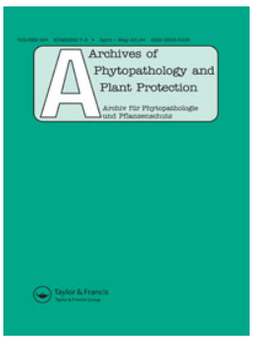

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Exploiting plant growth-promoting *Amycolatopsis* sp. for bio-control of charcoal rot of sorghum (*Sorghum bicolor* L.) caused by *Macrophomina phaseolina* (Tassi) Goid

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ABSTRACT

One strain of *Amycolatopsis* sp. BCA-696, a rare genus of actinomycete, demonstrated previously for its plant growth-promotion traits in chickpea and sorghum, was tested for its antagonistic potential against *Macrophomina phaseolina* (the causal agent of charcoal rot disease of sorghum) by dual culture assay, metabolite production assay, and in greenhouse and field screens. In the dual culture and metabolite production assays, BCA-696 inhibited the growth of *M. phaseolina*. When BCA-696 was tested for its antagonistic activity under greenhouse and field conditions (two seasons) against charcoal rot of sorghum by tooth pick method of inoculation, it significantly reduced the disease. Scanning electron microscope analysis revealed that the xylem and phloem tissues of the BCA-696 treated stem samples were intact compared to that of disease control plants. This study indicates that the selected *Amycolatopsis* sp. BCA-696 has the potential to manage charcoal rot of sorghum.

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KEYWORDS
Charcoal rot; sorghum; *Amycolatopsis* sp.; biocontrol; greenhouse and field conditions

Introduction

Charcoal rot, caused by *Macrophomina phaseolina* (Tassi) Goid., is one of the important soil-borne disease of post-rainy sorghum endemic to tropical and temperate regions of the world (Wyllie 1998). It has been reported to cause significant losses of yield (>60%) under conditions favouring the disease in sorghum in India (Das et al. 2008). *M. phaseolina* infect more than 500 plant hosts including legume and cereal crops and can cause up to 100% yield losses under favourable conditions (Patil and Kamble 2011). Though diseases of crops like sorghum can be best managed through host plant resistance, high level of resistance against

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charcoal rot is not available in the cultivated sorghum. *M. phaseolina* can be effectively managed with the application of fungicides such as carbendazim and thiram (Manjeet and Umesh 2013), however, with the ever increasing cost and concern over environmental degradation, efforts needs to be taken to develop environment-friendly methods of control. These include use of botanicals, antagonistic bacteria and/or PGP bacteria.

Actinobacteria are Gram-positive bacteria with high GC content in their genome and are found commonly in soils and vermicompost. They are known to produce secondary metabolites of commercial interest including antibiotics, antifungals, antivirals and insecticides (Berdy 2012). Actinobacteria play an important role not only in the growth-promotion of plants but also in the biological control of insect pests and pathogens of cereals and grain legumes (Gopalakrishnan et al. 2016). *Streptomyces* is the most predominant genus of actinobacteria followed by *Saccharopolyspora*, *Nocardia*, *Frankia*, *Mycobacterium*, *Microbispora*, *Micromonospora*, *Actinomadura*, *Actinoplanes* and *Amycolatopsis*.

The genus *Amycolatopsis* is described as aerobic, Gram-positive and non-motile actinobacteria that form branched hyphae and contains 66box 73% G + C (Stackebrandt et al. 1997; Tang et al. 2010). *Amycolatopsis* spp., belonging to the family *Pseudonocardiaceae*, are known to produce antibiotics such as rifamycin and vancomycin by *Amycolatopsis rifamycinica* and *Amycolatopsis orientalis*, respectively (Wink et al. 2003; Bala et al. 2004). *Amycolatopsis* has been reported to be isolated from river soil, stems of coastal plant *Dendranthema indicum*, petroleum-contaminated soil and deep sea sediments and helps in degrading polycarbonate and polycyclic aromatic hydrocarbons and producing vanillin (Pranamuda et al. 1999; Everest et al. 2013; Fleige et al. 2013; Xing et al. 2013; Ortega-Gonzalez et al. 2015; Zhang et al. 2016). Its importance in agricultural sector is not well known. Previously, we have demonstrated the potential of one strain of *Amycolatopsis* sp. BCA-696, isolated from chickpea rhizosphere, for PGP activities in chickpea and sorghum for the first time (Alekhya and Gopalakrishnan 2016); however, this strain was not tested for its biocontrol efficacy against sorghum diseases. Therefore, the objectives of this investigation were to further evaluate this strain for its biocontrol potential against charcoal rot disease, caused by *M. phaseolina* in sorghum under both greenhouse and field conditions.

