



# Deciphering the tri-dimensional effect of endophytic *Streptomyces* sp. on chickpea for plant growth promotion, helper effect with *Mesorhizobium ciceri* and host-plant resistance induction against *Botrytis cinerea*

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## ARTICLE INFO

### Keywords:

Endophytes  
chickpea  
*Streptomyces*  
*Botrytis cinerea*  
Host-plant resistance  
Antioxidants  
Nodulation

## ABSTRACT

A total of 219 endophytic actinobacteria, isolated from roots, stems and leaves of chickpea, were characterized for antagonistic potential against *Botrytis cinerea*, causal organism of *Botrytis* grey mold (BGM) disease, in chickpea. Among them, three most potential endophytes, AUR2, AUR4 and ARR4 were further characterized for their plant growth-promoting (PGP) and nodulating potentials and host-plant resistance against *B. cinerea*, in chickpea. The sequences of 16S rDNA gene of the three endophytes were matched with *Streptomyces* but different species. *In planta*, the isolate AUR4 alone was able to significantly enhance PGP traits including seed numbers (11.8 vs. 9.8/Plant), seed weight (8 vs. 6.8 g/Plant), pod numbers (13.6 vs. 11.5/Plant), pod weight (9.3 vs. 7.5 g/Plant) and biomass (10.9 vs. 8 g/Plant) over the un-inoculated control in chickpea genotype JG11. Interestingly, consortium of the selected endophytes, AUR2, AUR4 and ARR4 were found less effective than single inoculation. Co-inoculation of the selected endophytes with *Mesorhizobium ciceri* significantly enhanced nodulation and nitrogenase activity in five chickpea genotypes including ICCV2, ICCV10, ICC4958, Annigeri and JG11 over the un-inoculated control. The selected endophytes showed antagonistic potential *in planta* by significant reduction of disease incidence (28–52%) in both single inoculation and consortium treatments over the un-inoculated control across the genotypes ICC4954 (susceptible), ICCV05530 (moderately resistant) and JG11 (unknown resistance). Further, antioxidant enzymes such as superoxide dismutase, catalase, ascorbate peroxidase, guaiacol peroxidase, glutathione reductase, phenylalanine ammonia-lyase and polyphenol oxidase and phenolics were found induced in the leaves of chickpea inoculated with selected endophytes over un-inoculated control. Principal component analysis revealed that, the antioxidant enzymes and phenolics were found in the magnitude of ICC4954 < JG11 < ICCV05530 which correlates with their resistance level. The selected endophytes enhanced the plant growth and also host plant resistance against BGM in chickpea.

## 1. Introduction

Endophytes are present in all the plants without affecting the host and may be of either facultative or obligate in nature [1]. However, the endomicrobiome helps in influencing crop health by their specific traits including nutrient acquisition, toxin removal, host-plant resistance to biotic and abiotic stress factors. Their functional role in interactions with host and influencing phytochemical constituents is also highlighted [2]. Role of endophytes as biocontrol agents has long history from 4 decades ago, starting with the knowledge on symptom inhibition [3], to exploration of its multipartite functions as anti-insect and anti-phytopathogenic agents [4].

Many prokaryotic endophytes has been reported for crop health traits comprises gamma proteobacteria, alpha proteobacteria and beta proteobacteria with 56, 57 and 23 recognized genera, respectively; and the major reported genus are *Pseudomonas*, *Enterobacter*, *Burkholderia*, *Rhizobium* and *Bradyrhizobium* [2]. The gram positive endophytes belongs to the class Actinobacteria comprises diverse endophytes of 107 recognized genera with majorly reported genus of *Streptomyces*, *Microbacterium*, *Mycobacterium*, *Arthrobacter* and *Curtobacterium*. It is known that, actinobacteria majorly *Streptomyces* are the potential producers of bioactive metabolites for safeguarding plant, animal and human health [5].

Chickpea, the second largest cultivated legume in the world and

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**Table 1***In vitro* biocontrol and PGP traits of selected chickpea endophytic actinobacteria against *B. cinerea*.

Isolates	Antibiosis	Volatiles		Competitive molecules	Extracellular lytic enzymes					Growth hormone	Mineral mobilization			Stress reliever
		HCN	Ammonia		Siderophore	β-1,3-glucanase μ	Chitinase	Cellulase	Protease		Lipase	IAA#	P %	
AUR2	69 ± 3	1	+	3	5 <sup>b</sup> ± 0.2	3	–	0	4	12 <sup>a</sup> ± 0.2	–	–	400 ± 6	0.7 ± 0.1 <sup>b</sup>
AUR4	63 ± 2	1	–	2	4 <sup>b</sup> ± 0.3	4	3	1	3	5 <sup>b</sup> ± 0.8	38 ± 0.2	–	–	0.3 ± 0.1 <sup>b</sup>
ARR4	62 ± 1	2	+	1	8 <sup>a</sup> ± 0.4	3	2	4	2	2 <sup>b</sup> ± 0.3	–	–	107 ± 7	8.4 ± 0.6 <sup>a</sup>

Values are Mean  $\pm$  SE (n = 3). HCN- Hydrocyanic acid. IAA – Indole acetic acid. ACC – 1-amino cyclo propane-1-carboxylic acid.  $\mu$  - % inhibition,  $\mu$  - Units, One unit of  $\beta$ -1,3-glucanase activity was defined as the amount of enzyme that liberated 1  $\mu$ mol of glucose h<sup>-1</sup> at defined conditions. # -  $\mu$ g ml<sup>-1</sup>. % - P equivalents  $\mu$ g ml<sup>-1</sup>.  $\mu$  - % Solubilization efficiency.  $\mu$  - nmoles  $\alpha$ -ketobutyrate mg protein<sup>-1</sup> h<sup>-1</sup>. + and – indicates positive and negative for ammonia production. The rating scale for HCN production are 0 = no color change, 1 = light reddish brown, 2 = medium reddish brown and 3 = dark reddish brown. The rating scale for siderophore are 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. The rating scale for chitinase, cellulase, protease and lipase are 0 = no change, 1 = 1–6 mm, 2 = halo zone of 7–12 mm, 3 = halo zone of 19–24 mm and 4 = halo zone of 25–30 mm and above. Different superscript lowercase letters in the same column indicates significant difference (p < 0.05) as per Tukey's test.

