Streptomyces spp., a potential biocontrol agent of charcoal rot of sorghum caused by *Macrophomina phaseolina*

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Abstract

Seven strains of Streptomyces *spp. (BCA-546, BCA-659, BCA-667, BCA-689, BCA-698, CAI-8 and CAI-133; demonstrated previously to have potential for plant growth-promotion on sorghum and chickpea) were evaluated for their antagonistic potential against* Macrophomina phaseolina, *causal agent of charcoal rot disease of sorghum, by dual culture assay, secondary metabolite production assay and in greenhouse disease screen. All the seven strains inhibited* M. phaseolina *in both dual culture as well as secondary metabolite production assays but four of them (BCA-546, BCA-667, BCA-698 and CAI-8) were notable. When these selected four strains were tested for their antifungal activity in greenhouse on sorghum by tooth pick method, BCA-546 and CAI-8 significantly reduced the disease. The stem samples of the control and* Streptomyces *sp. treated plants were analysed under scanning electron microscope, where the xylem and phloem tissues of the* Streptomyces *spp. treated plants were found intact compared to that of infected control plants. This study indicates that the selected two* Streptomyces *strains, BCA-546 and CAI-8, have the potential to control charcoal rot disease in sorghum.*

Keywords: Charcoal rot, sorghum, *Macrophomina phaseolina*, *Streptomyces* spp., biocontrol

Introduction

Sorghum (*Sorghum bicolor* (L.) Moench) is one of the most important post rainy (*Rabi*) and rainy (*Kharif*) season crops of India, particularly in Maharashtra, Karnataka and Andhra Pradesh (Das *et al*., 2008), and other semi-arid tropical regions of the world (Wyllie, 1998). *Macrophomina phaseolina* (Tassi) Goid is one of the important pathogen of sorghum causing charcoal rot disease in the post rainy season, particularly when there is a long spell of drought. It is one of the soil-borne diseases of sorghum. *M. phaseolina* infection leads to rotting of roots followed by rotting of stalks, resulting in lodging of the plant at later stages. Charcoal rot disease has been reported to cause significant yield reduction in sorghum (up to 64%) in India under favourable conditions in *Rabi* sorghum (Das *et al*., 2008). Improved high yielding cultivars under good management practices have also been reported to be susceptible to the disease resulting in high yield losses (Mughogho and Pande, 1984). Management of charcoal rot can be done with the application of chemicals such as thiram, carbendazim and Topsin M (Gaikwad *et al*., 2002; Manjeet and Umesh, 2013), however, it is not economical and indiscriminate use of such chemicals results in negative impact on environment.

Biological control, using antagonistic bacteria, can be a safe and effective method to manage charcoal rot disease as these bacteria also enhance plant growth-promotion traits (Postma *et al*., 2003; Gopalakrishnan *et al*., 2011a). Further, *M. phaseolina* is a soil-borne pathogen, which is difficult to be controlled by chemicals; hence biological control offers a good alternative (Tonelli *et al*., 2010). Protection from fungal infection with microbial inoculants has been used as eco-safe management in several crops (Kishore *et al*., 2005; Fernando *et al*., 2007). Biological control of *M. phaseolina* using antagonistic bacteria and fungi have been reported in a wide range of host plants (Singh *et al*., 2002; Baird *et al*., 2003; Cardona and Rodrigue, 2006; Gopalakrishnan *et al*., 2011a; Karthikeyan *et al*., 2015). However, the use of actinomycetes is less known in the control of charcoal rot of sorghum.

Actinomycetes are a group of Gram-positive bacteria with high G + C content, belong to order Actinomycetales and are found commonly in rhizosphere and compost. They

also play an important role in the decomposition of organic matter and produce secondary metabolites of commercial interest. Actinomycetes have been widely reported to protect cash crops against plant pathogens (Doumbou *et al*., 2001a). However, not much reports are available for controlling charcoal rot of sorghum with actinomycetes. In our previous study, seven strains of *Streptomyces* spp., (BCA-546, BCA-659, BCA-667, BCA-689, BCA-698, CAI-8 and CAI-133, were demonstrated to have potential for plant growth-promotion on sorghum and chickpea (Alekhya and Gopalakrishnan, 2016a; 2016b). In the present study, these seven strains were further evaluated for their antagonistic potential against *M. phaseolina* by dual culture and secondary metabolite production assays and to control charcoal rot of sorghum following artificial inoculation in the greenhouse.

