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Evaluation of actinomycete isolates obtained from herbal vermicompost for the biological control of Fusarium wilt of chickpea

Subramaniam Gopalakrishnan*, Suresh Pande, Mamta Sharma, Pagidi Humayun, Bandru Keerthi Kiran, Dasyam Sandeep, Meesala Sree Vidya, Kanala Deepthi, Om Rupela

Global Theme-Crop Improvement, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru 502 324, Andhra Pradesh, India

* Corresponding author. Tel.: +91 40 3071 3610; fax: +91 40 3071 3074

E-mail address: s.gopalakrishnan@cgiar.org (S. Gopalakrishnan)

ABSTRACT

A total of 137 actinomycetes cultures, isolated from 25 different herbal vermicomposts, were characterized for their antagonistic potential against *Fusarium oxysporum* f. sp. *ciceri* (FOC) by dual-culture assay. Of the isolates, five most promising FOC antagonistic isolates (CAI-24, CAI-121, CAI-127, KAI-32 and KAI-90) were characterized for the production of siderophore, cellulase, protease, hydrocyanic acid (HCN), indole acetic acid (IAA) and antagonistic potential against *Rhizoctonia bataticola*, which causes dry root rot in chickpea (three strains viz. RB-6, RB-24 and RB-115) and sorghum (one strain). All of the five FOC antagonistic isolates produced siderophore and HCN, four of them (except KAI-90) produced IAA, KAI-32 and KAI-90 produced cellulase and CAI-24 and CAI-127 produced protease. In the dual-culture assay, three of the isolates, CAI-24, KAI-32 and KAI-90, also inhibited all three strains of *R. bataticola* in chickpea, while two of them (KAI-32 and KAI-90) inhibited the lonely strain in sorghum. When the FOC antagonistic isolates were evaluated further for their antagonistic potential in the greenhouse and wilt-sick field conditions on chickpea, 45-76% and 4-19% reduction of disease incidence were observed, respectively compared to the control. The sequences of 16S rDNA gene of the isolates CAI-24, CAI-121, CAI-127, KAI-32 and KAI-90 were matched with *Streptomyces tsusimaensis*, *S. caviscabies*, *S. setonii*, *S. africanus* and an identified species of *Streptomyces*, respectively using the BLAST searching. This study indicated that the selected actinomycete isolates have the potential for biological control of Fusarium wilt disease in chickpea.

*Keywords***:** Antagonistic actinomycetes; Biocontrol; Chickpea; Wilt; *Fusarium oxysporum* f. sp. *ciceri*; *Streptomyces* spp.

1. Introduction

Fusarium wilt, caused by *Fusarium oxysporum* Schl. emend. Snyd. and Hans. f. sp. *ciceri* (Padwick; FOC), is regarded as one of the most important diseases of chickpea. Other species and formae

speciales of *Fusarium* also cause wilt in chickpea and produce mycotoxins (Di Pietro et al., 2003; Gopalakrishnan and Strange, 2005; Gopalakrishnan et al., 2005). FOC may survive in soil and on crop residues as chlamydospores for up to six years in the absence of susceptible host, and spread by means of both soil and infected seed (Haware et al., 1978). FOC is a major threat to chickpea production in the Indian subcontinent and Mediterranean basin (Trapero-Casas and Jimenez-Diaz, 1985; Jalali and Chand, 1992). Fusarium wilt is prevalent in almost all chickpea-growing areas of the world, and its incidence varied from 14% to 32% in the different states of India (Dubey et al., 2010). This disease causes yield losses up to 100% under favorable conditions in chickpea (Anjaiah et al., 2003; Landa et al., 2004).

Management of *Fusarium* wilt of chickpea is difficult, as no single control measure is fully effective. Solarization of soil, advanced sowing date, use of FOC-free seed and fungicide-treated seed are some of the measures usually employed to control Fusarium wilt in chickpea, but with limited success (Jalali and Chand, 1992; Haware et al., 1996; Navas-Cortes et al., 1998). The use of resistant cultivar is the most economical and efficient control measure but the effectiveness of Fusarium wilt resistance is restricted by the occurrence of eight pathogenic races in FOC, due to evolution of variability in the pathogen (Haware and Nene, 1982; Jimenez-Gasco and Jimenez-Diaz, 2003).

Currently, biological control of this soil and seed-borne plant pathogenic fungi has been addressed using bacterial and fungal antagonists. Strains of *Pseudomonas* spp., *Bacillus* spp., *Trichoderma* spp. and non-pathogenic isolates of *F. oxysporum*, isolated from the rhizospheres of crop plants and composts, were shown effective not only to control plant pathogens but also in helping the plants to mobilize and acquire nutrients (Glick, 1995; Burr et al., 1998; Postma et al., 2003; Khan et al., 2004; Perner et al., 2006). Such novel microorganisms, with plant growth-promoting and biocontrol traits, are found in much higher levels in forest, pasture soils and herbal compost than in arable soils (Torsvik et al., 2002; Tinatin and Nurzat, 2006).

There is a growing interest in the use of secondary metabolites, such as toxins, proteins, hormones, vitamins, amino acids and antibiotics from microorganisms, particularly from actinomycetes, for the control of plant pathogens as these are readily degradable, highly specific and less toxic to nature (Doumbou et al., 2001). It is a well-known fact that actinomycetes are found most common in compost and play an important role not only in the decomposition of organic materials but also in their ability to produce

secondary metabolites of pharmacological and commercial interest. Hence, in the present investigation, several herbal vermicomposts were screened for actinomycetes that contain antagonistic potential against Fusarium wilt of chickpea. The objective of this study was to characterize and evaluate the actinomycetes isolated from vermicomposts for their ability to suppress FOC under both green house and field conditions.

2. Materials and methods

2.1. Preparation of herbal vermicompost samples

Foliages of 25 different plant species (*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 ± 2 ^{o}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 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 herbals were digested. When the herbal compost was ready, about 100 g of the sample was collected and stored in a refrigerator at 4 ^{o}C for further studies.

2.2. Isolation of actinomycete strains

Ten grams of herbal vermicompost from each sample were separately suspended in 90 ml of physiological saline (0.85% of NaCl) in a flask and placed on an orbital shaker (at 100 rpm) at room temperature (28 ± 2 ^{o}C) for 1 h. At the end of shaking, the vermicompost samples were serially diluted up to 10^6 dilutions with physiological saline. Dilutions 10^4–10^6 were plated on starch casein agar (SCA) by spread plate technique and incubated at 28 ± 2 ^{o}C for four days. The most prominent 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 ^{o}C for further studies.