India is the lead producer. Productivity of chickpea is stagnant for the last four decades, but it may be improved if the adverse effects of abiotic and biotic stresses are reduced. Among the various biotic stresses of chickpea, *Botrytis* grey mold (BGM), caused by *Botrytis cinerea* Pers. ex. Fr., a broad host range fungus can devastate the crop up to 100% [6]. Management of BGM is not easy as no single control measure including chemical control and use of resistant cultivar is completely effective [7]. However, plant growth-promoting (PGP) agents including essential oils and antagonistic microbes are reported to control BGM effectively [8,9]. Use of endophytic actinobacteria in the context of chickpea has been evaluated for the biocontrol of *Phytophthora* [10] and *Sclerotium rolfsii* [11,12]. However, there are no reports exploring endophytic actinobacteria for the biological control of BGM. The main objective of the present study was to explore chickpea endophytic actinobacteria for multipartite interactions including PGP, nodulation and nitrogen fixation and host-plant resistance induction against *B. cinerea* in chickpea.

## 2. Materials and methods

### 2.1. Sample collection and isolation of endophytic actinobacteria

Chickpea plants were collected during Nov 2014 across India (Supplementary Table 1) and kept for air drying at room temperature for 48 h. The samples were washed thoroughly with water including sonication which removes soil particles and organic matter. Chickpea plants were surface sterilized as per Coombs and Franco [13]. The sterilized plants were excised into root, shoot and leaves using sterile scalpel, crushed using sterile mortar and pestle and inoculated on tap water yeast extract agar and humic acid-vitamin B agar supplemented with Benomyl (50  $\mu$ g/mL). The plates were incubated at 28 °C for 2 weeks. Effect of surface sterilization was tested by inoculating surface-sterilized uncrushed plant parts into each isolation media and incubated as above. Presence of bacterial growth in these plates indicates inadequate sterilization and corresponding endophyte culture plates of such samples were discarded. The selected endophytic actinobacterial isolates were stored in actinomycete isolation agar plates at 4 °C.

### 2.2. Preliminary screening by antibiosis

All the isolates were screened for antibiosis property against *B. cinerea* (acquired from Legumes Pathology, ICRISAT, Patancheru, India) by dual culture assay. The isolates were streaked on glucose casamino acid yeast extract agar and incubated at 28 °C for 2 days. Later, a 6 mm disc of *B. cinerea* was placed on the centre of the plate and incubated at 19 °C for a week. The diameter of inhibition zones was measured and the % inhibition was calculated [14]. Endophytes with highest inhibitory activity were characterized further.

### 2.3. *In vitro* biocontrol and plant growth-promoting traits

The selected endophytes were evaluated for the production of siderophore [15],  $\beta$ -1,3-glucanase [16], chitinase [17], indole acetic acid (IAA) [18], cellulase [19], lipase and protease [20], hydrocyanic acid (HCN) [21], ammonia [22] and 1-aminocyclopropane-1-carboxylate (ACC) deaminase [23]. The selected endophytes were also evaluated for solubilization traits on phosphate [24], potassium [25] and zinc [26].

### 2.4. Molecular identification

The most potential antagonistic isolates against *B. cinerea* were identified by 16 S rDNA analysis at MacroGen Inc. Seoul, Korea [27]. The sequences were submitted to GenBank, NCBI and accession numbers were obtained.

### 2.5. Pot experiments

The selected isolates were evaluated *in planta* for PGP properties (on chickpea genotype JG11; experiment 1), co-inoculation effect (on five chickpea genotypes ICCV2, ICCV10, ICC4958, Annigeri and JG11 with reference strain *Mesorhizobium ciceri* ATCC 51585<sup>T</sup>; experiment 2) and antagonistic and induced host-plant resistance properties against *B. cinerea* (on chickpea genotypes ICC4954 [susceptible to BGM], ICCV05530 [moderately resistant to BGM] and JG11 [unknown resistance]; experiment 3) under controlled environmental conditions at ICRISAT, Patancheru, India. A randomized complete block design was used in all the experiments. The chickpea seeds were obtained from Genebank, ICRISAT, Patancheru, India.

#### 2.5.1. Experiment 1 – *In planta* PGP properties

The experiment was conducted during Oct 2015. It contained 5 treatments (control, the three best antagonistic isolates and their consortium, i.e., mix of three best antagonistic isolates) with 6 replications/treatment. Pot mixture was prepared with black soil, sand and farm yard manure (3:2:1), sterilized and filled in 8" plastic pots. The chickpea seeds were surface sterilized (2.5% sodium hypochlorite for 5 min) and subjected to seed bacterization (10<sup>8</sup> CFU/mL/h). The seeds were allowed to dry and sown in pots (4 seeds/pot, but thinned to 2 after a week). Booster doses of isolates (5 mL/seedling, 10<sup>8</sup> CFU/mL) were applied at 15 and 30 days after sowing (DAS) by soil drench method. Shoot length and dry weight, number of branches, flowers and pods, leaf area, SPAD, leaf dry weight, and root length, surface area, volume and dry weight were determined at 45 DAS. During harvest, seed number and weight, pod number and weight and total biomass were determined.

**Table 2**  
In planta PGP properties of selected endophytic actinobacteria on chickpea genotype JG11 under glasshouse conditions at 45 DAS.