Materials and methods

Evaluation of *Streptomyces* strains for their antagonistic potential

Seven strains of *Streptomyces* spp., BCA-546 (GenBank accession number: KF770898), BCA-659 (GenBank accession number: KF770889), BCA-667 (GenBank accession number: KF770888), BCA-689 (GenBank accession number: KF770899), BCA-698 (GenBank accession number: KF770900), CAI-133 (GenBank accession number KF770895) and CAI-8 (GenBank accession number: KF770890) were selected for this study. The pathogen, *M. phaseolina*, was isolated from infected sorghum plants collected from the disease nursery conducted at ICRISAT Patancheru, Telangana, India.

Laboratory assay. The seven *Streptomyces* strains were evaluated for their antagonistic potential against *M. phaseolina* by dual culture and metabolite production assays. For dual culture assay, at one end of the Petri plate containing glucose cassamino acid yeast extract agar (GCY), the test isolate (*Streptomyces* sp.) was streaked while at the other end of the Petri plate, fungal disc (4 mm diameter of *M. phaseolina*) was placed using a standard template and the plates were incubated for 5 days at 28 ± 2 °C. At the end of incubation, the zone of inhibition was measured between the pathogen and the test culture.

For metabolite production assay, the seven strains were grown on starch casein broth (SCB) for five days at 28 ± 2 °C. The cell free extract was collected, by centrifuging the culture at 10000*g* for 20 min, and their metabolites were extracted by solvent partitioning method using ethyl acetate (Westley *et al*., 1979). The resultant organic and aqueous fractions were collected and tested against *M. phaseolina* by poisoned food technique, for which GCY agar plates with 10% fractions were prepared. A fungal disc with 4 mm diameter of *M. phaseolina* was bored and placed at the centre of the plate. After 5 days of incubation at 28 ± 2 °C, the fungal growth was measured and compared with control plates, where no fractions of organic or aqueous extracts were added.

Greenhouse studies. The four most potential strains of *Streptomyces*, selected based on the dual culture and metabolite production assays, were further evaluated for their antagonistic potential against *M. phaseolina* under greenhouse conditions using toothpick method. For this, six treatments were tested which included four test strains, one positive control with only pathogen inoculation and one negative control without any test bioagent or pathogen inoculation. The experiment was conducted in completely randomized design with ten replications/treatment. Pot mixture comprising black soil, sand and farm yard manure in 3:2:1 ratio was sterilized and filled in 8 inch plastic pots. Seeds of charcoal rot susceptible sorghum line B296 were surface sterilized with 3% chlorax for 5 minutes and rinsed with sterilized water for 3-4 times. Surface sterilized seeds were soaked in *Streptomyces* spore suspension (at 10^7 cfu/ml; grown in SCB) and in sterilized water for control for one hour. The treated seeds (3/pot) were sown immediately in the pots at 3 cm depth. After germination, the plants were thinned to one per. Booster doses of *Streptomyces* strains (5 ml per seedling, 10^7 cfu/ml) were applied at 15, 30 and 45 days after sowing (DAS) by the soil drench method. The pathogen was grown on the toothpick for infecting the plant. For this, *M. phaseolina* culture was grown on potato dextrose agar (PDA) plates till proper sporulation. Peptone and honey (2:1) solution was prepared and sterilized separately. This solution was poured on the *M. phaseolina* culture and the fungal growth containing mycelium and conidia was gently removed by scraping with a plastic inoculation loop. The solution containing the pathogen was poured into bottles containing sterilized toothpicks. The bottles were sealed properly and incubated for 15 days at 28 ± 2 °C. At the end of incubation, the pathogen covered entire toothpick till the tip. The plants were artificially inoculated by inserting toothpick infested with inoculum of *M. phaseolina* into the second internode of the stalk at 10 days after 50% flowering. After crop maturation the disease severity was recorded by measuring the length of infection and number of nodes infected. The trial was repeated to confirm the results.

