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Exploiting plant growth-promoting *Amycolatopsis* sp. in chickpea and sorghum for improving growth and yield

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ABSTRACT

In an attempt to identify plant growth-promoting (PGP) actinomycetes other than *Streptomyces* sp., from rhizosphere soils of chickpea and sorghum, a total of 37 actinomycetes were isolated and evaluated for their PGP traits. Of which, one isolate BCA-696 was found to produce PGP traits including indole acetic acid (IAA), siderophore, cellulase, lipase, protease, chitinase, hydrocyanic acid and β-1,3-glucanase. BCA-696 was found to tolerate wide range of pH, temperature, NaCl concentrations and fungicides. BCA-696 was identified as *Amycolatopsis* sp. in 16S rDNA analysis. On chickpea, under greenhouse and field conditions, BCA-596 enhanced the root length, root volume, shoot weight, root weight, nodule number, pod number, seed weight, stover yield and grain yield over the un-inoculated control. BCA-696 also enhanced PGP traits on sorghum, under field conditions, including the leaf area, stem weight, root weight, plant weight, grain yield and stover yield over the un-inoculated control. The rhizosphere soils of both chickpea and sorghum were also found to enhance total N, available P and % organic C in BCA-696 treated plots over un-inoculated control plots. BCA-696 was found to colonize both chickpea and sorghum roots in scanning electron microscope analysis. This is the first report on the role of *Amycolatopsis* sp. in PGP on chickpea and sorghum.

Keywords: *Amycolatopsis* sp., Plant growth-promotion, Chickpea, Sorghum.

Chickpea (*Cicer arietinum* L.) and sorghum (*Sorghum bicolor* L.) are the third and fifth most important crop in the world, respectively. Chickpea is called poor man's meat as it is a rich source of protein and acts as a supplement to the cereal diet. Sorghum is a highly drought tolerant crop and extensively cultivated for food and production of ethanol, starch, adhesives and paper. Both the crops are normally grown in the areas of semi-arid tropics where annual rainfall is below 700 mm. The low-nutrient soils of semi-arid tropics, insects and pathogens can reduce the yield of these crops or loss of entire crop. The yield of chickpea and sorghum are enhanced by applying chemical fertilizers to fertilize the plants and pesticides to control insect-pests and pathogens. However, the use of chemical fertilizers and pesticides also enhances environmental contamination, human and animal health hazards, develops insect resistance to insecticides and reduces the natural beneficial organisms in soils (Mingma *et al*., 2014). The alternatives of inorganic farming are usage of biological options including application of animal wastes, botanicals, crop residues, entomopathogens, antagonistic microorganisms, endophytes and plant growth-promoting (PGP) microbes.

Microorganisms are widely used as growth-promoter of plants. PGP microorganisms improves plant growth either directly by producing growth hormones viz. IAA, siderophore and 1-Aminocyclopropane-1-Carboxylate (ACC) deaminase (Correa *et al*., 2004) or indirectly by producing chitinase, β-1,3-glucanase, antibiotics, fluorescent pigments and cyanide (Pal *et al*., 2001; Praveen *et al*., 2012). Rhizosphere microorganisms interact with plants and increase plant growth by enhancing nutrient and water uptake and also produce compounds that inhibit pathogens (Gamalero *et al*., 2009). Bacteria of diverse genera were identified for PGP, of which *Bacillus and Pseudomonas* are predominant. There are many recent reports on PGP by microorganisms such as *Paenibacillus dendritiformis* in potato (Lapidot *et al*., 2015), *Glomus intraradices* and *Trichoderma atroviride* in vegetables (Colla *et al*., 2015), *Penicillium menonorum* in cucumber (Babu *et al*., 2015) *Pseudomonas* spp. in chickpea (Gopalakrishnan *et al*., 2014) *Mesorhizobium ciceri* in chickpea (Sahai and Chandra, 2010; Chandra and Pareek, 2015) and *Bacillus* spp. in sorghum (Grover *et al*., 2014).