Treatments	Shoot traits			Leaf traits			Root traits			Reproductive traits		
	Length (cm)	Dry Weight (g)	Number of branches	Area (cm <sup>2</sup> )	SPAD	Dry Weight(g)	Length (cm)	Surface Area (cm <sup>2</sup> )	Volume (cm <sup>3</sup> )	Dry Weight (g)	Number of flowers	Number of pods
AUR2	26.6 ± 1.3	1.3 ± 0.1	9.8 ± 1.1	139.6 ± 10.4	65.4 ± 1.4	2.1 ± 0.2	4679.5 ± 314.9	968.1 ± 76.3	16.1 ± 1.5	0.6 ± 0.1	10.8 ± 1.6	2.9 ± 0.4
AUR4	29.6* ± 0.4	1.5 ± 0.1	11.0* ± 0.3	203.1* ± 8.0	69.5* ± 0.9	2.3 ± 0.2	4282.1 ± 154.2	1037.3* ± 45.8	17.7* ± 0.5	0.5 ± 0.1	12.8* ± 0.9	3.0 ± 0.3
ARR4	28.3* ± 0.5	1.4 ± 0.1	8.7 ± 0.7	202.1* ± 2.9	64.1 ± 1.3	2.3 ± 0.1	4404.3 ± 318.2	950.5 ± 53.7	16.4 ± 0.7	0.5 ± 0.0	8.8 ± 0.6	3.5* ± 0.5
Consortium	26.3 ± 0.5	1.4 ± 0.1	9.6 ± 0.7	179.4 ± 16.4	62.8 ± 1.0	2.2 ± 0.1	4411.5 ± 332.4	921.5 ± 68.0	15.4 ± 1.1	0.5 ± 0.0	10.2 ± 0.9	2.8 ± 0.3
Control	24.9 ± 0.8	1.4 ± 0.0	8.7 ± 0.4	168.6 ± 5.9	63.9 ± 1.2	2.2 ± 0.0	4100.6 ± 575.4	788.4 ± 91.4	14.1 ± 1.4	0.6 ± 0.1	9.3 ± 0.9	2.1 ± 0.2

Values are Mean ± SE (n = 6). AUR2 – *Streptomyces* sp. AUR2 treated. AUR4 – *Streptomyces* sp. AUR4 treated. ARR4 – *Streptomyces* sp. ARR4 treated. Consortium – AUR2 + AUR4 + ARR4 treated. \* - Values are statistically significant as per Dunnett's test ( $p < 0.05$ ) over the control.

### 2.5.2. Experiment 2 – In planta co-inoculation effect

The experiment contained five treatments (control, *M. ciceri*, best antagonistic isolate 1 + *M. ciceri*, best antagonistic isolate 2 + *M. ciceri*, best antagonistic isolate 3 + *M. ciceri*) with 6 replications/treatment and conducted during Nov 2016. Pot mixing, seed surface sterilization, seed bacterization and sowing were done as like experiment 1 and according to the treatments. *M. ciceri* was grown in yeast mannitol broth at 200 rpm, 28 °C for 5–7 days with the cell count of  $\sim 1 \times 10^9$  CFU/mL. For co-inoculation treatments, a cocktail consists of *M. ciceri* and selected endophyte in the ratio of 1:1 was used [28]. Booster dose of inoculum was added at 15 DAS by soil drench method. Plant growth responses were determined by shoot, root and nodule dry weight at 35 DAS. Nitrogenase activity was estimated by acetylene reduction activity in Agilent 7890 B gas chromatograph equipped with a flame ionization detector.

### 2.5.3. Experiment 3 – In planta antagonistic and induced host-plant resistance properties

The experiment was conducted during May 2016. Inoculum of *B. cinerea* was prepared as per the protocols of Pande et al. [29], with the cell count of  $\sim 3 \times 10^5$ /mL, using marigold flowers (*Tagetes erecta* L.). Chickpea seeds were planted in rows in plastic trays (35 × 25 × 8 cm) filled with sterilized sand and vermiculate (4:1). The plants were grown in glass house up to 10 days; later the seedlings were transferred to a controlled environment growth chamber maintained at  $15 \pm 2$  °C with approximately 1500 lux light intensity and 12 h photoperiod. The experiment was conducted with three replications and each replication contained 10 seedlings. Commercially available *Trichoderma harzianum* (ECOSOM®-TH) was used as reference strain. The experiment contained seven treatments including T1: control, T2: disease control (*B. cinerea* challenged), T3: best antagonistic isolate 1 treated, T4: best antagonistic isolate 2 treated, T5: best antagonistic isolate 3 treated, T6: consortium treated and T7: *T. harzianum* treated. In treatments T3-T7, the test inoculum were sprayed first, allowed to dry and sprayed with *B. cinerea* inoculum. Plant growth room was maintained with 24 h of 100% RH for first 10 days after pathogen inoculation (DAPI) followed by 8 h for another 2 days. Disease symptoms were monitored on 4 DAPI and recorded at 2 days interval till 12 DAPI. Disease severity was scored at a rating scale of 1–9 [30] and the area under disease progress curve (AUDPC) was calculated [31].

For host plant resistance properties, chickpea leaves were collected in liquid nitrogen at 4, 6, 8, 10 and 12 DAPI from all the three replications and the samples were stored at  $-80$  °C for further analysis. The oxidative damage to lipids was determined as malondialdehyde (MDA) content [32] and the results were expressed as nMoles MDA/g Fresh Weight (FW). For antioxidant enzyme analysis, soluble proteins of the leaf samples were extracted with 0.1 M phosphate buffer, pH 7.5 containing 1% polyvinylpyrrolidone, 1 mM EDTA and 10 mM mercaptoethanol in a pre-chilled mortar and pestle. For ascorbate peroxidase activity, the extraction buffer is supplemented with 1 mM ascorbic acid. The homogenate was centrifuged at  $10,000 \times g$  for 10 min at 4 °C and the supernatant was stored at  $-80$  °C until further analysis. The antioxidant enzymes studied include, superoxide dismutase (SOD) [33], catalase (CAT) [34], ascorbate peroxidase (APX) [35], guaiacol peroxidase (GPX) [36], glutathione reductase (GR) [37], phenylalanine ammonia-lyase (PAL) [38] and polyphenol oxidase (PPO) [39]. Total phenolic content (TPC) were estimated by Folin-Ciocalteu method [40] and the results were expressed as mg gallic acid equivalents (GAE)/g FW.