2.3. In vitro antifungal activity

Actinomycete isolates were evaluated for their antifungal activity against FOC (Race 1; causal agent of Fusarium wilt in chickpea), *Rhizoctonia bataticola* (Taub) Butter (three strains viz. RB-6, RB-24 and RB-115; causal agent of dry root rot in chickpea) and *Macrophomina phaseolina* (Tassi) Goid. (causal agent of charcoal rot in sorghum) by dual-culture assay. FOC and all the three strains of *R. bataticola* were acquired from the Legumes Pathology Division, ICRISAT, Patancheru, India and *M. phaseolina* was acquired from the Directorate of Sorghum Research, Hyderabad, India. A fungal disk (FOC/*R. bataticola*) of 6 mm diameter was placed on one edge (1 cm from the corner) of the glucose casamino acid yeast extract (GCY agar; Anjaiah et al., 1998) plate, and actinomycete isolate was streaked on the other edge of the plate (1 cm from the corner), followed by incubation at 28 ± 2 ^{o}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.

2.4. Enzymatic activities and secondary metabolite production by the actinomycete isolates

2.4.1. 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 ±

2 ^{o}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 0–4 rating scale as follows: 0 = no change; 1 = positive; 2 = halo zone of 1–3 mm; 3 = halo zone of 4–6 mm and 4 = halo zone of 7 mm and above.

2.4.2. Cellulase production

The standardized protocols of Hendricks et al. (1995) were used to evaluate the cellulase production. Actinomycetes were streaked on cellulose Congo red agar media and incubated at 28 ± 2 °C for four days. The plates were observed for halo zone around the actinomycete colonies, which indicate the presence of cellulase. Observations were recorded on a 0–4 rating scale as follows: 0 = no change; 1 = positive; 2 = halo zone of 1–3 mm; 3 = halo zone of 4–6 mm and 4 = halo zone of 7 mm and above.

2.4.3. Protease production

It was done as per the protocols of Bhattacharya et al. (2009). Actinomycetes were streaked on casein agar and incubated at 28 ± 2 °C for four days. At the end of the incubation, the plates were observed for halo zone around the colonies, which indicates the presence of protease. Observations were recorded on a 0–4 rating scale as follows: 0 = no change; 1 = positive; 2 = halo zone of 1–3 mm; 3 = halo zone of 4–6 mm and 4 = halo zone of 7 mm and above.

2.4.4. Hydrocyanic acid (HCN) production

HCN was estimated qualitatively by the sulfocyanate colorimetric method (Lorck, 1948). The actinomycetes were grown in 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 ± 2 °C for four days. Development of reddish brown color on the filter

paper indicated positive for HCN production. Observations were recorded (by a panel of three observers) on a 0–3 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.

2.4.5. 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 µg ml^{-1}) for four days. At the end of the incubation, the cultures were centrifuged at 10,000*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 ± 2 ºC for 30 min. At the end of the incubation, development of pink color indicated the presence of IAA. Quantification of IAA was done my measuring the absorbance in a spectrophotometer at 530 nm. A standard curve was plotted to quantify the IAA (µg ml^{-1}) present in the culture filtrate.

2.5. Evaluation of actinomycetes under greenhouse conditions

The five most potential antagonistic actinomycete isolates (CAI-24, CAI-121, CAI-127, KAI-32 and KAI-90) against FOC from the in vitro studies were evaluated individually for their antagonistic potential in pots in the greenhouse. Each actinomycete isolate was inoculated by five different methods viz. M1 = inoculation of the potting mixture with respective actinomycete culture along with FOC two weeks before sowing; M2 = inoculation of the seeds by soaking in the respective actinomycete culture for 1 h; M3 = inoculation of the sprouted seeds by soaking in the respective actinomycete culture for 1 h; M4 = inoculation of the potting mixture with actinomycete culture at the time of sowing (10 ml of well-grown actinomycete culture [10^8 CFU ml^{-1}] was applied on the seed and covered with soil) and M5 = inoculation of the seedlings after emergence with actinomycete culture (10 ml of well-grown actinomycete culture [10^8 CFU ml^{-1}] was applied on the seedlings after emergence). Thus the combination of actinomycetes isolates × five methods of inoculation constituted 25 independent treatments. Also, there were seven more treatments as various controls were included as follows: treatments 26–30: the potting mixtures inoculated with each

actinomycete isolate individually two weeks before sowing, 31: the potting mixtures inoculated only with FOC 2 weeks before sowing and 32: the potting mixture treated only with 200 ml sterilized water two weeks before sowing (negative control). The experiment had six replications.

FOC inoculum was mass-multiplied on chickpea grains (variety JG-62, highly susceptible to Fusarium wilt, acquired from the Legumes Pathology Division, ICRISAT) using 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 (w/w) and was filled in 8 inches plastic pots followed by inoculation with FOC inoculum (20% of pot weight, 200 g pot^{-1}). In the M1 treatment, respective actinomycetes (grown in starch casein broth for 4 days) were inoculated (20% of pot weight; 200 ml pot^{-1}, 10^8 CFU ml^{-1}) along with the FOC, whereas in the other treatment pots 200 ml of sterile water was added. Inoculum was thoroughly mixed with the pot mixture and the pots were covered with polythene sheets. The whole set-up was incubated at 26 ± 2 ^{o}C for 15 days to have Fusarium wilt sick conditions. Two weeks later, the seeds of chickpea variety JG-62 were surface-sterilized with 2.5% sodium hypochlorite solution for 5 min and rinsed with sterilized water (8 times). The surface-sterilized seeds were treated with respective actinomycetes and/or FOC (as discussed earlier). Six such seeds were sown in each pots and one week later thinned to retain three seedlings. Plants were irrigated once every two days with 20 ml sterilized distilled water. Incidence of *Fusarium* wilt disease (number of plants showing wilt symptoms to the total number of plants in a pot) was recorded on 5, 10, 15, 20 and 29 days after sowing (DAS). In addition, actinomycetes population was enumerated on day 29 in the negative control, in the actinomycetes control (where no FOC was inoculated) and in the treatments (where both FOC and actinomycetes were inoculated) on SCA plate, as explained earlier.

2.6. Evaluations of actinomycetes in Fusarium wilt sick field

The five most potential antagonistic actinomycetes from the in vitro and greenhouse studies were further evaluated individually for their antagonistic potential in Fusarium-infested field at ICRISAT, Patancheru, during 2009–10 cropping seasons. The field was maintained as wilt sick plot since 1980 (Nene et al., 1981). Each actinomycete isolate was inoculated by four different methods viz. M1 = inoculation of the seeds by soaking in the respective actinomycete culture for 1 h; M2 = inoculation of the sprouted seeds

by soaking in the respective actinomycete culture for 1 h; M3 = inoculation of the soil with respective actinomycete culture (5 ml per seed, 10^8 CFU ml^{-1}) at the time of sowing and M4 = inoculation of the seedlings after emergence with the respective actinomycete culture (5 ml per seedling, 10^8 CFU ml^{-1}). Thus, the combination of actinomycete isolates × four methods of inoculation constituted 20 independent treatments in addition to one positive control, where no actinomycete was inoculated. Each treatment was replicated three times in randomized complete block design (RCBD) and the plot size was 3 rows of 2 m long with a row spacing of 30 cm and a plant-to-plant spacing of 10 cm. Chickpea seeds of a Fusarium wilt highly susceptible cultivar JG-62 (acquired from the Legumes Pathology Division, ICRISAT), were surface-sterilized with sodium hypochlorite (2.5% for 5 min) and rinsed with sterilized water (eight times) before being sown into the field. During the cropping season, a maximum temperature range between 30.1 ^oC and 34.3 ^oC and a minimum temperature range of between 9.2 ^oC and 16.2 ^oC were recorded. Incidence of Fusarium wilt disease (number of plants showing wilt symptoms to total number of plants in a plot) was recorded on 17, 21, 24 and 28 DAS till the susceptible check showed 100% mortality. Actinomycete population was also enumerated, as explained earlier, from the rhizosphere soils at 28 DAS for all the treatments.

