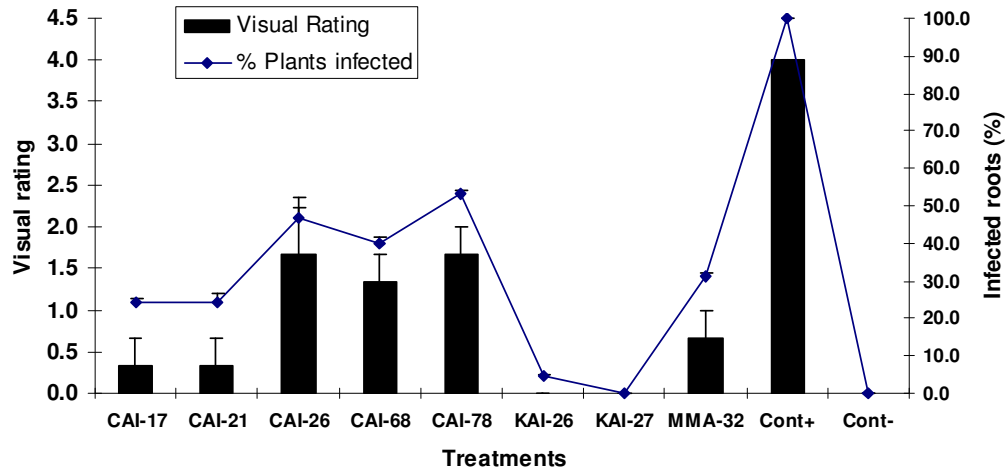


Figure 1. Influence of the eight most potential actinomycete isolates on *Macrophomina phaseolina* by blotting paper assay (Gopalakrishnan et al., 2011).

Table 3. Evaluation of the eight most potential actinomycetes for their antagonistic potential against *Macrophomina phaseolina*, in sorghum under green house conditions.

| Treatment | Root length (cm) | Root weight (g) | Shoot weight (g) | Shoot root ratio | % Increase over control | |
|-----------|------------------|-----------------|------------------|------------------|-------------------------|-------------|
| | | | | | Shoot weight | Root weight |
| CAI-17 | 174 | 0.86 | 2.58 | 3.01 | 28 | 14 |
| CAI-21 | 159 | 0.84 | 3.08 | 3.74 | 53 | 12 |
| CAI-26 | 171 | 0.91 | 2.92 | 3.24 | 45 | 21 |
| CAI-68 | 136 | 0.79 | 2.9 | 3.7 | 44 | 5 |
| CAI-78 | 137 | 0.81 | 2.82 | 3.52 | 40 | 8 |
| KAI-26 | 167 | 0.83 | 2.88 | 3.5 | 43 | 10 |
| KAI-27 | 169 | 0.85 | 2.8 | 3.3 | 39 | 13 |
| MMA-32 | 153 | 0.85 | 2.68 | 3.17 | 33 | 13 |
| Control | 150 | 0.75 | 2.01 | 2.74 | | |
| Mean | 159 | 0.83 | 2.74 | 3.35 | 40 | 11 |
| SE± | 15.5*** | 0.061*** | 0.195* | 0.220*** | | |
| LSD (5%) | 4.4 | 0.173 | 0.554 | 0.624 | | |
| CV% | 23 | 17 | 17 | 17 | | |

Values are means of six replications and data calculated per plant after 60 DAS. * = statistically significant at 0.05; *** = statistically significant at < 0.001; SE = standard error; LSD = least significant difference; CV = coefficient of variance.

17) and shoot root ratio (except CAI-21, CAI-26 and MMA-32) were also found greater in comparison to the control (Table 4). Among the eight actinomycetes, MMA-32 increased all the parameters by at least 25%, that is shoot dry mass (27%), root dry mass (57%), root length (60%) and root volume (64%), over the control (Table 4).

Effect of culture filtrates of actinomycetes on fungal pathogens

Culture filtrates of three promising actinomycetes, CAI-21, CAI-26 and MMA-32, were partitioned against ethyl acetate, and the resultant organic and aqueous fractions

were evaluated against *M. phaseolina*. Organic fractions of CAI-21, CAI-26 and MMA-32, at 0.5% concentration inhibited the growth of *M. phaseolina* by 55 to 65%, whereas aqueous fractions enhanced the growth of the fungus by 33 to 64%, over the control (Figure 2).