### 2.6. Statistical analysis

The data of (i) *in-vitro* PGP traits, (ii) chickpea growth responses and nodulation, and (iii) disease incidence and antioxidant parameters were subjected to one-way analysis of variance (ANOVA) and the significant difference between mean values was determined by Tukey's and

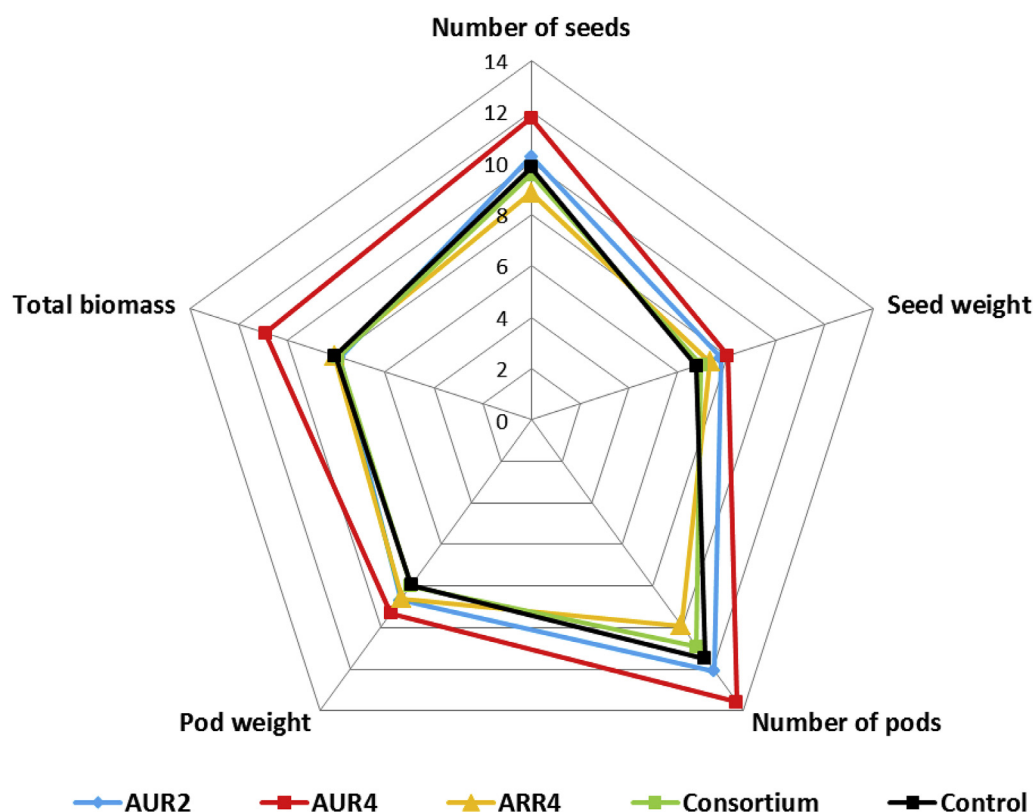


Fig. 1. Yield components of chickpea JG11 towards selected endophytic actinobacterial treatment under glasshouse conditions.

Values are Mean  $\pm$  SE (n = 6). AUR2 – *Streptomyces* sp. AUR2 treated; AUR4 – *Streptomyces* sp. AUR4 treated; ARR4 – *Streptomyces* sp. ARR4 treated; Consortium – AUR2 + AUR4 + ARR4 treated.

Dunnett's test using Statistical Package for the Social Sciences (SPSS) 13.0 (SPSS Inc., Chicago, Illinois, USA). Pearson correlation coefficient has been calculated between *in vitro* and *in planta* growth promoting traits, and between the *in planta* antioxidant parameters using SPSS. Principal component analysis (PCA) on chickpea growth responses and antioxidant parameters has been done using R statistical package 3.2.5 (R Foundation for Statistical Computing). Significant relationship between the antioxidant parameters in the context of genotype, treatment, time and its interactions were tested through one-way and two-way ANOVA by GENSTAT 14.0 (VSN International Ltd., Hemel Hempstead, UK).

### 3. Results

#### 3.1. Preliminary screening against *B. cinerea* and *in vitro* PGP traits

The chickpea plants collected across India yielded 219 endophytic actinobacteria in which roots possess highest number of endophytes (129) followed by leaves (47) and stems (43) (Supplementary Table 1). In the preliminary screening for antibiosis against *B. cinerea*, 15 endophytic isolates were found to have 50% and above antagonistic activity (Data not shown). Among the 15 antagonistic isolates, 10 were of root endophytes while 4 and 1 were of leaves and stem endophytes, respectively. Among the 15 endophytes, three root endophytes, AUR2, AUR4 and ARR4 having 62–69% antagonistic activity were selected for further characterization (Table 1). These root endophytes were found to produce HCN, siderophore,  $\beta$ -1,3-glucanase, chitinase, lipase, IAA and ACC deaminase. Other traits including production of ammonia, cellulase, protease and P and Zn solubilization were noticed in either of the isolates. However, none of the isolates solubilized K. Consortium of these three promising endophytes (1:1:1 ratio) is evaluated for their antagonistic activity against *B. cinerea*. Inhibitory activity of 73% was