Materials and methods

Actinomycete used in this study

One strain of *Amycolatopsis* sp. BCA-696 (GenBank accession number: KM191337), previously reported to have capacity for PGP in chickpea

and sorghum (Alekhya and Gopalakrishnan 2016), was selected for the present investigation.

In vitro *antifungal activity*

The actinomycete, *Amycolatopsis* sp. BCA-696, was tested for its antagonistic activity against *M. phaseolina* (acquired from cereal pathology, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India) by dual culture assay as described earlier (Alekhya et al. 2016).

For metabolite production assay, BCA-696 was grown on starch casein broth (SCB) at 28 ± 2 °C for five days. The cells free extract was collected and their metabolites were extracted by solvent partitioning method (Westley et al. 1979). The resultant organic and aqueous fractions were tested against *M. phaseolina* by modified poisoned food technique as described earlier (Grover and Moore 1962; Alekhya et al. 2016).

In vivo *antifungal activity*

Determination of *in vivo* antifungal activity of the *Amycolatopsis* sp. BCA-696 against *M. phaseolina* was done by blotter paper assay technique (Nene et al. 1981; Gopalakrishnan et al. 2011a). In brief, two-week-old seedlings of sorghum (296 B- susceptible to charcoal rot) were dipped in the inoculum of *M. phaseolina* (grown separately in potato dextrose broth (PDB) at 28 ± 2 °C) for 30 min followed by dipping in BCA-696 (grown separately in starch casein broth; SCB) and placed side by side on a blotter paper (45 × 25 cm) so that only the roots were covered. Positive and negative controls were made by inoculating the plants only with pathogen (*M. phaseolina*) and sterile water, respectively. Fifteen plants per replicate with three replications were assayed for each treatment. The blotter paper was kept moist with sterilised water all the time and incubated for 8 days at 28 ± 2 °C (with a 12-h day length provided by fluorescent lights; 120 µ mol m^{-2} s^{-1}). At the end of the incubation, the disease symptoms of charcoal rot (black-colored infection and microselerotia on the root surface) in the 0 – 4 rating scale (0 represents no visible charcoal rot symptom, while 4 represents maximum disease symptoms; Nene et al. 1981; Gopalakrishnan et al. 2011a) were recorded. The percentage of infected roots in BCA-696 inoculated treatment compared with control was also calculated.

Greenhouse studies

Amycolatopsis sp. BCA-696 was evaluated in greenhouse for its antagonistic potential against *M. phaseolina* by tooth pick method. The pathogen (*M. phaseolina*) was grown on toothpick as described earlier (Alekhya et al. 2016). A total of three treatments [BCA-696 inoculated + *M. phaseolina* inoculated, *M. phaseolina* inoculated (positive control), water inoculated (negative control)] were evaluated with ten replications. The experiment was conducted in completely randomised design. Pot mixture comprising of black soil, sand and farm yard manure in 3:2:1 ratio was filled in 8 inch plastic pots. Seeds (296B; susceptible to charcoal rot of sorghum) were surface sterilised (with 3% chlorax for 5 min and rinsed with sterilised water for four times) and soaked in BCA-696 spore suspension (at 10^7 cfu ml^{-1}; grown in SCB) or in sterilised water (for negative control) for one hour. The treated seeds were sown immediately at 3 cm depth in the pots (three pot^{-1}). After germination, the plants were thinned to one plant pot^{-1}. Booster doses of BCA-696 (5 ml $seedling^{-1}$, 10^7 cfu ml^{-1}) was applied at 15, 30 and 45 days after sowing (DAS) by the soil drench method. The plants (all minus negative control) 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.