Actinomycetes also plays significant role in PGP. Actinomycetes produce different kinds of PGP substances, secondary metabolites and biologically active substances such as enzymes and antibiotics (Adegboye and Babalola, 2012; Goudjal *et al*., 2014). These substances play a major role in disease reduction also (Yandigeri *et al*., 2015). Actinomycetes produce spores which make them resistant to desiccation and nutrient stress (Yandigeri *et al*., 2015), hence can be used in a wide range of soils and environmental conditions. The use of actinomycetes for PGP are widely reported, for instance, *Streptomyces* spp. by Gopalakrishnan *et al*. (2014) in rice, Gopalakrishnan *et al*. (2015a) in chickpea, Goudjal *et al*. (2014) in tomato and Poovarasan *et al*. (2013) in pomegranate. However, PGP by other members of actinomycete family are rarely reported. The main objective of the present study was to isolate, characterize and evaluate actinomycetes other than *Streptomyces* spp. for their PGP traits in chickpea and sorghum.

MATERIALS AND METHODS

The chickpea and sorghum rhizosphere soils (0-15 cm) were collected from ICRISAT, Patancheru fields. Ten grams of soil sample was suspended in 90 ml of sterilized physiological saline (0.85% of NaCl) and kept on shaker for 1 h. At the end of incubation, samples were serially diluted up to 10^7 dilutions with physiological saline. Dilutions 10^4"10^6 were plated (0.1 ml) on actinomycetes isolation agar (AIA) by spread plate technique. The plates were incubated at 28 ± 2 °C for five days. Colonies with different morphologies of actinomycetes were picked and their pure cultures were maintained in AIA slants.

All the actinomycete isolates were evaluated for their PGP and biocontrol traits including IAA, siderophore, cellulase, lipase, protease, chitinase, hydrocyanic acid (HCN) and β-1,3-glucanase. The production of IAA was estimated by Patten and Glick (1996), siderophore by Schwyn and Neilands (1987), cellulase by Hendricks *et al*. (1995), lipase and protease by Bhattacharya *et al*. (2009), chitinase by Hsu and Lockwood (1975), HCN by Lorck (1948) and Gopalakrishnan *et al*. (2011) and β-1,3-glucanase by Singh *et al*. (1999). Each PGP trait was replicated thrice and experiments were repeated three times.

The physiological properties such as pH, temperature and salinity tolerance were studied for all the actinomycete isolates. For pH, temperature and salinity, the test isolates were streaked on Bennet agar, adjusted to different pH (5, 7, 9 and 11), temperatures (20°C, 30°C and 40°C; for 50°C, Bennet broth was used) and saline concentrations (0"12% at the interval of 2%) and incubated at 28 °C for 5 days. The fungicide tolerance of the test actinomycetes was evaluated on AIA plates amended with fungicides including Bavistin, Thiram, Benlate, Captan and Ridomil at field application levels of 2500, 3000, 4000, 3000, and 3000 ppm, respectively (Gopalakrishnan *et al*., 2012). At the end of 5-day incubation, the growth of the traits was recorded on a scale of 0 to 3.

The pure culture of the most potential PGP actinomycete was grown in starch casein broth (SCB) until log phase and genomic DNA was isolated and identified by 16S rDNA sequencing. The 16S rDNA gene was amplified using universal primers 1492R (5'-TAC GGY TAC CTT GTTACG ACT T-3') and 27F (5'- AGA GTT TGA TCM TGG CTC AG-3') as per the protocol by Pandey *et al*. (2005). The PCR product was sequenced at Macrogen Inc. Seoul, Korea. The sequences obtained was 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 tree inferred using the neighbor-joining method (Saitou and Nei, 1987). The nucleotide sequences of the actinomycete were submitted to GenBank, NCBI and the accession number was obtained.