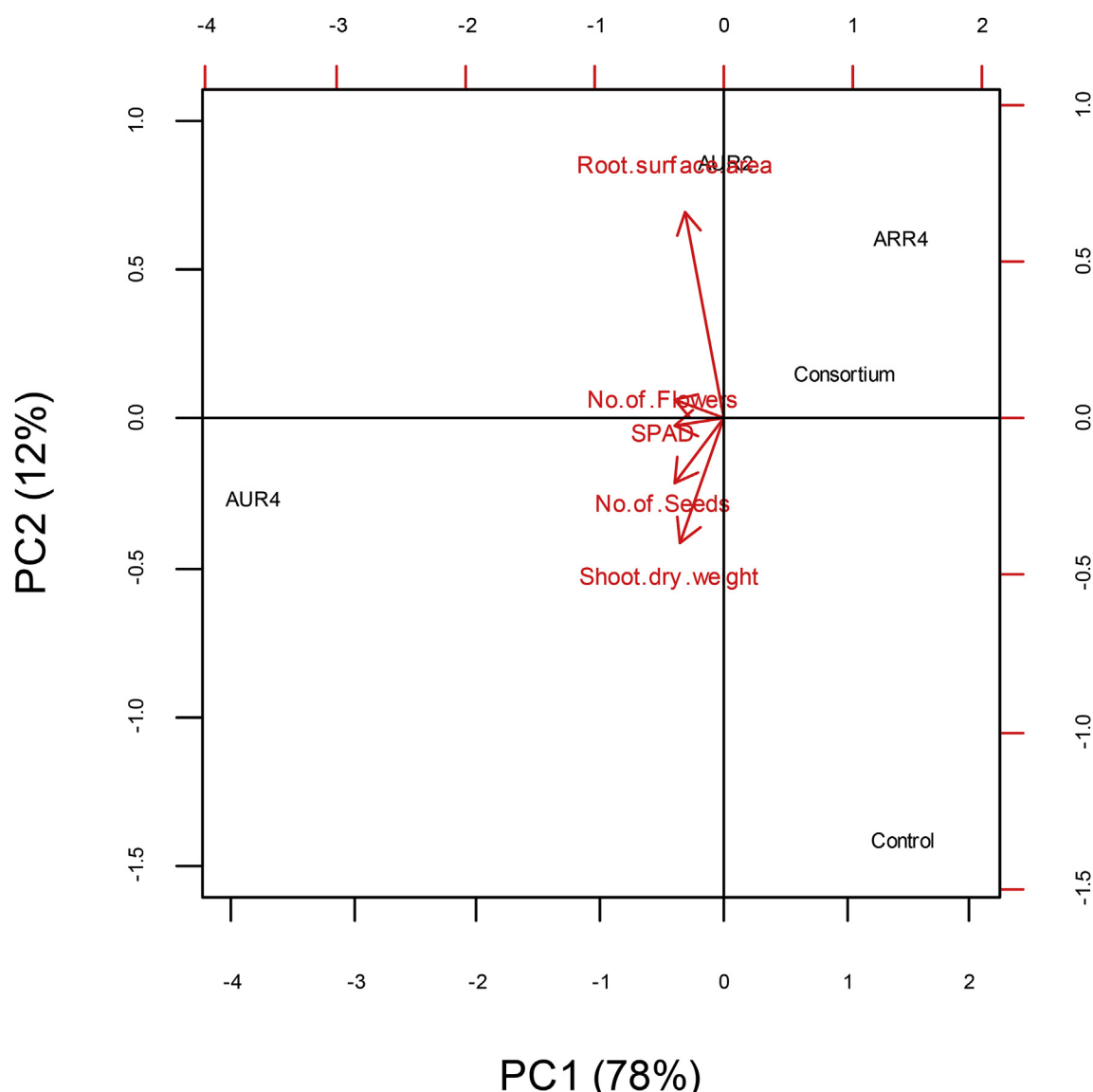
noted (data not shown) which gives a hint for their compatibility and enhanced inhibitory activity on *B. cinerea*.

#### 3.2. Molecular identification of the selected antagonistic endophytic actinobacteria

The selected root endophytes AUR2, AUR4 and ARR4 were identified as *Streptomyces* sp. and their phylogenetic relationship is depicted in Supplementary Fig. 1. Partial sequences were submitted to GenBank, NCBI and accession numbers obtained as *Streptomyces* sp. AUR2 (KX427130), *Streptomyces* sp. AUR4 (KX427131) and *Streptomyces* sp. ARR4 (KX427132).

#### 3.3. *In planta* PGP properties

The selected antagonistic endophytes, AUR2, AUR4 and ARR4, were evaluated for their PGP traits *in planta*. At 45 DAS, traits including shoot length, number of branches, leaf area, SPAD, root surface area, root volume and number of flowers were significantly influenced by endophyte AUR4 over the control plants. The other traits were not influenced by any of the root endophyte treatment (Table 2). Further, consortium was found to be less effective compared to single inoculations. At harvest, seed number (11.8 vs. 9.8/Plant), seed weight (8 vs. 6.8 g/Plant), pod number (13.6 vs. 11.5/Plant), pod weight (9.3 vs. 7.5 g/Plant) and biomass (10.9 vs. 8 g/Plant) were influenced only by the endophyte AUR4 (Fig. 1). Pearson correlation coefficient analysis between *in vitro* PGP traits of root endophytes and *in planta* growth responses of chickpea showed significant positive correlations between P solubilization vs. total biomass, Zn solubilization vs. root length and protease vs. pod number (Supplementary Table 2). The observed chickpea growth responses have highest positive correlation with minimum of 2 and maximum of 6 *in vitro* PGP traits. PCA for the



**Fig. 2.** Principal component analysis on chickpea growth responses towards endophytic actinobacteria treatment. AUR2 – *Streptomyces* sp. AUR2; AUR4 – *Streptomyces* sp. AUR4; ARR4 – *Streptomyces* sp. ARR4; Consortium - AUR2 + AUR4 + ARR4 treated.

selected chickpea growth responses shows the contribution of 78% and 12% variance for PC1 and PC2, respectively (Fig. 2). This further confirms that, single inoculations with AUR4 shows highest growth responses than consortium and/or other selected root endophytes.

### 3.4. In planta co-inoculation effect

Single inoculations of chickpea rhizobia *M. ciceri* and co-inoculation of *M. ciceri* + selected antagonistic root endophytes significantly ( $p < 0.05$ ) induced nodulation and nitrogen fixation over the un-inoculated control plants irrespective of the chickpea genotypes (Fig. 3). Since single inoculation of endophytes didn't produce any nodules in experiment 1, they were not included in this experiment. Among the microbial treatments, there was no significant difference between single inoculation of *M. ciceri* and co-inoculation of *M. ciceri* + root endophytes in nodulation and nitrogenase activity; though some fold increases were seen in *M. ciceri* + AUR4 treatment across the genotypes by about 3–46% nodulation and 27–113% nitrogenase activity. In chickpea genotype ICC4958, single inoculation of *M. ciceri* showed highest values than co-inoculation with root endophytes. Genotypes ICCV2, ICC4958, Annigeri and JG11 showed minute nodule like