Host-plant resistance traits

The shoot samples of BCA-696 treated plants and controls (both positive and negative) from the greenhouse experiment, after crop maturation, were collected in liquid nitrogen and stored at –80 °C. The samples were analysed for antioxidant traits including superoxide dismutase (SOD; Martinez et al. 2001), catalase (CAT; Aebi 1984), ascorbate peroxidase (APX; Nakano and Asada 1981), guaiacol peroxidase (GPX; Srivastava and Van Huystee 1977), glutathione reductase (GR; Schaedle and Bassham 1977), phenylalanine ammonia-lyase (PAL; Brueske 1980), polyphenol oxidase (PPO; Gauillard et al. 1993) and total phenolic content (TPC; Singh et al. 2013).

Scanning electron microscopy

The shoot samples of *Amycolatopsis* sp. BCA-696 treated plants and both controls from the greenhouse experiment, after crop maturation, were examined for colonisation and any morphological changes occurred

because of treatments by SEM analysis (Bozzolla and Russel 1999; Alekhya et al. 2016). The morphological changes and the size of the cells were measured under scanning electron microscopy (Model: JOEL-JSM 5600) at the required magnifications using standard procedures at RUSKA Lab, College of Veterinary Science, SVVU, Rajendranagar, Hyderabad, India.

Field studies

The actinomycete, *Amycolatopsis* sp. BCA-696, was also evaluated for its antagonistic potential against *M. phaseolina* under field conditions at ICRISAT, Patancheru, during 2016–2017 cropping season. During the cropping seasons, a maximum temperature range of 25.0–39.4 °C and a minimum temperature range of 7.3–23.0 °C was recorded. The experiment was conducted by tooth pick method, as explained for greenhouse conditions. Sorghum seeds of a highly susceptible cultivar to charcoal rot (genotype 296B) were sown in the field. A total of two treatments (BCA-696 + *M. phaseolina*) inoculated and only *M. phaseolina* inoculated (positive control). For the first treatment, BCA-696 was coated on the seed before sowing and booster doses of BCA-696 (5 ml seedling^{-1}, 10^7 cfu ml^{-1}) was applied at 15, 30 and 45 days after sowing (DAS) by the soil drench method. In both treatments, 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. Each treatment was replicated three times in a randomised complete block design (RCBD) and the plot size was three rows of 2 m long with a row spacing of 75 cm and a plant-to-plant spacing of 10 cm. After crop maturation, the disease severity was recorded by measuring the length of infection and number of nodes infected.

Statistical analysis

The data were analysed statistically by ANOVA (Genstat 10.1 version) to evaluate the efficiency of *Amycolatopsis* sp. BCA-696. Mean values were compared at 5% level of significance.

Results

In vitro *and in vivo antifungal activity*

Amycolatopsis sp. BCA-696 inhibited *M. phaseolina* in both dual culture as well as metabolite production assays. It showed a maximum inhibition of 12.7 mm in the dual culture assay. In the metabolite production assay, only the organic fraction inhibited (60%) the pathogen. In the *in vivo*

Table 1. *In vitro* and *in vivo* antifungal activity of *Amycolatopsis* sp. BCA 696 against *M. phaseolina*.

Treatment	Dual culture Assay@	Metabolite Production assay#	Blotter paper assay	
			Visual rating	% inhibition
BCA-696 + *M. phaseolina*	12.7	60	1	83
M. phaseolina (+ve control)	–	–	4	3
Water (-ve control)	0.0	0	0	100
Mean	6.3	30	2	62
SE±	0.47**	0.2***	0	1.9***
LSD (5%)	2.87	1.4	0	7.6

Note: SE: standard error; LSD: least significant differences; CV: coefficient of variation.
**Statistically significant at 0.01.
***Statistically significant at 0.001; @ = zone of inhibition in mm, # = Percent inhibition of organic fraction.

blotter paper assay, very little disease symptoms (rating 1) and lesser root infection (17%) was observed in BCA-696 treated roots, whereas disease symptoms on the rating scale of 4 and much higher root infection (97%) in the pathogen inoculated control was observed (Table 1; Figures 1(a,b) and 2).