2.7. Molecular identification of the isolates

Pure cultures of potential FOC antagonistic actinomycetes were grown in starch casein broth until log phase (four days) and genomic DNA was isolated according to Bazzicalupo and Fani (1995). The amplification of 16S rDNA gene was done using universal primers 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3') and 27F (5'- AGA GTT TGA TCM TGG CTC AG-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 (Alschul 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 using MEGA version 4 program (Tamura et al., 2007) for 1000 replicates was performed to attach confidence estimates for the tree topologies.

2.8. Statistical analysis

All the greenhouse experiments were arranged in completely randomized block design with six replications. Field experiment was conducted in a completely randomized block design with three replications. Dual-culture assay was conducted in completely randomized design with three replications [For FOC: 411 Petri plates were used (137 actinomycetes x 3 replications); for *M. phaseolina* (1 strain) and *R. bataticola* (3 strains): 60 plates were used (5 actinomycetes x 3 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 biocontrol agent's application in the greenhouse studies. Significance of differences between the treatment means was tested at $P = 0.01$ and 0.05.

3. Results

3.1. Selection of antagonistic actinomycete isolates

A total of 137 actinomycetes, the most prominent isolates (the ones which were found abundantly, produced pigments and inhibited the adjacent colonies) in the SCA plate, were isolated from the 25 different herbal vermicompost samples and further screened for their antagonistic potential against FOC by *in vitro* dual-culture assay. Of the 137 actinomycete isolates, only 33 of them were found to have the antagonistic potential against FOC. When the five most potential FOC antagonistic actinomycetes [compost actinomycete isolate (CAI) -24, CAI-121, CAI-127, and Karnataka actinomycete isolate (KAI) -32 and KAI-90; all originated from the 25 different herbal vermicomposts] were evaluated further for their antagonistic potential against *M. phaseolina* and *R. bataticola* in the dual-culture assay, two isolates (KAI-32 and KAI-90) inhibited *M. phaseolina*, while three isolates (CAI-24, KAI-32 and KAI-90) inhibited all the three strains (RB-6, RB-24 and RB-115) of *R. bataticola* (Table 1).

3.2. Enzymatic activities and secondary metabolite production by the actinomycetes isolates

When the five FOC antagonistic actinomycetes were evaluated for their enzymatic activities and secondary metabolite production, all the five isolates produced siderophore, HCN and IAA (except KAI-90), whereas two isolates produced cellulase (KAI-32 and KAI-90) and protease (CAI-24 and CAI-127) (Table 1). Isolate CAI-24 produced the maximum IAA with 43.7 µg ml^{-1} of culture filtrate, 8–10 times higher than the other positive isolates (Table 1).

3.3. *Evaluation of actinomycetes in greenhouse conditions*

When the five FOC antagonistic actinomycetes were evaluated for their antagonistic potential in greenhouse, 45–76% reduction in Fusarium wilt incidence was observed at 29 DAS over the FOC-inoculated control (Fig. 1). In the FOC-inoculated control, 100% disease incidence was observed within 20 DAS itself. Reduction of wilt incidence was found the maximum in CAI-24 (76%) followed by CAI-127 (72%), CAI-121 (67%), KAI-32 (56%) and KAI-90 (45%) (Fig. 1). CAI-24 acted well with only M1, M2 and M5 methods of actinomycete inoculation (22–76% reduction of disease incidence over the control), whereas the other isolates acted well with all the five methods of actinomycete inoculation (CAI-121, CAI-127, KAI-32 and KAI-90) reduced the disease incidence by 22–67%, 11–72%, 22–56% and 22–45%, respectively (Fig. 1). Actinomycetes population in the rhizosphere soils of both the treatments (where FOC was inoculated and not inoculated) were about 8 Log_{10} values (Fig. 2).

3.4. Evaluation of actinomycetes in Fusarium wilt sick field

When the five potential FOC antagonistic actinomycetes were further evaluated in wilt stick field conditions, a reduction of Fusarium wilt incidence (4–19%) was observed at 28 DAS over the control, where no actinomycetes were inoculated (Fig. 3). In the control, 100% disease incidence could be noticed by 20 DAS itself. Reduction of wilt disease incidence was found maximum with CAI-24 that was up to 25% at 24 DAS and 15% at 28 DAS. The next one being KAI-90 with 22% reduction at 24 DAS and 19% at 28 DAS with the M1 (seed inoculation) method. The other three isolates (CAI-121, CAI-127 and KAI-32) showed lower levels of reduction of wilt disease incidence (up to 18% at 24 DAS and 10% at 28 DAS)

over the control (Fig. 3). At 30 DAS, when the population of actinomycetes was enumerated from the rhizosphere soils, no actinomycete was found in the control plots, whereas actinomycetes (up to 10^6 values) were found in actinomycete-inoculated plots (Fig. 4).

3.5. Molecular identification of the actinomycete isolates

In order to determine the identity of the five potential FOC antagonistic actinomycetes, its 16S rDNA was sequenced and analyzed. A neighbor-joining dendrogram was generated using the sequence from the five FOC antagonistic actinomycetes (1,400 bp) and representative sequences from the databases. Phylogenetic analysis of 16S rDNA sequences of the five isolates showed that CAI-24 had maximum sequence similarity (98%) with *Streptomyces* spp. M10 and *S. tsusimaensis*, whereas CAI-121, CAI-127 and KAI-32 showed maximum sequence similarity (98%) with *S. caviscabies*, *S. setonii* and *S. africanus*, respectively (Fig. 5). The fifth isolate, KAI-90, was identified only up to the genus level, *Streptomyces* spp., as its sequences did not match with any of the species in the databases (Fig. 5).

4. Discussion

Of the 137 actinomycete isolates obtained from the herbal vermicomposts, only 33 of them (24%) were found to have the antagonistic potential against FOC. The isolates which showed more than 10 mm inhibition zone (CAI-24, CAI-121, CAI-127, KAI-32 and KAI-90) were further evaluated for siderophore, HCN, cellulase, protease and IAA production. Siderophores are usually produced by various soil microbes including actinomycetes to bind Fe^{3+} 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 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, all the five FOC antagonistic actinomycetes produced

siderophore and HCN and hence it can be concluded that actinomycetes have good biocontrol potential (Table 1).

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, four out of five FOC antagonistic actinomycetes (except KAI-90) produced IAA (Table 1), suggesting that these isolates could be used for plant growth promotion. Of the five isolates studied, CAI-24 produced 8–10 times higher IAA than the other isolates and thus can be exploited by the host plants.