Scanning electron microscopy (SEM) analysis of stalk samples

The samples of *Streptomyces* treated plants and the controls from the greenhouse experiment were examined for colonization and any morphological changes occurred because of *Streptomyces* strains by SEM analysis as per the protocols of Bozzola and Russell (1999). For this, small pieces of the infected portion of the stalk were cut, fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 24 h at 4°C and post fixed in 2% aqueous osmium tetroxide for 4 h. The shoot samples were dehydrated with a series of graded alcohols and dried to a critical point with a critical point drying (CPD) unit. The processed samples were mounted over the stubs with double-sided carbon conductivity tape, and the samples were coated with a thin layer of gold using an automated sputter coater (Model - JEOL JFC-1600) for 3 min and scanned under SEM (Model: JOEL-JSM 5600) at the required magnifications using standard procedures at RUSKA Lab, College of Veterinary Science, SVVU, Rajendranagar, Hyderabad, India. Upon viewing the morphological changes the size of the cells were measured.

Statistical analysis

The data were analysed statistically by ANOVA (Genstat 10.1 version) to compare the efficiency of biocontrol agents. Mean values were compared at 5% level of significance.

Results and discussion

In the present study, seven strains of *Streptomyces* which were previously reported to enhance plant growth-promotion on sorghum and chickpea (Alekhya and Gopalakrishnan *et al*., 2016a; 2016b) were evaluated for their antagonistic potential against charcoal rot of sorghum. In the dual culture assay, all the seven strains were found to inhibit growth of *M. phaseolina*. Of the seven test strains, BCA-546 and BCA-667 recorded inhibition of more than 10 mm (Table 1). This inhibition could be due to the production of hydrolytic enzymes or antibiotics by the tested *Streptomyces* strains which were dispersed through the media. Hence, it was decided to evaluate the secondary metabolite production potential of the selected *Streptomyces* strains. When the organic fraction of the culture filtrates of the *Streptomyces* strains were evaluated for their antagonistic potential, all but one (BCA-689) were found to inhibit *M. phaseolina,* whereas none of the aqueous fractions inhibited the pathogen. Of the six positive strains, two (BCA-546 and CAI-8) were found to have 70% inhibition of *M. phaseolina* (Table 1).

The selected seven strains of *Streptomyces* were reported previously by us for the production of siderophore, hydrocyanic acid (HCN), lipase, protease, chitinase, indole acetic acid (IAA) and β-1,3-glucanase (Alekhya and Gopalakrishnan, 2016a). Many studies have demonstrated the production of extracellular substances having direct effect on soil-borne pathogens. Dey *et al*., (2004) reported the role of siderophore in control of plant root pathogens. HCN was also reported to help in disease suppression (Haas *et al*., 1991). Chitinase is known for its ability to degrade fungal cell walls (Shapira *et al.,* 1989). IAA is known to help the host plants to stimulate seed germination, root formation and root elongation and indirectly control plant pathogens (Ahemad and Kibret, 2014). Production of lytic enzymes and their role in lysis of pathogenic fungal cell wall was reported by Lima *et al*., (1998) and Singh *et al*., (1999). Hence, it is concluded that the control of *M. phaseolina* is attributed to the production of hydrolytic enzymes/antibiotics by the *Streptomyces* strains. Based on the results of both dual culture and metabolite production

Table 1. Evaluation of seven *Streptomyces* strains for their antagonistic potential against *M. phaseolina* by dual culture and metabolite production assays

Treatments	Dual culture assay (zone of inhibition in mm)	Metabolite production (organic fraction) assay (per cent inhibition)
BCA-546	10.0	70
BCA-659	5.7	50
BCA-667	10.7	55
BCA-689	7.3	13
BCA-698	7.0	58
CAI-8	6.3	70
CAI-133	8.3	49
Control	0.0	0

assays, four most potential strains (BCA-546, BCA-667, BCA-698 and CAI-8) were further evaluated for their antagonistic potential against *M. phaseolina* to control charcoal rot under greenhouse conditions.