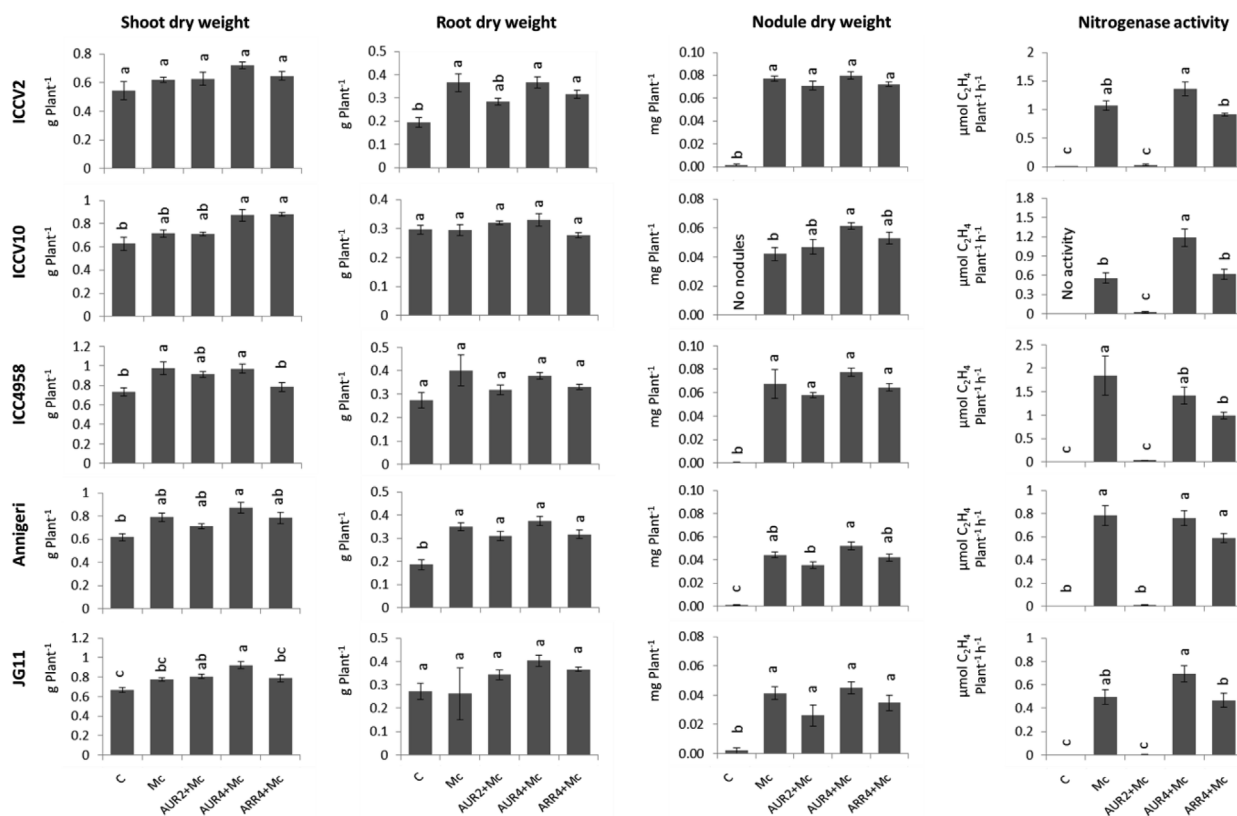
structures in control, in spite of sterile soil used in the experiment, however, they are devoid of nitrogenase activity. Significant increases of shoot dry weight over the control were noticed in *M. ciceri* + AUR4 inoculation in all the genotypes except ICCV2. In contrast, significant root dry weight was seen only in ICCV2 and JG11. This was further confirmed by ANOVA, where root dry weight was non-significant between genotypes and also in genotype  $\times$  treatment interaction (Supplementary Table 3).

### 3.5. In planta antagonistic and induced host-plant resistance properties

#### 3.5.1. Effect on disease severity

The disease severity depicted as AUDPC in Fig. 4 shows that, ICCV05530 (30–58) and ICC4954 (42–75) were medium resistant and susceptible to *B. cinerea* respectively; while JG11 (31–65) was intermittent between them. The severity was highest in disease control treatments by about 58–75; whereas, microbial treatments significantly ( $p < 0.05$ ) reduced the severity to 31–50. Within the selected endophyte treatments there was no significant difference between individual inoculums, consortia and *T. harzianum* in reducing disease severity of all the three chickpea genotypes. AUR4 alone showed





**Fig. 3.** Effect of co-inoculation on selected endophytic actinobacteria and *Mesorhizobium ciceri* on nodulation and nitrogen fixation in five genotypes of chickpea. Values for bars are Mean ( $n = 6$ ). Error bar indicates SE. Bars within a graph not sharing the same letter are significantly different as per Tukey's test ( $p < 0.05$ ). C – Control; Mc – *Mesorhizobium ciceri* ATCC 51585<sup>T</sup> treated; AUR2 + Mc – *Streptomyces* sp. AUR2 and *M. ciceri* treated; AUR4 + Mc – *Streptomyces* sp. AUR4 and *M. ciceri* treated; ARR4 + Mc – *Streptomyces* sp. ARR4 and *M. ciceri* treated.

significantly lower disease severity than other microbial treatments JG11 alone.

### 3.5.2. Effect on primary antioxidants

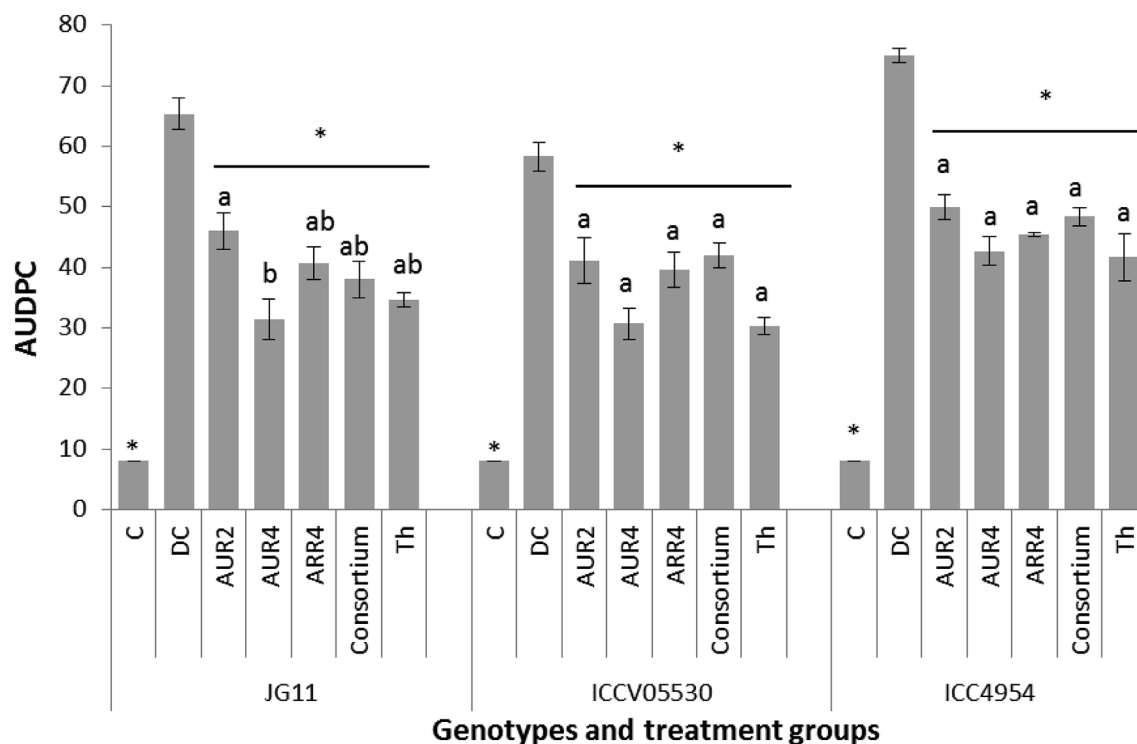
MDA was observed to increase with time irrespective of the chickpea genotype studied in the range of 21–43, 17–37 and 29–48 nMoles/g FW in JG11, ICCV05530 and ICC4954, respectively. The highest contents were noticed in disease control groups and reduction of peroxidation by about 24–53% was observed by microbial treatments in which *T. harzianum* registered the least MDA content (Supplementary Fig. 2).