In the present study, it was also noticed that two of the five FOC antagonistic actinomycetes produced cellulase (KAI-32 and KAI-90) and protease (CAI-24 and CAI-127). Cellulose is the most abundant polysaccharide (20–50%) present in the plant biomass which can be degraded by microbial enzymes such as cellulase (Lynd et al., 2002). Hence, protease and cellulase-producing microorganisms play an important role in the decomposition of organic matter, nutrient mineralization and promoting plant growth. In addition, these also act as biocontrol agents on cellulose and protein cell wall-bearing pathogens such as *Phytophthora* and *Pythium* spp. (Lima et al., 1998).

When the five actinomycetes were further evaluated for their antagonistic potential against *M. phaseolina* and *R. bataticola* in the dual-culture assay, two isolates (KAI-32 and KAI-90) inhibited *M. phaseolina*, while three isolates (CAI-24, KAI-32 and KAI-90) inhibited all the three strains of *R. bataticola* (Table 1). From these results, it can be concluded that KAI-32 and KAI-90 have good antagonistic potential as these inhibited the three tested pathogens. Further, the 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.

In the greenhouse experiment, the five actinomycetes showed 45–76% reduction in Fusarium wilt incidence at 29 DAS over the FOC-inoculated control, where 100% disease incidence was observed within 20 DAS (Fig. 1). In order to know whether the introduced actinomycetes were able to colonize in the chickpea rhizosphere, actinomycetes were enumerated at 30 DAS. Actinomycetes count in the treatments

where FOC was inoculated (FOC+) was equally good (8 $\log_{10}$) and comparable with the counts from the treatments where FOC was not inoculated (FOC-), indicating that the introduced actinomycetes survived, competed with the FOC, colonized in the rhizosphere and controlled the disease (Fig. 2).

It has been shown in a number of studies that effective suppression of disease can only be obtained under low to moderate disease pressure or environmental conditions moderately favorable for disease development. For instance, Hervas et al. (1997, 1998) reported that *Pseudomonas putida* WCS358, *P. fluorescens* WCS374, non-pathogenic *F. oxysporum* Fo47 and *F. oxysporum* Fo 90105 reduced Fusarium wilt of chickpea when the disease incidence and the pathogen inoculum density were low. Ryder and Jones (1993) reported that the ability of the antagonistic microorganism 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 root-colonizing ability.

In the present study, the field evaluation was conducted in a 30-year-old wilt sick field where environmental conditions were highly conducive for Fusarium wilt development but the five FOC antagonistic actinomycetes were able to delay the disease onset and control the disease to a greater extent. The five FOC antagonistic actinomycetes showed 4–19% reduction in wilt incidence at 28 DAS over the control (where 100% disease incidence was observed within 20 DAS itself), and in the rhizosphere soils (top 15cm soil at 30 DAS) no actinomycete was found in the control plots, whereas actinomycetes up to 10^6 log values were found in the treatment plots (Figs. 3 and 4). Hence, it can be concluded that no native actinomycetes were present in the wilt sick rhizosphere soils, while the artificially inoculated actinomycetes survived in the field conditions, colonized in the chickpea rhizosphere soils, competed with the native FOC (that has been well established since 30 years) and controlled the wilt of chickpea to a greater extent. In a similar kind of field experiment in wilt sick plot, Landa et al. (2004) reported that biocontrol agents, *B. subtilis* GB02 and *P. fluorescencs* RG26, when applied alone and in combination with non-pathogenic *F. oxysporum* Fo 90105 delayed the disease onset and suppressed Fusarium wilt. However, field evaluation of antagonistic actinomycetes against Fusarium wilt is not reported anywhere.

Phylogenetic analysis of 16S rDNA sequences of the five FOC antagonistic actinomycetes showed that all the isolates belong to genus *Streptomyces* but different species (Fig. 5). *Streptomyces* spp. have been shown to protect many crop plants against pathogenic fungi (Liu et al., 1996). For instance, *S. hygroscopicus* antagonized *Rhizoctonia solani*, the causal organism of pea root rot (Rothrock and Gottlieb, 1984), and *Streptomyces* spp. strain g10 exhibited strong antagonism towards *F. oxysporum* f. sp. *cubense*, causal organism of wilt in banana (Getha et al., 2005). *Streptomyces* spp. were also found to control FOC, for example *S. rochei* and *S. 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 *Sclerotium rolfsii* have been shown to belong to *Streptomyces* spp. (Khamna et al., 2009). Non-streptomycete actinomycetes are also reported in the literature as biocontrol agents of soil-borne fungal plant pathogens and as plant growth promoters (El-Tarabily and Sivasithamparam, 2006); however, none of them was reported for controlling FOC.

The biological degradation and conversion of agricultural wastes by vermicomposting is becoming a preferable method of recycling wastes. Application of vermicompost and/or compost prepared from herbs not only promotes plant growth, as it contain beneficial microorganisms that help the plants to mobilize nutrients (Perner et al., 2006; Yin et al., 2011), but also inhibits plant pathogens e.g. vermicompost at 25% controlled damping off in Patience plant (*Impatiens wallerana*) caused by *R. solani* (Asciutto et al., 2006). In the present investigation, the five actinomycetes isolated from the vermicompost showed persistent antimicrobial activity against FOC in both greenhouse and wilt sick field conditions. The broad range of antifungal activity of the five antagonistic actinomycetes demonstrates the multiple mechanisms of action (antibiosis, HCN, siderophore, IAA and cell wall-degrading enzymes; Table 1) and hence may involve more than one antifungal metabolite. Therefore, 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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References

Alschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403-410.

Anjaiah, V., Cornelis, P., Koedam, N., 2003. Effect of genotype and root colonization in biological control of *Fusarium* wilts in pigeonpea and chickpea by *Pseudomonas auruginosa* PNAI. Can. J. Microbiol. 49, 85-91.

Anjaiah, V., Koedam, N., Nowak-Thompson, B., Loper, J.E., Hofte, M., Tambong, J.T., Cornelis, P., 1998. Involvement of phenazines and anthranilate in the antagonism of *Pseudomonas aeruginosa* PNA1 and Tn5 derivative toward *Fusarium* spp. and *Pythium* spp. Mol Plant Microbe Interact 11, 847-854.

Asciutto, K., Rivera, M.C., Wright, E.R., Morisigue, D., Lopez, M.V., 2006. Effect of vermicompost on the growth and health of *Impatiens wallerana*. Int. J. Exp. Bot. 75, 115-123.

Bashar, M.A., Rai, B., 1994. Antagonistic potential of root region microflora of chickpea against *Fusarium oxysporum* f. sp. *ciceri*. Bangl. J. Bot. 23, 13-19.

Bazzicalupo, M., Fani, R., 1995. The use of RAPD for generating specific DNA probes for microorganisms. In: Clap, J.P. (Ed.) Methods in molecular biology, species diagnostic protocols: PCR and other nucleic acid methods. Humana Press Inc, Totowa NJ, pp. 112-124.

Bhattacharya, A., Chandra, S., Barik, S., 2009. Lipase and protease producing microbes from the environment of sugar beet field. Ind. J. Agric. Biochem. 22, 26-30.