Greenhouse studies

When *Amycolatopsis* BCA-696 was evaluated for its antagonistic potential, charcoal rot disease severity was reduced significantly when compared to positive control (*M. phaseolina*). The infection was observed in only 1–1.3 nodes and length of infection was reduced to 1.5–3.8 cm (in both first and second years) in BCA-696 treated plants, whereas in the positive control, four nodes were infected with up to 20 cm length of infection (Table 2 and Figure 3).

Host plant resistance traits

Shoot samples treated with both BCA-696 and *M. phaseolina* exhibited significantly enhanced antioxidant traits including superoxide dismutase (by 16% and 23%), catalase (by 85% and 99%), ascorbate peroxidase (by 22% and 75%), guaiacol peroxidase (by 55% and 76%), glutathione reductase (by 56% and 55%), phenylalanine ammonia lyase (by 58% and 57%), polyphenol oxidase (by 24% and 11%) and total phenolic contents (by 8% and 22%) over shoot samples treated with only *M. phaseolina* (positive) and control (negative control), respectively (Table 3).

Scanning electron microscopy

In the SEM, the xylem and phloem tissues size and morphology were found almost normal and intact in BCA-696 and *M. phaseolina* treated

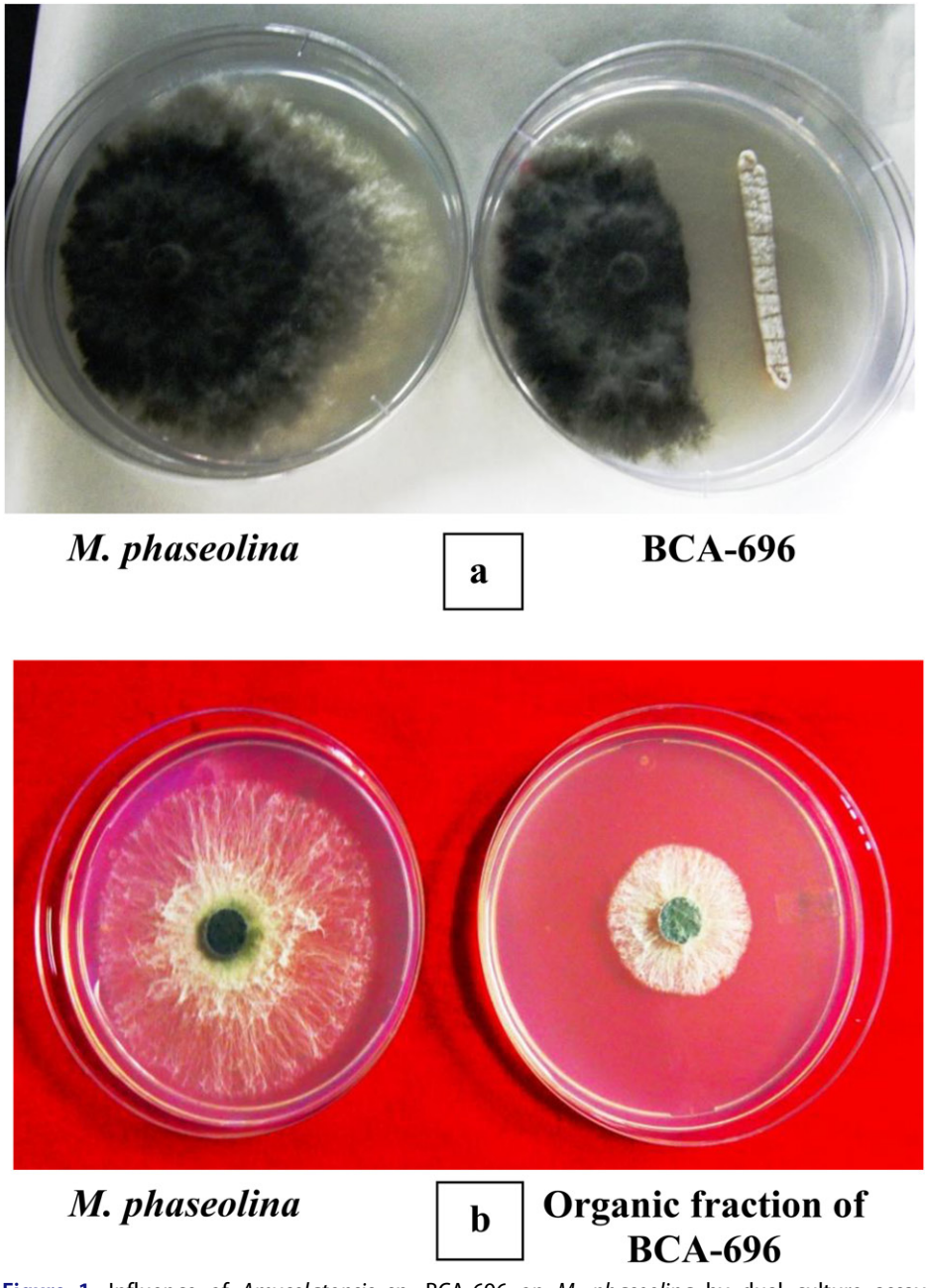

Figure 1. Influence of *Amycolatopsis* sp. BCA-696 on *M. phaseolina* by dual culture assay (Figure 1(a)) and metabolite production assay (Figure 1(b)).

shoot samples compared to *M. phaseolina* treated positive control, where most of the stem tissues were found damaged. The xylem and phloem tissues in the non-infected (water control), infected with both BCA-696 and *M. phaseolina* and infected with only *M. phaseolina* (positive) ranged up to 92 µm, 90.7 µm and 73 µm, respectively (Figure 4).