All the selected endophytes, consortium and *T. harzianum* significantly enhanced the SOD activity in chickpea plantlets over the control and disease control with nearly similar activity units in JG11 (4–35 U/g FW) and ICCV05530 (7–39 U/g FW) (Supplementary Fig. 3). CAT activity observed in the range of 88–102, 95–112 and 64–107  $\mu\text{mol H}_2\text{O}_2$  decomposed/min/g FW in control groups of JG11, ICCV05530 and ICC4954, respectively shows the basal CAT activity of chickpea plants. Induction of CAT towards microbial treatment was similar to induction of SOD (Supplementary Fig. 4). Irrespective of the microbial treatments, all the chickpea genotypes induced the APX activity till 12 DAPI with 27–89  $\mu\text{mol}$  ascorbate oxidized/min/g FW in JG11 and ICCV05530; and 17–65  $\mu\text{mol}$  ascorbate oxidized/min/g FW in ICC4954 (Supplementary Fig. 5). The microbial treatments have increased the GPX activity up to 35–117%, 39–133% and 27–173% over the control groups in JG11, ICCV05530 and ICC4954, respectively (Supplementary Fig. 6). Control plants showed GR activity in the range of 18–27, 25–32 and 12–18 U/g FW in JG11, ICCV05530 and ICC4954, respectively during the 12 day study period. It was also induced by both *B. cinerea* and endophytes up to 8 and 12 DAPI, respectively (Supplementary Fig. 7). Changes of PAL activity over the time course

with the activity units of 4.2–7.8, 4.4–8.5 and 3.6–6.9 mMol *t*-cinnamic acid/g FW was documented in which the lower and higher units was registered by disease control and *T. harzianum* treatment respectively in JG11, ICCV05530 and ICC4954, respectively (Supplementary Fig. 8). PPO activity was observed lower in ICC4954 (0.1–0.8 U/g FW) when compared to ICCV05530 (0.4–2.5 U/g FW) and JG11 (0.2–1.7 U/g FW) (Supplementary Fig. 9). Phenolics were found to increase in both disease control (74–226%) and selected endophytes and *T. harzianum* treatments (142–813%) up to 12 DAPI (Supplementary Fig. 10).

### 3.5.3. PCA and correlation analysis for induced host-plant resistance properties

The influence of selected endophytes on the tested antioxidant parameters across the chickpea genotypes were studied by PCA (Fig. 5). PC1 (70%) is associated with the effect of selected antagonistic endophytes and *T. harzianum*; and disease control, as the highest difference is being contributed by *B. cinerea* challenged and *T. harzianum* treatments; while PC2 (17%) is associated with the effect of pathogen, as the highest difference is being observed between control and disease control groups. PC1 and PC2 also decipher the role of disease resistance spectrum with the highest difference between ICCV05530 and ICC4954 in PC1; and JG11 and ICCV05530 with ICC4954 in PC2. This further confirms the sensitiveness of ICC4954 towards *B. cinerea*. Importance of all the tested antioxidant enzymes in protecting the plants was shown by the arrangement of arrows in the biplot (Fig. 5) and further confirmed by their significant positive correlations (Table 3). PCA on individual chickpea genotype JG11, ICCV05530 and ICC4954 provides an overview on highest antioxidant expression which was shown by consortium and *T. harzianum* treatment across the genotypes (Supplementary Figs. 11–13). ANOVA on Table 4 shows the significance of antioxidants between the genotypes except CAT, GR and



**Fig. 4.** Disease response of chickpea genotypes against *B. cinerea* towards endophytic actinobacteria treatment.

Values for bars are Mean AUDPC (n = 3). Error bar indicates SE. \* Values are statistically significant against DC as per Dunnett's test ( $p < 0.05$ ). Bars having different lowercase letters within a genotype indicate the significant difference among the endophytes and *Th* treated groups as per Tukey's test ( $p < 0.05$ ). C – Control; DC – Disease Control (*Bc* challenged); AUR2 – *Streptomyces* sp. AUR2 treated; AUR4 – *Streptomyces* sp. AUR4 treated; ARR4 – *Streptomyces* sp. ARR4 treated; Consortium – AUR2 + AUR4 + ARR4 treated; *Th* – *Trichoderma harzianum* treated.

TPC which shows non-significance. On the other hand, treatment and time has significance on all of the tested antioxidants. Two-way ANOVA observed that, time has no significance on genotypes in influencing antioxidants. Still, time has effect on treatments and treatment has its effect on genotypes except PAL on both the interactions.

#### 4. Discussion

Endophytes usually obtain their nutritional requirements and protection against various environmental stress factors from host plants. In return, they provide enhanced crop growth-promoting traits through multi-partite interactions such as nitrogen fixation, phyto-hormone production, biocontrol of phytopathogens and induction of systemic resistance [41]. Understanding the functions of endophytic actinobacteria in the context of legumes and chickpea in particular is superficial. Hence, the current study was intended to isolate chickpea endophytic actinobacteria and to characterize their role in PGP and host-plant resistance induction.