The PGP potential of the most promising actinomycete was evaluated under greenhouse conditions on chickpea (variety ICCV 2). Pot mixture containing black soil, sand and farm yard manure (3:2:1) was filled in plastic pots (8"). One treatment (the most potential PGP isolate) and a control (without inoculum) with three replications each were maintained. Chickpea seeds were surface-sterilized and incubated with the actinomycete isolate (10^7 cfu ml^{-1}) for 1 h before sowing. In each pot, three seeds were sown and thinned to one plant after germination. At 15, 30 and 45 days after sowing (DAS), booster doses of actinomycete isolate was applied along with irrigation. The growth parameters including the root length, root volume, shoot weight, leaf dry weight, root dry weight, leaf area and nodule number were recorded at 45 DAS and growth and yield parameters including shoot weight, root weight, pod number and pod weight were recorded at harvest.

The most potential actinomycete was further evaluated for its PGP potential under field conditions in chickpea and sorghum. The field trials were performed in 2012 Rabi (post-rainy) season at ICRISAT, Patancheru in the Telangana State of India. The plot sizes of 4 × 3m ridges in a randomized complete block design (RCBD) were prepared and three replications per treatment were maintained. The selected actinomycete was grown in SCB for five days, soaked with chickpea seeds (ICCV 2) and sorghum seeds (SPV1411) for 1 h and sown by hand at 5 cm depth. Booster doses of the test isolate (10^8 cfu ml^{-1}) were applied to soil at an interval of 15 DAS until flowering stage. The control plot contained no test isolate. For chickpea, at 30 DAS, the growth parameters such as nodule number, root weight and shoot weight were recorded and at 60 DAS, plant height, leaf area, leaf weight, pod number, stem weight and root weight were recorded. At final harvest, the growth and yield parameters including the pod weight, seed weight, stover yield, grain yield and total dry matter were recorded. For sorghum, at 60 DAS, growth parameters including the plant height, leaf area, leaf weight, stem weight, root weight and total plant weight were recorded. During the final harvest, the yield parameters including the 1000 seed weight, grain yield, stover yield and total dry matter were recorded. For both chickpea and sorghum trials, soil samples (from 0–15 cm soil profile) were collected at harvest and analysed for % organic carbon, available P and total N using the standardized protocols described by Nelson and Sommers (1982), Olsen and Sommers (1982) and Novozamsky *et al*. (1983), respectively. The data were analysed through analysis of variance (ANOVA) with the SAS GLM (General Linear Model) procedure (SAS Institute 2002-08, SAS version 9.3). The isolate means were tested for significance and compared using Fisher's protected least-significant difference.

The colonization of the test actinomycete isolate on the roots of chickpea and sorghum was demonstrated by Scanning Electron Microscope (SEM) analysis. For this, the chickpea seed (ICCV 2) and sorghum seeds (SPV1411) were surface sterilized, germinated and grown in light

chambers for 15 days as per the protocols of Gopalakrishnan *et al*. (2015a). After 15 days, the plants were taken out and the roots were processed for SEM analysis as per the protocols of Bozzola and Russell (1998). The samples were scanned under Electron Microscope (SEM - Model: JOEL-JSM 5600) at required magnifications as per the standard procedures at RUSKA Lab's, College of Veterinary Science, SVVU, Rajendranagar, Hyderabad, India.

RESULTS AND DISCUSSION

Isolation and characterization of actinomycetes - Actinomycetes are widely employed for PGP of many crops because of their ability to produce wide range of metabolites and PGP traits (Venkatachalam *et al*., 2010; Talwinder *et al*., 2013). Among the actinomycetes, only *Streptomyces* spp., are reported widely to be used in PGP, however, PGP by other members of actinomycete family including *Micromonospora* spp., *Nocardia* spp., *Actinomadura* spp., *Microbispora* spp., *Actinoplanes* spp. and *Amycolatopsis* spp. are rarely reported (Takahashi and Omura, 2003; Coombs *et al*., 2004; Dalal and Kulkarni, 2014). Hence, in the present study, an attempt was made to isolate and screen actinomycetes other than *Streptomyces* spp., from rhizosphere soils, and further evaluate for their PGP potentials in chickpea and sorghum. A total of 37 actinomycetes were isolated based on their colony morphology and pigment production capabilities. All the isolates were found to be Gram positive but the morphology and production of pigment varied from one to another. The mechanisms by which actinomycetes promote plant growth include production of plant growth regulators. Isolate BCA-696 was found to produce all the tested PGP traits including IAA (107 µg ml^{-1}), siderophore, cellulase, lipase, protease, chitinase, HCN and β-1,3-glucanase. BCA-696 was also tolerant to physiological traits including NaCl concentrations of 0%6%, pH of 5%11, temperatures of 20%40°C and a wide range of recommended fungicides including Bavistin (2500 ppm), Thiram (3000 ppm), Benlate (4000 ppm), Captan (3000 ppm) and Ridomil (3000 ppm) at field application levels (Table 1).