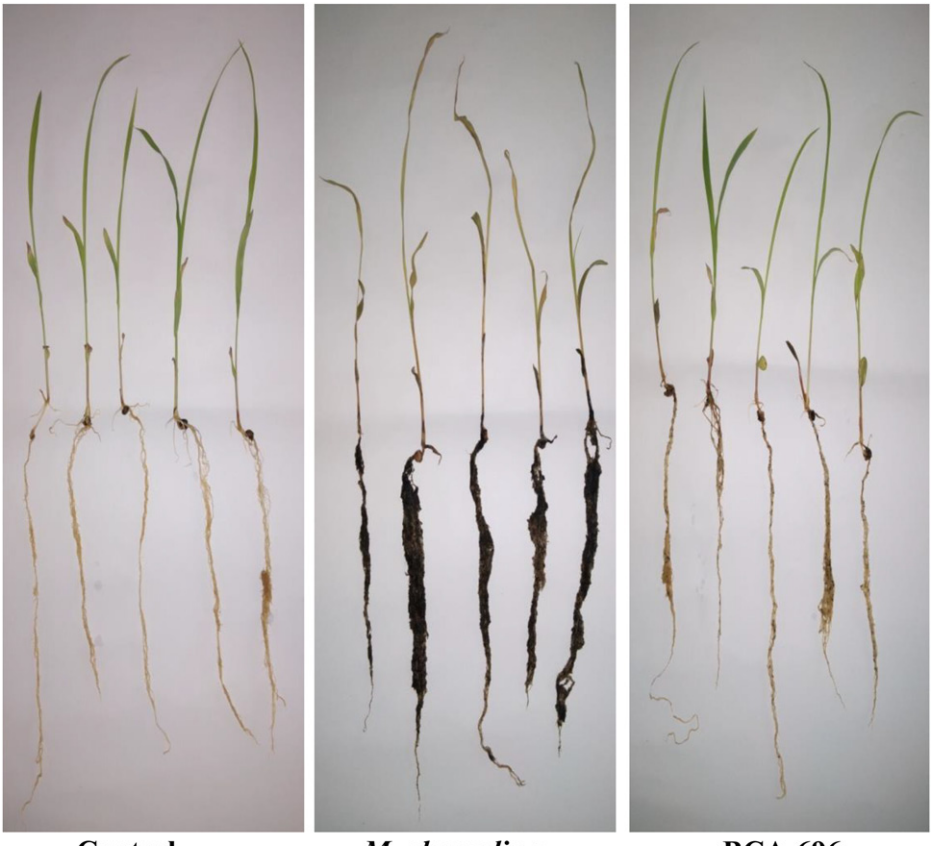

Figure 2. Influence of *Amycolatopsis* sp. BCA-696 on *M. phaseolina* by blotter paper assay.

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

	First year		Second year	
Treatment	Number of nodes infected	Length of infection (cm)	Number of nodes infected	Length of infection (cm)
BCA-696 + *M. phaseolina*	1.0	1.5	1.3	3.8
M. phaseolina (+ve control)	4.0	12.0	4.0	19.5
Water (-ve control)	0.0	0.0	0.0	0.0
Mean	1.7	4.5	1.8	7.8
SE±	0.33**	0.97**	0.22***	1.43***
LSD (5%)	1.31	3.8	0.76	4.94
CV%	35	37	25	37

Note: SE: standard error; LSD: least significant differences; CV: coefficient of variation.
**Statistically significant at 0.01.
***Statistically significant at 0.001.

Field studies

In the field, the charcoal rot disease severity was reduced significantly in BCA-696 treated plants when compared to positive control (only *M.*

Figure 3. Antagonistic potential of *Amycolatopsis* sp. BCA-696 against *M. phaseolina* under greenhouse conditions.

phaseolina). The infection was observed in only 3.2 nodes and length of infection was reduced to 10.6 cm in BCA-696 treated plants whereas in the positive control, 5.6 nodes were infected with up to 19.6 cm length of infection (Table 4 and Figure 5).

Discussion