Molecular identification of the actinomycete isolates

In order to determine the identity of the eight potential PGP and antagonistic (against *M. phaseolina*) actinomycetes, its 16S rDNA was sequenced and analyzed. A neighbor joining dendrogram was generated using the sequences from the eight actinomycetes (1400 bp) and

Table 4. Evaluation of the eight most potential actinomycetes for their plant growth promoting potential, in sorghum under green house conditions.

| Treatment | Shoot weight (g) | Root weight (g) | Root length (cm) | Root volume (cm ⁻³) | Shoot root ratio | % Increase over control | | | |
|-----------|---------------------|---------------------|--------------------|---------------------------------|----------------------|-------------------------|-------------|-------------|-------------|
| | | | | | | Shoot weight | Root weight | Root length | Root volume |
| CAI-17 | 6.67 | 0.75 | 431 | 14.6 | 9.0 | 19 | 19 | 7 | 0 |
| CAI-21 | 7.36 | 0.88 | 421 | 17 | 8.6 | 31 | 40 | 4 | 16 |
| CAI-26 | 6.16 | 0.81 | 575 | 21.2 | 7.7 | 10 | 29 | 42 | 45 |
| CAI-68 | 6.44 | 0.64 | 405 | 15.5 | 10.5 | 15 | 2 | 0 | 6 |
| CAI-78 | 6.52 | 0.72 | 611 | 19.2 | 9.8 | 16 | 14 | 51 | 31 |
| KAI-26 | 7.50 | 0.67 | 472 | 16.1 | 12.2 | 34 | 6 | 17 | 10 |
| KAI-27 | 6.25 | 0.68 | 483 | 16 | 8.9 | 11 | 8 | 20 | 10 |
| MMA-32 | 7.14 | 0.99 | 647 | 23.9 | 7.2 | 27 | 57 | 60 | 64 |
| Control | 5.61 | 0.63 | 404 | 14.6 | 8.9 | | | | |
| Mean | 6.69 | 0.74 | 486 | 17.3 | 9.5 | 21 | 20 | 23 | 20 |
| SE± | 0.256 ^{NS} | 0.063 ^{**} | 65.17 [*] | 2.490 ^{NS} | 0.851 ^{***} | | | | |
| LSD (5%) | 0.728 | 0.181 | 185.13 | 7.074 | 2.420 | | | | |
| CV% | 9 | 21 | 33 | 35 | 22 | | | | |

Values are means of six replications and data calculated per plant after 60 DAS. * = statistically significant at 0.05; ** = statistically significant at <0.01; *** = statistically significant at <0.001; SE = standard error; LSD = least significant difference; CV = coefficient of variance.

representative sequences from the databases. Phylo-genetic analysis of 16S rDNA sequences of the eight actinomycete isolates matched with *Streptomyces* but it was not possible to identify up to species level (Figure 3).

DISCUSSION

Vermicomposts have been widely used in agriculture not only for its beneficial effects on soil structure and biota but also for its ability to inhibit plant pathogens, for example, Ascituo et al. (2006) reported that vermicompost at 25% controlled *Rhizoctonia solani* that causes damping off in Patience-plant (*Impatiens walleriana*). Hence, in the present investigation, various herbal vermicomposts were screened for isolating

antagonistic actinomycetes against *M. phaseolina*. Of the 137 actinomycetes obtained from the herbal vermicomposts, 79 (58%) of them were found to have the antagonistic potential (in the dual-culture assay) against *M. phaseolina*. This inhibition could be due to the production of hydrolytic enzymes or antibiotics by the actinomycetes which were disseminated through the media. Therefore, it can be concluded that production of antifungal substances may be involved in the inhibition of hyphal growth of fungal pathogens, as there was no direct contact between fungal pathogens and actinomycete colonies in the dual-culture assay. The eight most promising isolates (CAI-17, CAI-21, CAI-26, CAI-68, CAI-78, KAI-26, KAI-27 and MMA-32) (Table 2) were further characterized for siderophore, HCN, IAA, chitinase and protease. Siderophores

are usually produced by various soil microbes including actinomycetes to bind Fe³⁺ from the environment and make it available for its own growth; plants also utilize these as an iron source (Wang et al., 1993). Actinomycetes (*Streptomyces* spp.) isolated from rhizosphere soil have been reported to produce siderophores and inhibit the growth of phytopathogens (Tokala et al., 2002). Actinomycetes found in the rhizosphere, the need to compete with other rhizosphere plant pathogens for iron, hence competition for iron is also a possible mechanism to control the phytopathogens. HCN production is also reported to play a role in disease suppression (Wei et al., 1991), for instance, Haas et al. (1991) reported HCN production by strains of *Pseudomonas fluorescens* that helped in suppression of black root rot of tobacco. In the present investigation,