Burr, A., Ortuno, A., Armero, T., 1998. Phosphate solubilizing effect of *Aspergillus niger* and *Pseudomonas*. Microbiol. Espanola 30, 113.

Di Pietro, A., Madrid, M.P., Caracuel, Z., Delgado-Jarana, J., Roncero, M.I.G., 2003. *Fusarium oxysporum*: exploring the molecular arsenal of a vascular wilt fungus. Molec. Plant Pathol. 4, 315-325.

Doumbou, C.L., Salove, M.K., Crawford, D.L., Beaulieu, C., 2001. Actinomycetes, promising tools to control plant diseases and to promote plant growth. Phytoprotection 82, 85-102.

Dubey, S.C., Singh, S.R., Singh, B., 2010. Morphological and pathogenic variability of Indian isolates of *Fusarium oxysporum* f. sp. *ciceri* causing chickpea wilt. Arch. Phytopathol. PFL 43, 174-190.

El-Tarabily, K.A., Sivasithamparam, K., 2006. Non-streptomycete actinomycetes as biocontrol agents of soil-borne fungal plant pathogens and as plant growth promoters. Soil Biol. Biochem. 38, 1505-1520.

Getha, K., Vikineswary, S., Wong, W.H., Seki, T., Ward, A., Goodfellow, M., 2005. Evaluation of *Streptomyces* spp. strain g10 for suppression of *Fusarium* wilt and rhizosphere colonization in pot-grown banana plantlets. J. Ind. Microbiol. Biotechnol. 32, 24-32.

Glick, B.R., 1995. The enhancement of plant growth by free-living bacteria. Can. J. Microbiol. 41, 109-117.

Gopalakrishnan, S., Beale, M.H., Ward, J.L., Strange, R.N., 2005. Chickpea wilt: identification and toxicity of 8-O-methl-fusarubin from *Fusarium acutatum*. Phytochemistry 66, 1536-1539.

Gopalakrishnan, S., Strange, R.N., 2005. Identity and toxicity of *Fusarium* species isolated from wilted chickpea. Phytopathol. Mediterr. 44, 180-188.

Gupta, C.P., Dubey R.C., Maheswari, D.K., 2002. Plant growth enhancement and suppression of *Macrophomina phaseolina* causing charcoal rot of peanut by fluorescent *Pseudomonas*. Biol. Fertil. Soils 35, 399-405.

Haas, K., Keel, C., Laville, J., Maurhofer, M., Oberhansli, T.F., Schnider, U., Voisard, C., Wuthrich, B., Defago, G., 1991. Secondary metabolites of *Pseudomonas fluorescens* strain CHAO involved in the suppression of root diseases. In: Hennecks, H., Verma, D.P.S. (Eds.) Advances in molecular genetics of plant-microbe interactions. Interlaken, Switzerland. pp. 450-456.

Haware, M.P, Nene, Y.L., 1982. Races of *Fusarium oxysporum* f. sp. *ciceri*. Plant Dis. 66, 809-810.

Haware, M.P., Nene, Y.L., Natarajan, M., 1996. Survival of *Fusarium oxysporum* f. sp. *ciceri* in soil in the absence of chickpea. Phytopathol. Mediterr. 35, 9-12.

Haware, M.P., Nene, Y.L., Rajeswari, R., 1978. Eradication of *Fusarium oxysporum* f. sp. *ciceri* transmitted in chickpea seed. Phytopathology 68, 1364-1367.

Hendricks, C.W., Doyle, J.D., Hugley, B., 1995. A new solid medium for enumerating cellulose-utilizing bacteria in soil. Appl. Environ. Microbiol. 61, 2016-2019.

Hervas, A., Landa, B., Jimenez-Diaz, R.M., 1997. Influence of chickpea genotype and *Bacillus* spp. on protection from *Fusarium* wilt by seed treatment with non-pathogenic *Fusarium oxysporum*. Eur. J. Plant Pathol. 103, 631-642.

Hervas, A., Landa, B., Datnoff, L.E., Jimenez-Diaz, R.M., 1998. Effects of commercial and indigenous microorganisms on *Fusarium* wilt development in chickpea. Biol. Control 13, 166-176.

Jalali, B.L., Chand, H., 1992. Chickpea wilt. In: Singh, U.S., Mukhopadhayay, A.N., Kumar, J., Chaube, H.S. (Eds.) Plant Diseases of International Importance. Vol. 1. Diseases of Cereals and Pulses. Prentice Hall. Englewood Cliffs, NJ, pp. 429-449.

Jimenez-Gasco, M.M., Jimenez-Diaz, R.M., 2003. Development of a specific polymerase chain reaction-based assay for the identification of *Fusarium oxysporum* f. sp. *ciceri* and its pathogenic races 0, 1A, 5 and 6. Phytopathology 93, 200-209.

Khamna, S., Yokota, Lumyong, S., 2009. actinomycetes isolated from medicinal plant rhizosphere soils: diversity and screening of antifungal compounds, indole-3-acetic acid and siderophore production. World J. Microbiol. Biotechnol. 25, 649-655.

Khan, M.R., Khan, S.M., Mohiddin, F.A., 2004. Biological control of *Fusarium* wilt of chickpea through seed treatment with the commercial formulation of *Trichoderma harzianum* and/ or *Pseudomonas fluorescens*. Phytopathol. Mediterr. 43, 20-25.

Landa, B.B, Navas-Cortes, J.A., Jimenez-Diaz, R.M., 2004. Integrated management of *Fusarium* wilt of chickpea with sowing date, host resistance and biological control. Phytopathology 94, 946-960.

Lima, L.H.C., Marco, J.L., Felix, J.R., 1998. Enzimas hidroliticas envolvidas no controle biologico por miciparasitisma. In: Melo, I.S., Azevedo, J.L., (Eds.). Controle biologico. Jaguraiuna: EMBRAPA-Meio Ambiente 11, pp. 263-304.

Liu, D., Anderson, N.A., Kinkel, L.K., 1996. Selection and characterization of strains of *Streptomyces* suppressive to the potato scab pathogen. Can. J. Microbiol. 42, 487-502.

Lorck, H., 1948. Production of hydrocyanic acid by bacteria. Physiol. Planta. 1, 142-146.

Lugtenberg, B.J.J., Dekkers, L.C., 1999. What makes *Pseudomonas* bacteria rhizosphere competent? Environ. Microbiol. 1, 9-13.

Lynd, L.R., Weimer, P.J., Van, Z.W.H., Pretorius, I.S., 2002. Microbial cellulose utilization: fundamentals and biotechnology. Microbiol. Mol. Biol. Rev. 66, 506-577.

Navas-Cortes, J.A., Hau, B., Jimenez-Diaz R.M., 1998. Effect of sowing date, host cultivar and races of *F. oxysporum* f. sp. *ciceri* on development of *Fusarium* wilt of chickpea. Phytopathology, 88, 1338-1346.

Nene, Y.L., Haware, M.P., Reddy, M.V., 1981. Chickpea diseases: resistance-screening techniques. Information Bulletin No.10. ICRISAT, pp. 5-7.