Amycolatopsis is well documented for its antibiotic producing traits (Wink et al. 2003; Bala et al. 2004) and thus is an obvious focal point for drug discovery programmes. However, its importance in agricultural

Table 3. Antioxidant parameters of sorghum shoot samples against *M. phaseolina* by *Amycolatopsis* sp. under greenhouse conditions.

Treatments	Superoxide dismutase (SOD)	Catalase (CAT)	Ascorbate peroxidase (APX)	Guaiacol peroxidase (GPX)	Glutathione reductase (GR)	Phenylalanine ammonia lyase (PAL)	Polyphenol oxidase (PPO)	Total Phenolic content (TPC)
BCA-696 + *M. phaseolina*	148	114	4.407	0.011	0.845	0.099	19.8	213
M. phaseolina (+ve control)	124	17	3.420	0.005	0.373	0.041	15.0	196
Water (-ve control)	113	1	1.099	0.003	0.377	0.043	17.7	167
Mean	128	44	2.975	0.006	0.532	0.061	17.5	192
SE±	6.8*	0.5***	0.005***	0.0004***	0.0105***	0.0005***	0.65*	1.7***
LSD (5%)	26.6	2.1	0.020	0.0015	0.0413	0.0019	2.53	6.8
CV%	9	2	1	11	3	1	6	2

Note: SE: standard error; LSD: least significant differences; CV: coefficient of variation; The units for the antioxidant parameters are as follows: SOD, mol units mg^{-1} protein; CAT, APX, GPX and GR: $unit^{-1}min^{-1}g$ fresh weight; PAL and PPO, units ml^{-1} enzyme; TPC, mg g^{-1} fresh weight.

*Statistically significant at .05.