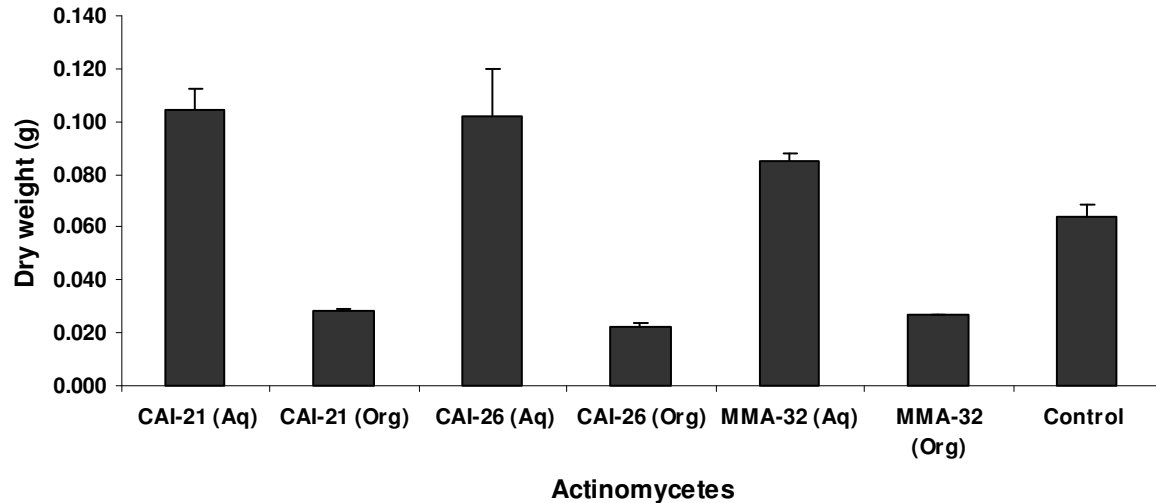


Figure 2. The effect of organic (Org) and aqueous (Aq) fractions (of solvent partitioning) of the culture filtrates of actinomycetes (CAI-21, CAI-26 and MMA-32) on the growth of *Macrophomina phaseolina*, compared to the control (Gopalakrishnan et al., 2011).

all the eight antagonistic actinomycetes produced HCN, while seven of them produced siderophore (except CAI-78). Hence, it can be concluded that the isolates have good biocontrol potential (Table 2).

IAA-producing microorganisms are known to promote root elongation and plant growth (Patten and Glick, 2002). By producing the plant hormones, microorganisms stimulate plant growth in order to increase the production of plant metabolites which can be beneficial for their growth. In the present study, all the eight actinomycetes produced IAA (Table 2), suggesting that these isolates could be used for plant growth promotion. Of the eight isolates studied, MMA-32 produced the highest IAA ($4.66 \mu\text{g ml}^{-1}$) and thus can be exploited by the host plants. In the present study, it was also noticed that seven actinomycetes produced protease (except KAI-27) but only four produced chitinase (CAI-26, KAI-26, KAI-27 and MMA-32). Protease and chitinase-producing microorganisms play an important role in the decomposition of organic matter, nutrient mineralization and promoting plant growth in addition to act as biocontrol agents on protein cell wall-bearing pathogens such as *Phytophthora* and *Pythium* spp. (Lima et al., 1998). When the eight potential actinomycete isolates, against *M. phaseolina* in the dual culture assay were evaluated by blotter paper assay on sorghum plants, KAI-26 and KAI-27 completely inhibited the growth of *M. phaseolina* (no infection or charcoal-rot disease symptoms were found in the sorghum roots), while all the other isolates inhibited the fungus greatly (47 to 77% lesser infection compared to the control) (Figure 1). In the green house experiment, where the eight isolates were evaluated for their antagonistic and PGP potential on sorghum plants, shoot dry mass (up to 34% increase), root dry mass (up to 57% increase), root length (up to 60% increase) and root

volume (up to 64% increase) were found higher in comparison with the control (Table 4). The mechanism by which the actinomycetes enhanced sorghum seedlings vigour could be its PGP attributes (IAA and siderophore production). As in the present study, all the actinomycetes isolates produced IAA and siderophore (except CAI-78) (Table 2). Root colonization is very much essential to deliver the beneficial microbes at the right place and time on the root, as poor root colonization may result in decreased biocontrol activity (Schippers et al., 1987). Ryder and Jones (1993) reported that the ability of the antagonistic microorganisms to maintain a sufficient population density in the rhizosphere for a sufficient length of time is critical for the success of any biocontrol method. Lugtenberg and Dekkers (1999) suggested that selection of any potent strain useful for biocontrol must be based on the rhizosphere competence, antagonistic ability and the root-colonizing ability.