Table 1. PGP and physiological traits of potential actinomycete isolate BCA-696

Traits	Units/Rating
PGP	
Indole acetic acid (IAA; µg ml^{-1})	107
Siderophore	2
Cellulase	2
Lipase	3
Protease	2
Chitinase	3
Hydro cyanic acid (HCN)	3
β-1,3-glucanase (mg ml^{-1})	0.08
Salinity (%)	
0	3
2	3
4	3
6	1
pH	
3	0
5	3
7	3
9	3
11	3
13	0
Temperature (°C)	
20	3
30	3
40	2
50	0
Fungicide Tolerance#	
Bavistin (2500 ppm)	3
Thiram (3000 ppm)	2
Benlate (4000 ppm)	2
Captan (3000 ppm)	2
Ridomil (3000 ppm)	2

Note: The rating scales for siderophore, chitinase, cellulase, lipase and protease were given as follows: 0 = no halo zone; 1 = halo zone of <1 mm; 2 = halo zone of 2"3 mm; 3 = halo zone of 4"6 mm, 4 = halo zone of 7"9 mm; 5 = 10 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. A standard curve was plotted to quantify the IAA (µg ml^{-1}) present in the culture filtrate. One unit of β-1,3-glucanase activity was defined as the amount of enzyme that liberated 1 µmol of glucose $hour^{-1}$ at defined conditions. The responses of pH, temperature, salinity and fungicide tolerance were recorded as follows: 0 = no growth; 1 = poor growth; 2 = medium growth; 3 = good growth. #= field application levels

IAA is the member of phytohormone and considered as the most important native auxin, which regulates plant development (including organogenesis and tropic responses) and cellular responses (including cell expansion, division, differentiation and gene regulation) (Ryu and Patten, 2008; Kaur and Khanna, 2014). Hence, production of IAA directly enhances the plant growth. BCA-696 was also found to produce siderophore. Several studies had demonstrated the usefulness of siderophore in controlling plant root pathogens (Dey *et al*., 2004). The potential to produce siderophores by microorganisms in improving iron availability to plants was also reported (Sharma *et al*., 2003). In the present study, BCA-696 was found to produce hydrolytic enzymes such as cellulase, lipase and protease. These enzymes degrade the cellulose and lipids, providing nutrition as well as posing antagonistic effect to other organisms. The production of cellulase, lipase and protease by bacteria and their role in PGP was reported by Siddikee *et al*. (2010). These enzymes help in preventing the crops from plant pathogens by degrading their cell walls. In the present study, BCA-696 was also found to produce

chitinase, HCN and β-1,3-glucanase. Chitin is a linear β-1, 4-linked homopolymer of N-acetylglucosamine and abundant in nature. The fungal (pathogenic) cell wall is composed of chitin (Yandigeri *et al*., 2015). Microbial chitinolytic enzymes have been considered important in the biological control of many plant pathogens because of their ability to degrade fungal cell walls (Shapira *et al.*, 1989). HCN is a volatile gas that plays an indirect role in biocontrol. The production of HCN and its role in PGP and biocontrol was reported in sugarcane by Bhosale *et al*. (2015) and in sorghum by Gopalakrishnan *et al*. (2011). In addition, cell-wall-degrading enzymes such as β-1,3-glucanase have also been implicated in the biological control of soil-borne fungal pathogens (Singh *et al*., 1999). It has been reported that β-1,3-glucanase suppress fungal growth and indirectly promote plant growth (De Boer *et al*., 1998). The growth hormones produced by microorganisms increase growth rates and improve yields of the host plants (Vinodrai *et al*., 2014). All these direct and indirect PGP traits make BCA-696 the best isolate of choice for PGP in chickpea and sorghum.