***Statistically significant at 0.001.

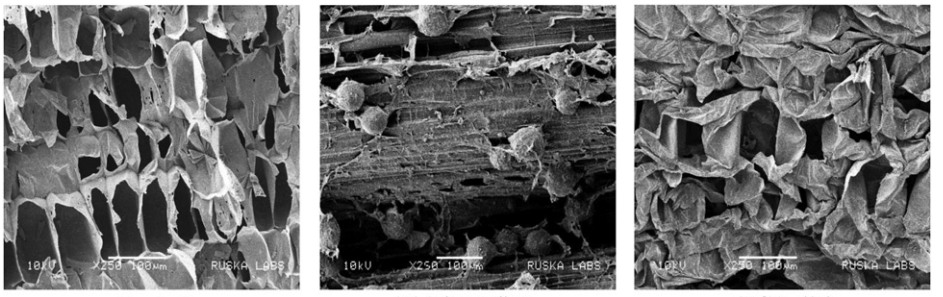

Figure 4. Scanning electron microscopy photographs of the *Amycolatopsis* sp. BCA-696 showing morphological changes in the stalks of sorghum, grown under greenhouse conditions.

Table 4. Evaluation of *Amycolatopsis* sp. for their antagonistic potential against *M. phaseolina* under field conditions.

Treatment	Number of nodes infected	Length of infection (cm)
BCA-696 + *M. phaseolina*	3.2	10.6
M. phaseolina (+ve control)	5.6	19.5
Water (-ve control)	0.6	2.3
Mean	3.1	10.8
SE±	0.34***	0.99***
LSD (5%)	1.31	3.92

Note: SE: standard error; LSD: least significant differences; CV: coefficient of variation.
***Statistically significant at 0.001.

sector is not well known. Perhaps, we were the first one to report for its usefulness as PGP in chickpea and sorghum (Alekhya and Gopalakrishnan 2016). In the present investigation, we wanted to further investigate the usefulness of *Amycolatopsis* BCA-696 for its antagonistic potential against charcoal rot disease in sorghum, caused by *M. phaseolina*.

In both dual culture and metabolite production assays, BCA-696 inhibited *M. phaseolina*. In the dual culture assay, the inhibition of *M. phaseolina* by *Amycolatopsis* BCA-696 could be due to the production of hydrolytic enzymes or antibiotics which were dispersed through the media. Bacteria isolated from rhizosphere are known to produce growth hormones such as auxins, siderophores and hydrolytic enzymes, such as chitinase, cellulase and β-1,3-glucanase and help plants to inhibit pathogens either directly or indirectly (Pal et al. 2001; Correa et al. 2004). *Amycolatopsis* BCA-696 has been reported to produce biocontrol and PGP traits including siderophore, hydrocyanic acid (HCN), chitinase, protease, cellulase, β-1,3-glucanase, lipase and IAA (107 µg ml^{-1}) under *in vitro* conditions (Alekhya and Gopalakrishnan 2016). Siderophores, HCN, cellulase, chitinase and β-1,3-glucanase are reported widely to play a role in disease suppression (Wei et al. 1991; Lima et al. 1998; Singh

Figure 5. Antagonistic potential of *Amycolatopsis* sp. BCA-696 against *M. phaseolina* under field conditions.

et al. 1999; Tokala et al. 2002). The assay results of secondary metabolite production by BCA-696, in the present study, further concluded this hypothesis. The organic fraction of the culture filtrate of BCA-696 inhibited *M. phaseolina* up to 60%. Similar results were obtained when BCA-696 was evaluated by *in vivo* blotter paper assay on sorghum plants; it inhibited *M. phaseolina* up to 83%. Hence, it is concluded that *Amycolatopsis* BCA-696 produces hydrolytic enzymes or antibiotics that inhibits *M. phaseolina*.