Nonoh, J.O., Lwande, W., Masiga, D., Herrmann, R., Presnail, J.K., Schepers, E., Okech, M.A., Bagine, R., Mungai, P., Nyende, A.B., Boga, H.I., 2010. Isolation and characterization of *Streptomyces* spp. with antifungal activity from selected national parks in Kenya. Afr. J. Microbiol. Res. 4, 856-864.

Pandey, P., Kang, S.C., Maheswari, D.K., 2005. Isolation of endophytic plant growth-promoting *Burkholderia* spp. MSSP from root nodules of *Mimosa pudica*. Curr. Sci. 89, 177-180.

Patten, C., Glick, B.R., 1996. Bacterial biosyntehsis of indole-3-acetic acid. Can. J. Microbiol. 42, 207-220.

Patten, C., Glick, B.R., 2002. Role of *Pseudomonas putida* in indole acetic acid in development of the host plant root system. Appl. Environ. Microbiol. 68, 3795-3801.

Perner, H., Schwarz, D., George, E., 2006. Effect of mycorrhizal inoculation and compost supply on growth and nutrient uptake of young leek plants grown on peat-based substrates. Hort. Sci. 41, 628-632.

Postma, J., Montanari, M., Van den Boogert, P.H.J.F., 2003. Microbial enrichment to enhance disease suppressive activity of compost. Eur. J. Soil Biol. 39, 157-163.

Rothrock, C.S., Gottlieb, D., 1984. Role of antibiosis in antagonism of *Streptomyces hygroscopicus* var. *geldanus* to *Rhizoctonia solani* in soil. Can. J. Microbiol. 30, 1440-1447.

Ryder, M.H., Jones, D.A., 1993. Biological control of crown gall. In: Hornby, D. (Ed.). Biological control of soil-borne plant pathogens. CAB international, UK, pp. 45-63.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406-425.

Schwyn, B., Neilands, J.B., 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. 160, 47-56.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596-1599.

Thompson, J.D., Gibsom, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The clustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24, 4876-4882.

Tinatin, D., Nurzat, T., 2006. Biodiversity of *Streptomyces* of high-mountainous ecosystems of Kyrgystan and its biotechnological potential. Antonie Leeuwenhoek 89, 325-328.

Tokala, R.K., Strap, J.L., Jung, C.M., Crawford, D.L., Salove, M.H., Deobald, L.A., Bailey., J.F., Morra, M.J., 2002. Novel plant-microbe rhizosphere interaction involving *Streptomyces lydicus* WYEC108 and the pea plant (*Pisum sativum*). Appl. Environ. Microbiol. 68, 2161-2171.

Torsvik, V., Ovreas, L., Thingstad, T.F., 2002. Prokaryotic diversity: magnitude, dynamics and controlling factors. Science 296, 1064-1066.

Trapero-Casas, A., Jimenez-Diaz, R.M., 1985. Fungal wilt and root rot diseases of chickpea in southern Spain. Phytopathol. 75, 1146-1151.

Wang, Y., Brown, H.N., Crowley, D.E., Szaniszlo, P.J., 1993. Evidence for direct utilization of a siderophore, ferrioxamine B, in axenically grown cucumber. Plant Cell Environ. 16, 579-585.

Wei, G., Kloepper, J.W., Sadik, T., 1991. Induction of systemic resistance of cucumber to *Colletotrichum orbiculare* by select strains of plant growth-promoting rhizobacteria. Phytopathol 81, 1508-1512.

Yin, S., Dong, Y., Xu, Y., Huang, Q., Shen, Q., 2011. Upland rice seedling wilt and microbial biomass and enzyme activities of compost-treated soils. Biol. Fertil. Soils, DOI 10.1007/s00374-010-0537-x.

Table 1

Enzymatic activities and secondary metabolite production by the five most potential antagonistic actinomycetes against *Fusarium oxysporum* f. sp. *ciceri* (FOC) [a].

	Production score for					Antagonistic against (inhibition zone in mm)				
Isolate	Siderophore	Cellulase	Protease	HCN	IAA (µg ml^{-1})	FOC	MP	RB-6	RB-24	RB-115
CAI-24	3	0	3	3	5.9	10	0	9	10	1
CAI-121	3	0	0	2	43.7	10	0	0	0	0
CAI-127	4	0	3	3	3.5	10	0	0	0	0
KAI-32	3	3	0	3	2.3	11	9	7	10	11
KAI-90	3	3	0	3	0	12	5	11	9	7
LSD (5%)	1.29	0.97	0.89	0.97	0.44	5.11	0.97	2.53	1.03	0.54
SE±	0.39^{NS}	0.30^{***}	0.27^{***}	0.30^{**}	0.14^{***}	1.57^{NS}	0.30^{***}	0.77^{***}	0.32^{***}	1.76^{***}
CV%	21	41	40	21	2	26	19	24	9	26

[a] HCN = hydrocyanic acid; IAA = indole acetic acid; MP = *Macrophomina phaseolina*; and RB-6, RB-24 and RB-115 are the three different strains of *Rhizoctonia bataticola*. The rating scales for siderophore, cellulase and protease were as follows: 0 = no halo zone; 1 = halo zone of <1 mm; 2 = halo zone of 1−3 mm; 3 = halo zone of 4−6 mm and 4 = halo zone of 7 mm and above. For HCN production, the following rating scale was used: 0 = no color change; 1 = light reddish brown; 2 = medium reddish brown; 3 = dark reddish brown; LSD = least significant difference, SE = standard error; CV = coefficient of variance; ** = statistically significant at 0.01 (*P* values) and *** statistically significant at 0.001 (*P* values).

Figure legends

Fig. 1. Evaluation of five most potential antagonistic actinomycetes against *Fusarium oxysporum* f. sp. *ciceri* (FOC) in wilt sick pots in the greenhouse conditions. Data were expressed in % reduction of disease incidence (DI) over FOC-inoculated control, where M1 = application of the actinomycete along with FOC, 2 weeks before sowing in the pot mixture; M2 = application of the actinomycete on the seeds; M3 = application of the actinomycete on the sprouted seeds; M4 = application of the actinomycete on the pot mixture, at the time of sowing; and M5 = application of the actinomycete on the pot mixture after emergence of seedling. The error bars are the standard errors.

Fig. 2. Population of the actinomycetes at the end of greenhouse experiment in the pot mixture of negative control [where no *Fusarium oxysporum* f. sp. *ciceri* (FOC) was inoculated], water control (where neither FOC nor actinomycetes were inoculated) and positive control (where 2 weeks before the start of the experiment FOC and actinomycetes were inoculated). The error bars are the standard errors. CV = coefficient of variance.

Fig. 3. Evaluation of five most potential antagonistic actinomycetes against *Fusarium oxysporum* f. sp. *ciceri* (FOC) in the wilt sick field conditions. Data were expressed in % reduction of disease incidence (DI) over positive control. M1 = application of the actinomycete on the seeds; M2 = application of the actinomycete on the sprouted seeds; M3 = application of the actinomycete on the soil, at the time of sowing; and M4 = application of the actinomycete after emergence of seedling. The error bars are the standard errors.