Molecular identification of the most promising actinomycete - The most promising PGP actinomycete was identified by 16S rDNA analysis. Neighbor-joining dendrogram was generated using the sequences of the isolate and other sequences from the database. Based on the maximum similarity, the actinomycete isolate was identified as *Amycolatopsis* sp. (Fig. 1) and the nucleotide sequences were submitted to GenBank, NCBI. The accession number is KM191337.

Amycolatopsis sp., is reported to a member of the family *Pseudonocardiaceae and* produce vancomycin-like glycopeptide antibiotic balhimycin (Nadkarni *et al*., 1998). Kenji *et al*. (1993) isolated a PGP substance called Amidenin from *Amycolatopsis* sp. but not further characterized. Recently, Ningthoujam *et al*. (2016) isolated and characterized *Amycolatopsis* spp. from the rhizosphere of upland rice.

Evaluation of *Amycolatopsis* sp. for its PGP traits in chickpea and sorghum under greenhouse and field conditions - Under greenhouse conditions, at 45 DAS, *Amycolatopsis* sp. exhibited enhancements in the growth parameters including the root length (up to 38%), root volume (up to 40%), shoot weight (up to 28%), leaf dry weight (up to 18%), root dry weight (up to 38%), leaf area (up to 28%) and nodule number (up to 45%) when compared to the un-inoculated control. At harvest, the *Amycolatopsis* sp. enhanced the growth and yield parameters including the shoot weight (up to 29%), root weight (up to 16%), pod number (up to 42%) and pod weight (up to 41%) over the un-inoculated control (Table 2).

Under field conditions, at 30 DAS, the *Amycolatopsis* sp. -treated plots significantly enhanced the nodule number (up to 43%), root weight (up to 10%) and shoot weight (up to 31%) whereas at 60 DAS, plant height (up to 4%), leaf weight (up to 14%), pod number (up to 48%), stem weight (up to 36%) and root weight (up to 50%) when compared over the un-inoculated control plots (Table 3). At harvest, the *Amycolatopsis* sp.-treated plots enhanced the pod weight (up to 4%), seed weight (up to 5%), stover yield (up to 6%), grain yield (up to 3%), total dry matter (up to 5%), soil total N (up to 8%), available P (up to 3%) and % organic C (up to 6%) when compared with the un-inoculated control plots (Table 4). Under field conditions, in sorghum, at 60 DAS, the *Amycolatopsis* sp. enhanced PGP parameters such as the plant height (up to 3%), leaf area (up to 11%), leaf weight (up to 21%), stem weight (up to 25%), root weight (up to 18%) and total plant weight (up to 24 %) and at harvest, 1000 seed weight (up to 4%), grain yield (up to 28%), stover yield (up to 6%), total dry matter (up to 12%), available P (up to 18%) and % organic C (up to 11%) over the un-inoculated control plots (Table 5).

In both chickpea and sorghum, the *Amycolatopsis* sp. enhanced the growth and yield parameters including shoot weight, root weight pod number, pod weight stover yield, grain yield and total dry matter when compared over the un-inoculated control plots. The mechanism by which the *Amycolatopsis* sp. consistently enhanced the PGP traits on both chickpea and sorghum could be attributed to their ability to produce siderophores, IAA and β-1,3-glucanase activities (Table 1). PGP in chickpea by bacteria such as *Pseudomonas geniculata* (Gopalakrishnan *et al*., 2015b),

Table 2. The role of *Amycolatopsis* sp. BCA-696 on PGP traits (pot experiment) in chickpea