In the present study, under greenhouse conditions, the *Amycolatopsis* BCA-696 significantly reduced the charcoal rot disease severity when compared to positive control. The infection was observed only in one node and length of infection was reduced to <3.8 cm whereas in the positive control, four nodes were infected with up to 20 cm length of infection. Furthermore, when the shoot samples from treated plants with BCA-696 were analysed for antioxidant traits, it significantly enhanced all the tested biochemicals including SOD, CAT, APX, GPX, GR, PAL, PPO and TPC over shoot samples treated with only *M. phaseolina* and water control. Plenty of evidences in literature for enhancement of antioxidants due to inoculation of beneficial organisms that helps in host plant resistance against variety of plant pathogens are available. For instance, *Harpophora oryzae* against *Magnaporthe oryzae* in rice (Su et al. 2013); *Pseudomonas* sp. and *Methylobacterium* sp. against *Pseudomonas atrosepticum* in potato (Mittler 2002; Pavlo et al. 2011); *Bacillus subtilis* against *Rhizoctonia solani* in rice (Nagendran et al. 2014); *Streptomyces* sp. against *Sclerotium rolfsii* in chickpea (Singh and Gaur 2016); *Streptomyces* spp. against *R. solani* in tomato (Singh et al. 2016); *Streptomyces* sp. against *Botrytis cinerea* in chickpea (Vijayabharathi et al. 2018). It is concluded that the enhancement of the antioxidants upon inoculation of BCA-696 would have enhanced the charcoal rot disease resistance levels in sorghum against *M. phaseolina*.

Colonisation of biocontrol potential bacteria at the right place and time on the root, sufficient numbers and rhizosphere competence are essential as poor colonisation results in reduced biocontrol activity (Schippers et al. 1987; Ryder and Jones 1993; Lugtenberg and Dekkers 1999). Therefore, in the present investigation, booster dozes of the *Amycolatopsis* BCA-696 was applied both in the greenhouse as well as field studies at regular intervals. When the shoot samples, of greenhouse experiment, were observed under SEM, the xylem and phloem tissues were found almost normal and intact in BCA-696 treated while in positive control (only *M. phaseolina* inoculated), most of the stem tissues were found damaged. This may be due to enhanced effect of defense of plant due to *Amycolatopsis* BCA-696 treatment.

In the present investigation, *Amycolatopsis* BCA-696 was also evaluated under field conditions for its antagonistic potential against *M. phaseolina*. The charcoal rot disease reduced significantly in BCA-696 treated plants when compared to *M. phaseolina* only (positive control) treatment. Charcoal rot of sorghum was reported to be controlled by *Acinetobacter tandoii*, *Bacillus altitudinis*, *Brevibacterium antiquum*, *Enterobacter ludwigii*, *Pseudomonas monteilii*, *Pseudomonas*

plecoglossicida and *Pseudomonas chlororaphis* under greenhouse conditions (Das et al. 2008; Gopalakrishnan et al. 2011a). Actinomycetes were also earlier reported to control charcoal rot in sorghum. For instance, a set of seven *Streptomyces* spp. isolated from herbal vermicompost and rhizosphere soils were reported to control charcoal rot in sorghum under greenhouse conditions (Gopalakrishnan et al. 2011b; Alekhya et al. 2016). However, as of our knowledge, there are no reports of any biocontrol agents evaluated under field conditions. Hence, it can be concluded that BCA-696 has the potential to control charcoal rot in sorghum.

In the present study, the usefulness of *Amycolatopsis* BCA-696, for biocontrol of charcoal rot disease in sorghum, was demonstrated at both greenhouse and field conditions. However, such trials need to be conducted at multi-locations in order to demonstrate its usefulness under various soils and climatic conditions. In our previous study, we had reported the tolerance of BCA-696 on wide range of pH (5–11), temperatures (20–40 °C), NaCl concentrations (0–6%) and fungicides (including Bavistin up to 2500 ppm, Thiram up to 3000 ppm, Benlate up to 4000 ppm, Captan up to 3000 ppm and Ridomil up to 3000 ppm) (Alekhya and Gopalakrishnan 2016). These traits could help BCA-696 to survive in harsh environments under natural conditions and thus this bio agent can be used in the integrated disease management programmes. Furthermore, *Amycolatopsis* BCA-696 needs to be formulated as bio-inoculant and used for biocontrol of charcoal rot in other crops also. The secondary metabolite(s) responsible for inhibition of *M. phaseolina* needs to be identified and characterised. In the absence of high level of genetic resistance in high-yielding varieties, *Amycolatopsis* BCA-696 could be effective in controlling charcoal rot disease and related loss in grain and stover quality of sorghum.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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