Observation on root colonization was not done in this study; however, upon looking at the data on biomass of plants (up to 34% increase in shoot mass and up to 64% increase in root mass in actinomycete inoculated treatments) it can be hypothesized that actinomycetes might have multiplied and colonized on sorghum roots, a property desirable for survival and functioning of a biocontrol agent. The eight actinomycetes used in this study were apparently well adapted to the sorghum rhizosphere environment, as it not only controlled the charcoal-rot disease in sorghum but also enhanced the plant growth. Adhikari et al. (2001) reported that bacterial strains isolated from rice rhizosphere have the potential to control the seedling disease of rice (caused by *Achlya klebsiana* and *Pythium spinosum*) and to promote plant growth. Similar results were obtained with the plant growth-promoting *Bacillus subtilis* BN1 from the

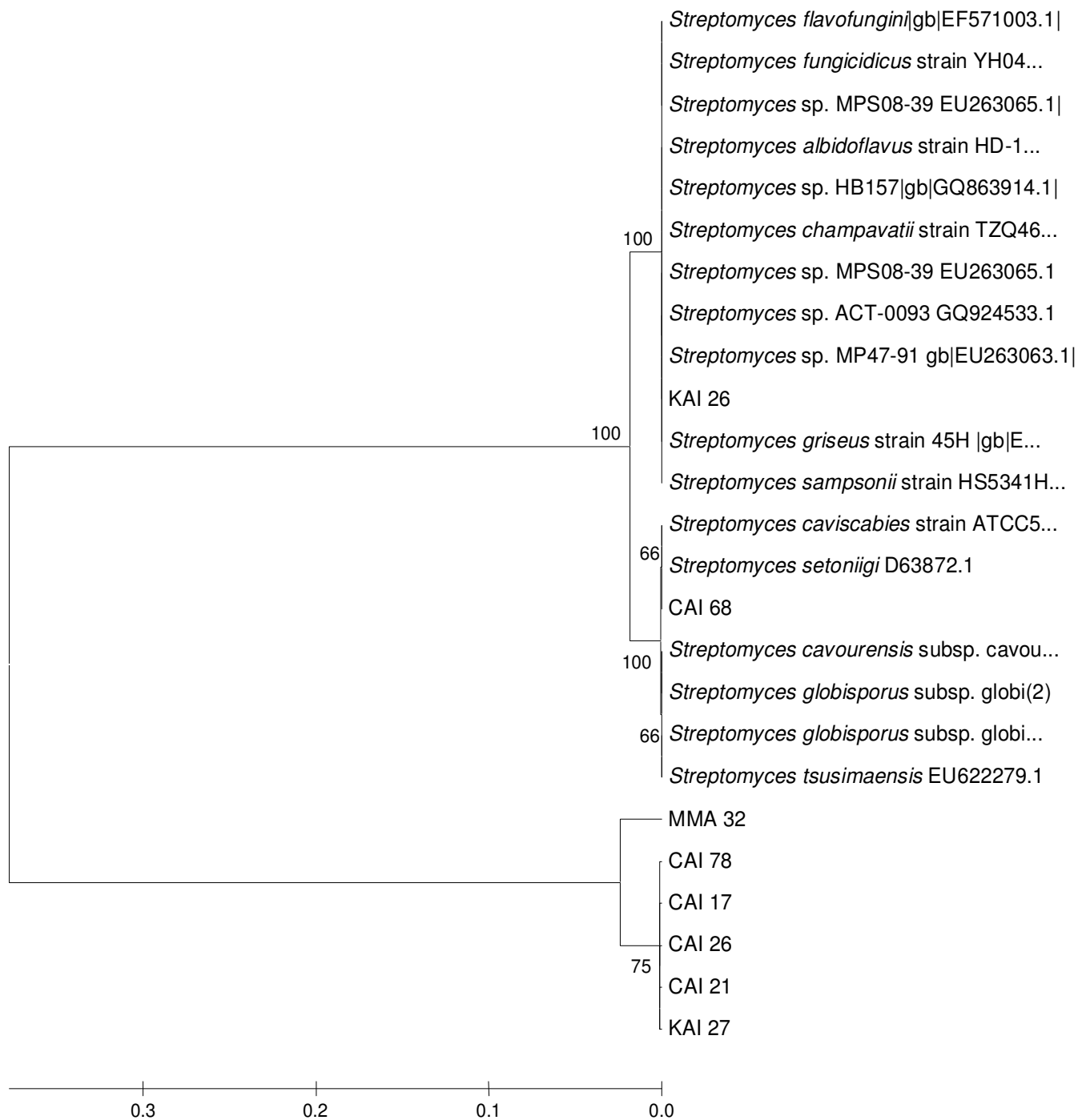


Figure 3. Phylogenetic relationship between actinomycete isolates and representative species based on full length 16S rDNA sequences constructed using the neighbor-joining method. The scale bar indicates the genetic distance and the numbers shown next to each node indicate the bootstrap values from 500 replications (Gopalakrishnan et al., 2011).

rhizospheres of chir pine (*Pinus roxburghii*) (Singh et al., 2008), fluorescent *Pseudomonas* GRC₂ from potato rhizosphere (Gupta et al., 2002) and *Pseudomonas chlororaphis* SRB 127 from sorghum rhizosphere (Das et al., 2008) that showed strong antagonistic effect against *M. phaseolina*, a charcoal-rot pathogen of peanut and

sorghum. However, there were no reports of actinomycetes isolated from vermicompost on *M. phaseolina*. The organic fractions of the culture filtrates (ethyl acetate extract) of the three promising actinomycetes, CAI-21, CAI-26 and MMA-32, at 0.5% inhibited the growth of *M. phaseolina* by 55 to 65%, whereas the aqueous fractions

of the culture filtrates enhanced the growth of the pathogen by 33 to 64% (Figure 2). Based on these results, it can be concluded that the organic fractions of the culture filtrates contain secondary metabolites that are capable of inhibiting the pathogen, while the aqueous fractions contain metabolites that are capable of enhancing the growth of the pathogen. Remya and Vijaykumar (2008) reported that ethyl acetate extract of *Streptomyces* strain RM 42 showed maximum activity against *E. coli*, followed by *Salmonella typhi*, *Candida albicans* and *B. subtilis*. The culture filtrates of *Streptomyces hygroscopicus* were found to inhibit *S. rolfsii* and *Colletotrichum gloeosporioides*, which cause collar rot and anthracnose, respectively in a wide variety of crops (Prapagdee et al., 2008).