Fig. 4. Population of the actinomycetes at the end of the field experiment in the Fusarium-infested field at ICRISAT) in the actinomycetes-inoculated soils over the positive control (where no actinomycetes were inoculated). M1 = seed treatment; M2 = sprouted seed treatment; M3 = soil applications and M4 = after seedling emergence. The error bars are the standard errors.

Fig. 5. Phylogenetic relationship between the five potential *Fusarium oxysporum* f. sp. *ciceri* (FOC) antagonistic actinomycetes and representative species based on full-length 16S rDNA sequences constructed using the neighbor-joining method. The numbers in the branches are bootstrap percentages.

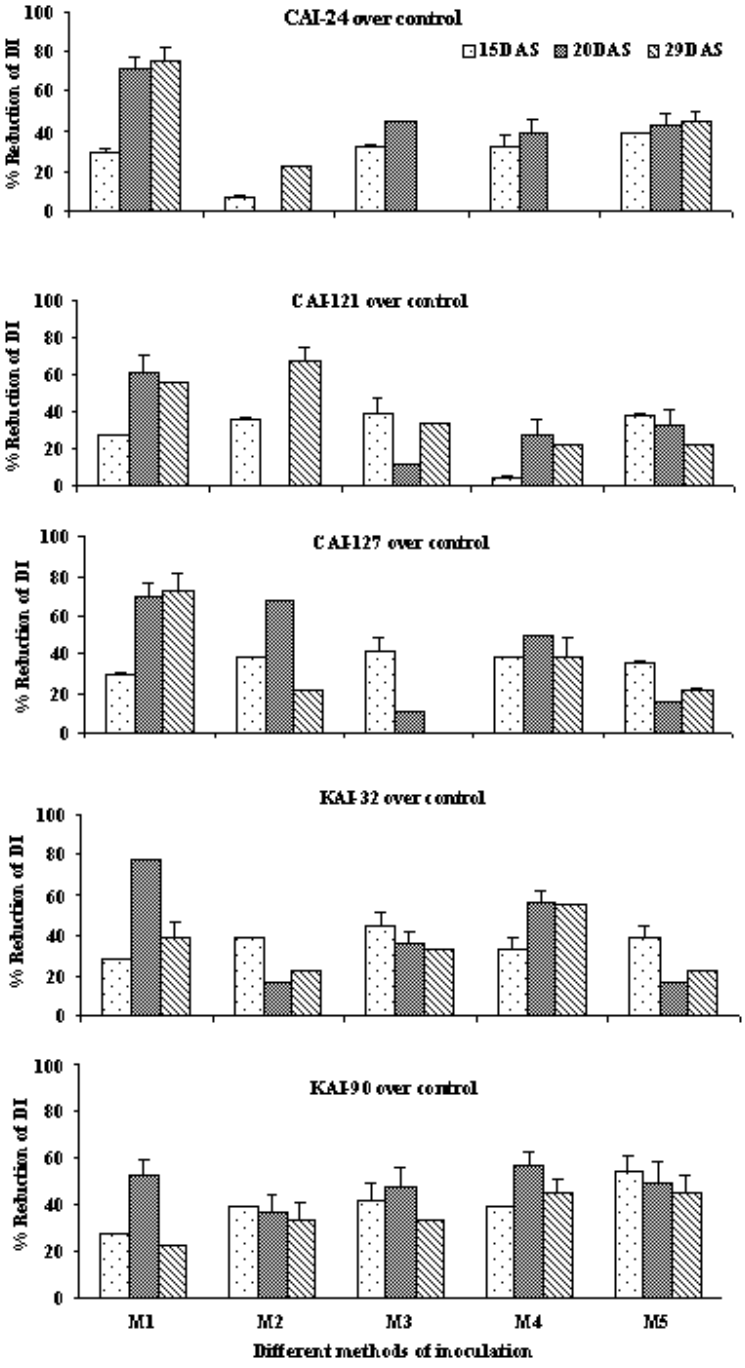
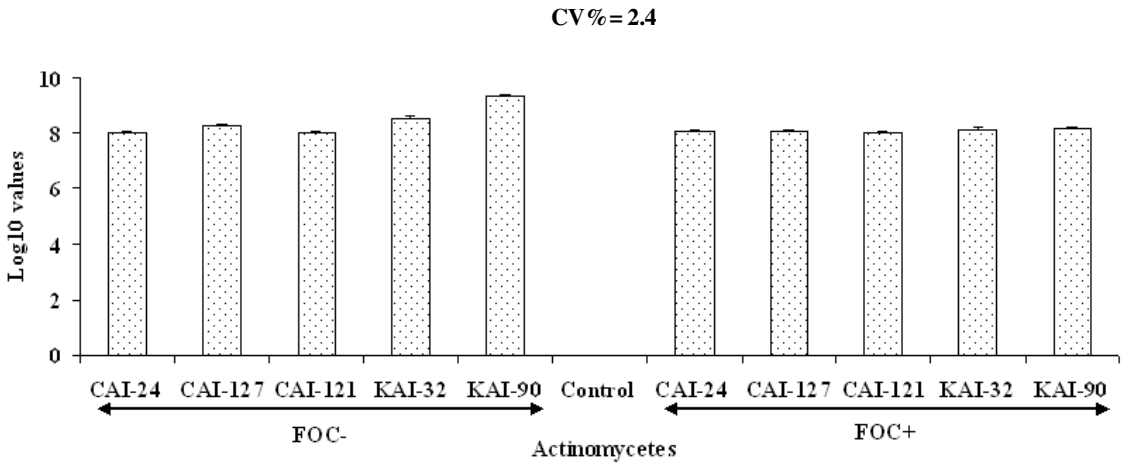


Fig. 1 Gopalakrishnan et al. (2010)