Under greenhouse conditions, three of the four *Streptomyces* strains, BCA-546, BCA-698 and CAI-8, reduced the charcoal rot disease severity significantly when compared to positive control. Of the three positive strains, BCA-546 and CAI-8 were found most promising as they reduced the disease severity by four fold. In these two most promising strains, the infection was observed only in one node and length of infection was reduced to 3 cm, whereas in the positive control, 5 nodes were infected with up to 21 cm length of infection (Table 2). Miller *et al*., (1990), Doumbou *et al*., (2001b) and Barakate *et al*., (2002) 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. Hence, in the present study, the *Streptomyces* strains were not only used as seed treatment before sowing but also applied at 15 days interval after sowing.

Actinomycetes are widely reported for the disease control in many plants. Of all the actinomycetes, *Streptomyces* appear to be good candidates to find new approaches to control plant pathogens (Behal, 2000). For incidence, they are known to control plant pathogens such as *Sclerotinia minor* (El-Tarabily *et al*., 2000), *Pythium aphanidermatum* (El-Tarabily *et al*., 2009), *Fusarium oxysporum* f. sp. *ciceri* (Gopalakrishnan *et al*., 2011c), *Phytophthora infestans* and *Sclerotium rolfsii* (Khushboo *et al*., 2014) and *Rhizoctonia solani* **(**Goudjal *et al*., 2014). As far as charcoal rot of sorghum is concerned, only few biocontrol bacteria are known and reported to be effective against this disease. *Streptomyces* (Das *et al*., 2008; Gopalakrishnan *et al*., 2011b) and non-actinomycetes such as *Bacillus*, *Pseudomonas*, *Brevibacterium*, *Enterobacter* and *Acinetobacter* (Gopalakrishnan *et al*., 2011a) were reported to control charcoal rot disease in sorghum, but these were mostly based on *in vitro* and/or blotter paper assay studies. Hence, it can be concluded that *Streptomyces*, particularly the strains BCA-546, BCA-698 and CAI-8 has the potential to control charcoal rot disease of sorghum.

The stem samples of CAI-8 and BCA-546 treated plants were observed under SEM and compared with that of controls. In the negative control, where no pathogen was inoculated, and in the CAI-8 and BCA-546 treatments, where both pathogen as well as *Streptomyces* were inoculated, the xylem and phloem size and morphology were found almost normal and intact. In contrast, in the positive control, where only pathogen was inoculated, most of stem tissues were found damaged. The xylem and phloem tissues in the non-infected controls ranged up to 104 µm and in BCA-546 up to 100, followed by CAI-8 up to 99 µm, whereas in the infected controls, the cell size ranged up to 64 µm (Table 3, Fig. 1). Hence it can be concluded that the two most promising strains of *Streptomyces viz*., CAI-8 and BCA-546 have the potential to control charcoal rot in sorghum.

Actinomycetes especially *Streptomyces* spp. are profoundly known for production of antifungal substances because of which they become the best choice organisms for biocontrol. In the present study, three of the seven *Streptomyces* strains *viz*., CAI-8, BCA-546 and BCA-698

Table 2. Evaluation of potential *Streptomyces* strains for their antagonistic potential against *M. phaseolina* under greenhouse conditions

	Trial 1		Trial 2	
Treatments	Number of nodes infected	Length of infection (cm)	Number of nodes infected	Length of infection (cm)
BCA-546	1.0	2.7	1.0	3.0
BCA-667	5.0	15.0	3.0	9.1
BCA-698	1.0	1.9	1.7	3.8
CAI-8	1.0	3.0	1.0	2.1
Negative control	0.0	0.0	0.0	0.0
Positive control	4.0	12.0	5.0	21.2
Mean	2.0	5.8	1.9	6.5
SEm ±	0.35***	0.50***	0.27***	1.13***
LSD (5%)	1.10	1.58	0.86	3.56

*** = Statistically significant at P = 0.001

demonstrated the antagonistic potentials in both *in vitro* as well as *in vivo* conditions. Further experiments are needed to determine the effectiveness of these strains under field conditions. Also, determination of the exact mechanisms of action of these promising biocontrol agents can assist in furthering the use of eco-friendly bio-fungicides.