Isolate	45 days after sowing							At harvest			
	Root length (cm)	Root volume (cm^{-3})	Shoot weight (g $plant^{-1}$)	Leaf dry weight (g $plant^{-1}$)	Root dry weight (g $plant^{-1}$)	Leaf area (cm^{-2} $plant^{-1}$)	Nodule number ($plant^{-1}$)	Shoot weight (g $plant^{-1}$)	Root weight (g $plant^{-1}$)	Pod number ($plant^{-1}$)	Pod weight (g $plant^{-1}$)
BCA-696	3463	8.50	1.11	1.77	0.50	203	44	4.09	1.13	33	7.09
Control	2129	5.14	0.98	1.46	0.31	147	24	2.89	0.95	19	4.16
Mean	2796	6.82	1.04	1.62	0.41	175	34	3.49	1.04	26	5.63
LSD (5%)	745.1	3.087	0.049	0.155	0.165	30.1	5.2	0.438	0.180	11.4	2.925
CV%	8	13	1	3	12	5	4	4	5	12	15

LSD = least significant differences; CV= coefficients of variation

Table 3. The role of *Amycolatopsis* sp. BCA-696 on PGP traits (field experiment) in chickpea

Isolate	At 30 Days After Sowing				At 60 Days After Sowing				
	Nodule number (plant^{-1})	Root weight (g plant^{-1})	Stem weight (g plant^{-1})	Plant height (cm)	Leaf area (cm^{-2} plant^{-1})	Leaf weight (g plant^{-1})	Pod number (plant^{-1})	Stem weight (g plant^{-1})	Root weight (g plant^{-1})
BCA-696	21	0.20	1.97	52	691	5.36	82	5.15	0.88
Control	12	0.18	1.35	50	670	4.59	43	3.32	0.44
Mean	17	0.19	1.66	51	680	4.98	62	4.24	0.66
LSD (5%)	9	0.033	0.250	1.4	176.5	1.815	5.2	1.479	0.152
CV%	15	5	4	1	7	10	2	10	7

Mesorhizobium spp. (Imen *et al*., 2015), *Enterobacter aerogenes* PS16 and *Rhizobium ciceri* (Singh *et al*., 2013) and fungus by *Penicillium citrinum* (Sreevidya *et al*., 2015) were reported. PGP in chickpea by actinomycetes were also reported (Jida and Assefa, 2012; Alekhya and Gopalakrishnan, 2014; Gopalakrishnan *et al*., 2015a,b,c). PGP in sorghum were reported by *Azospirillum brasilense* (Grover *et al*., 2014; Mounde *et al*., 2015) and by actinomycetes, particularly *Streptomyces* spp. (Gopalakrishnan *et al*., 2011, 2013; Alekhya and Gopalakrishnan, 2014) was previously reported. Though, Ningthoujam et al. (2016) isolated and characterized *Amycolatopsis* sp. from upland rice but was not evaluated for their PGP traits under field conditions. Perhaps this is the first study where *Amycolatopsis* sp. was demonstrated for its PGP traits under field conditions. Hence, *this genus of actinomycetes can be exploited for its PGP of cereals and legumes crops.*

The colonizing ability of the *Amycolatopsis* sp. to the root surface of chickpea and sorghum was observed under SEM. *Amycolatopsis* sp. was found to colonize the root surface of both chickpea and sorghum as demonstrated by SEM analysis. When observed under SEM, extensive colonization was observed. Both mycelial growth and sporulation were observed without damaging the root surface (Fig. 2). Ruanpanun *et al*. (2010), Gopalakrishnan *et al*. (2014) and Gopalakrishnan *et al*. (2015a,b,c) have reported the colonization of many plant roots by beneficial actinomycetes but none reported earlier for *Amycolatopsis* sp.

The actinomycete, *Amycolatopsis* sp. BCA-696, can be formulated as bio-inoculant and used for PGP in other crops also. Multi-location trials needs to be conducted in order to understand the usefulness of these in the chickpea and sorghum growing areas. Since the isolate was tested for both PGP and biocontrol traits this study can be further extended for exploiting of biocontrol properties under field conditions. In addition, the secondary metabolite(s) responsible for the PGP needs to be identified and further characterized.

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