Phylogenetic analysis of 16S rDNA sequences of the eight *M. phaseolina* antagonistic actinomycetes showed that all the isolates belong to the genus *Streptomyces* but it was not possible to identify up to the species level (Figure 3). *Streptomyces* spp. has been shown to protect many crop plants against pathogenic fungi (Liu et al., 1996). For instance, *S. hygroscopicus* antagonized *R. solani*, the causal organism of pea root rot (Rothrock and Gottlieb, 1984) and *Streptomyces* spp. strain g10 exhibited strong antagonism towards *Fusarium oxysporum* f. sp. *cubense*, causal organism of wilt in banana (Getha et al., 2005). *Streptomyces* spp. were also found to control FOC, for example *Streptomyces rochei* and *Streptomyces rimosus* isolated from the chickpea rhizosphere were found to have antagonistic potential against FOC (Bashar and Rai, 1994) and *Streptomyces* spp. isolated from national parks in Kenya were shown to have antifungal activity against FOC (Nonoh et al., 2010). The actinomycetes present in the rhizosphere soil that are antagonistic against the plant pathogens *Alternaria brassicicola*, *Collectotrichum gloeosporioides*, *F. oxysporum*, *Penicillium digitatum* and *S. rolfsii* have been shown to belong to *Streptomyces* spp. (Khamna et al., 2009).

The present study was successful in selecting effective isolates of actinomycetes, from herbal vermicomposts, that can be a useful component of integrated disease management. All the eight actinomycetes could be used as biocontrol agents for the control of charcoal-rot of sorghum. The broad range of antifungal activity of the eight antagonistic actinomycetes demonstrates the multiple mechanisms of action (antibiosis, HCN, siderophore, IAA and cell wall degrading enzymes) (Table 2) and hence may involve more than one antifungal metabolite. Ethyl acetate extract of the culture filtrates of CAI-21, CAI-26 and MMA-32 contains secondary metabolites that are capable of inhibiting *M. phaseolina*. Hence, these actinomycetes are likely to be the potential candidates for discovery of novel secondary metabolites for various biocontrol applications. Determination of the exact mechanisms of action of these biocontrol agents can assist in furthering the use of eco-friendly bio-fungicides.

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