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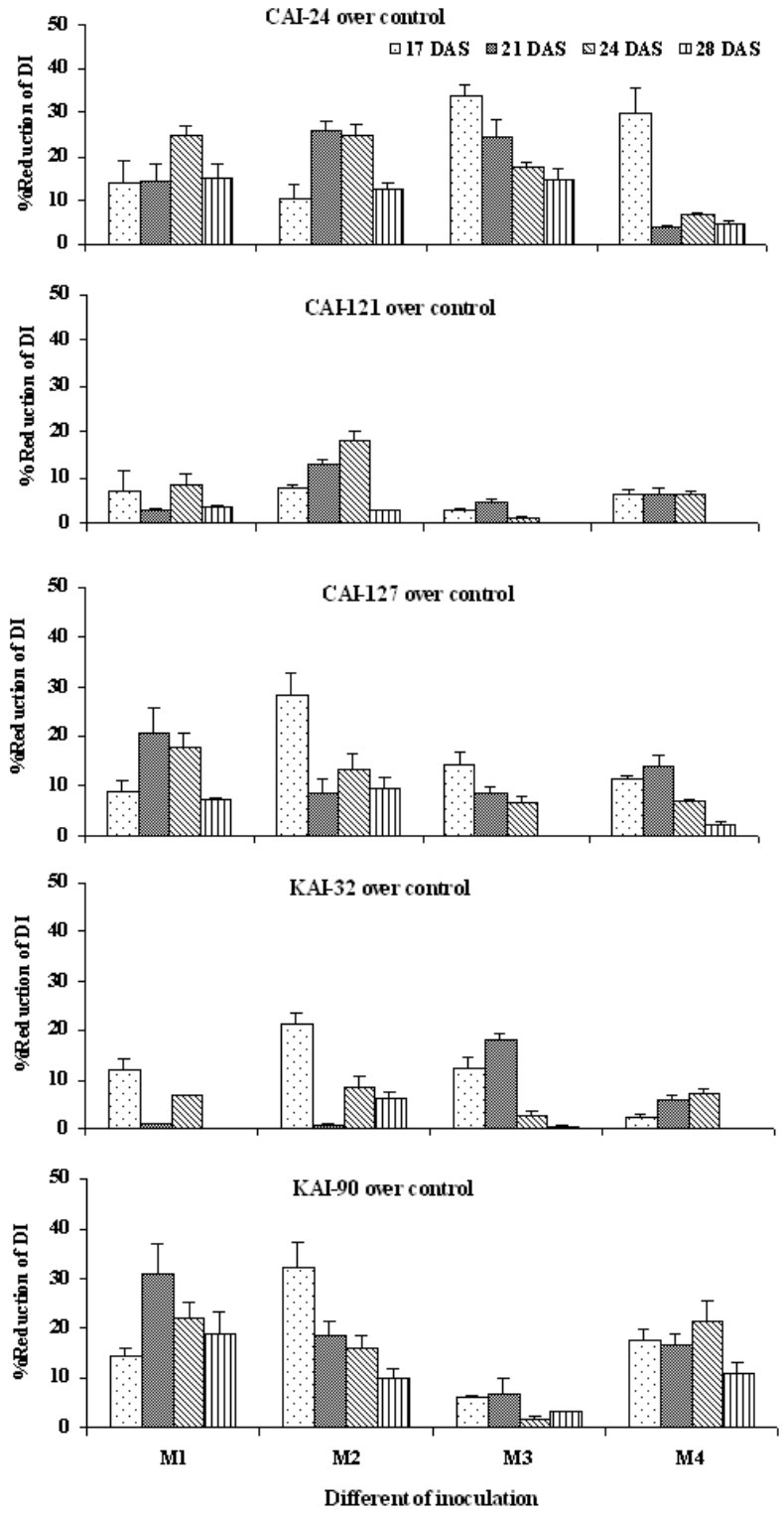

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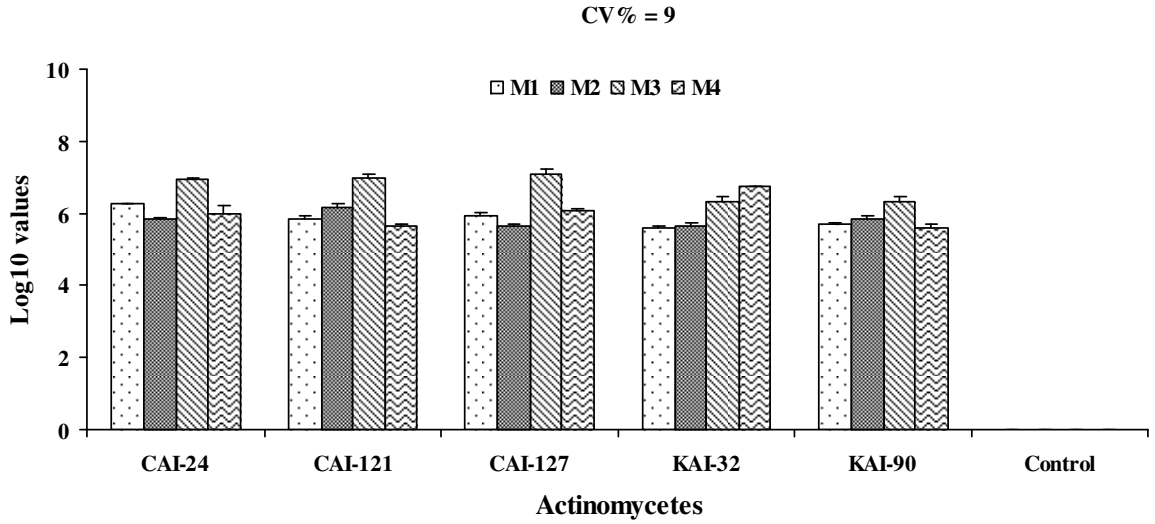

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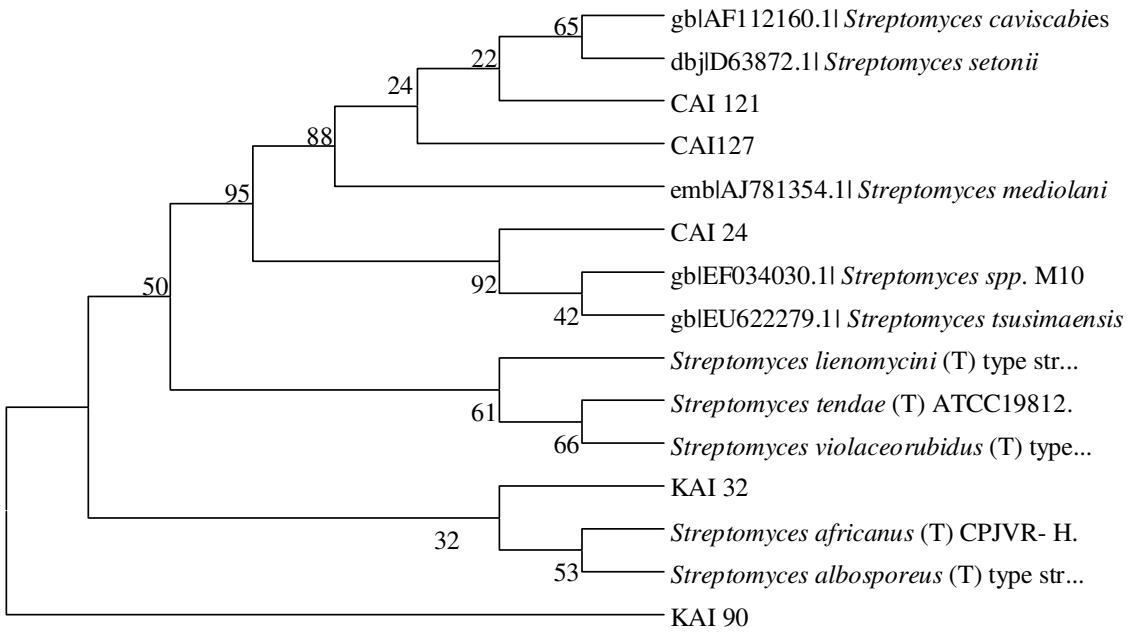

Fig. 5 Gopalakrishnan et al. (2010)