Table 3. SEM analysis of the stalk samples from greenhouse

Treatments	Cell diameter (in µm)
BCA-546	100
CAI-8	99
Positive control	64
Negative control	104
Mean	92
SE±	3.1***
LSD (5%)	10.9

*** = Statistically significant at P = 0.001

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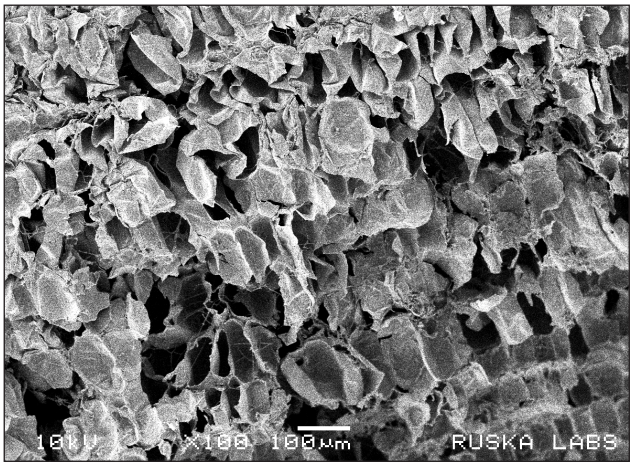
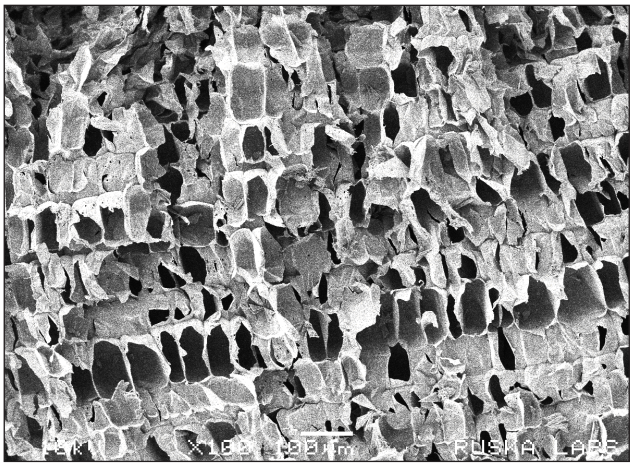
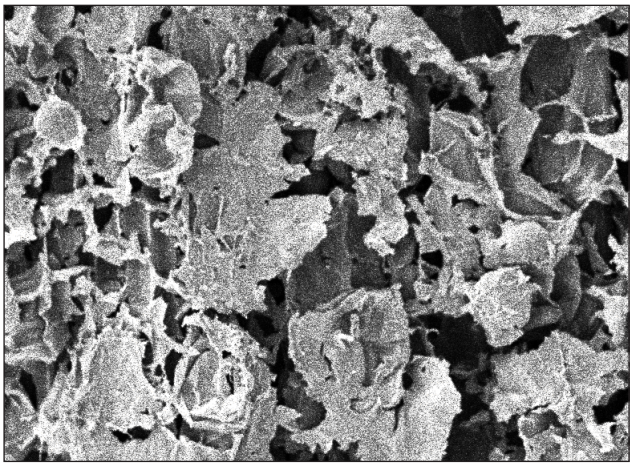


BCA-546

CAI-8



Negative control



Positive control

Figure 1. Scanning electron microscopy photographs of the two most potential *Streptomyces* strains showing morphological changes in the stalks of sorghum

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