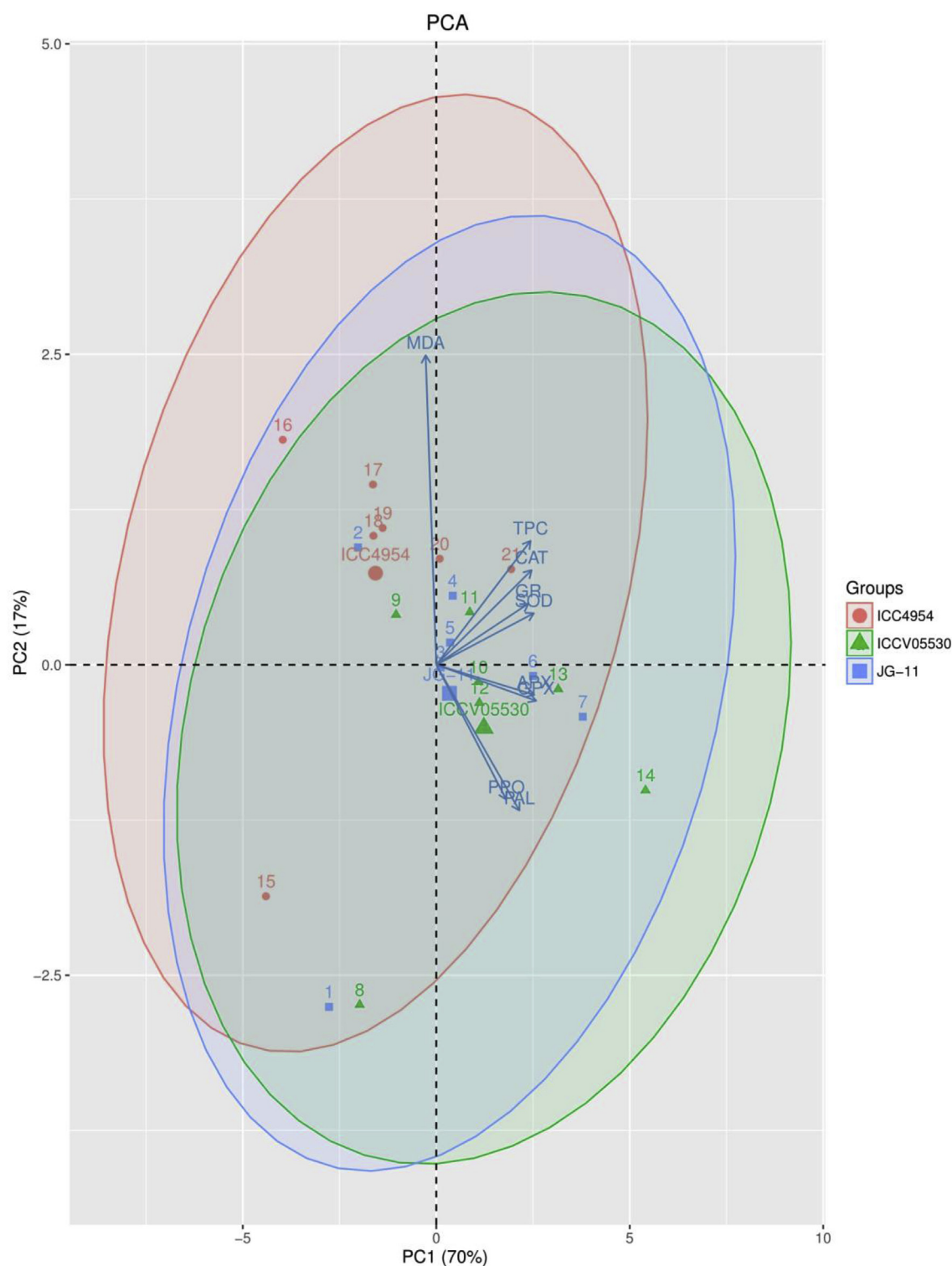
Among the 219 chickpea endophytes isolated, 15 were found to have more than 50% inhibitory activity towards *B. cinerea*. The three most potential isolates, AUR2, AUR4 and ARR4, were identified as *Streptomyces* sp. and observed to produce known antibiosis molecules including HCN, ammonia, siderophores,  $\beta$ -1,3-glucanase and chitinase. Endophytic *Streptomyces* from wilt resistant and susceptible tomato cultivars were reported to have antibiosis through siderophores and cell-wall degrading enzymes [42]. In the present study, IAA production of 2–12  $\mu\text{g/mL}$  by endophytic *Streptomyces* corroborates with Shutsrirung et al. [43] who reported an average IAA production of about 1.4–140.3  $\mu\text{g/mL}$  by the endophytes *Streptomyces*, *Nocardia*, *Nocardiopsis*, *Spirillospora*, *Microbispora* and *Micromonospora*.

Under wild conditions, microbes live as consortium, rather than single strain. Hence, the present study evaluated the *in planta* effects of selected root endophytes, AUR2, AUR4 and ARR4, as single inoculum

and also as consortium. Under glasshouse conditions, root endophyte AUR4 showed highest growth responses than other endophytes and/or consortium. Shutsrirung et al. [43] also reported enhanced shoot height, shoot weight and root weight of mandarin seedlings by 20–49% and 14–53% and 2–102% respectively, over the un-inoculated control by endophytes such as *Streptomyces*, *Nocardia*, *Nocardiopsis*, *Spirillospora*, *Microbispora* and *Micromonospora*. It is known that, higher IAA contributes to higher shoot and root growth; whereas lower IAA results in induced root growth rather than shoot. In the current study, the lower IAA production was associated with root growth which was indicated by positive and negative correlations with root and shoot traits respectively. This is highly correlating with Shutsrirung et al. [43] who noticed highest shoot growth by endophyte *Nocardia*, the higher IAA producing strain (62–222  $\mu\text{g/mL}$ ); whereas lower shoot growth promotion by endophyte *Microbispora*, the lower IAA-producing strain (0.3–3  $\mu\text{g/mL}$ ).

Helper effect of PGP bacteria with the simultaneous infection of nodulating rhizobia has increased nodulation and crop growth [28]. In this study, our endophytes along with the co-inoculation of *M. ciceri* have induced nodulation and nitrogenase activity to some extent across the five chickpea genotypes. Saini et al. [44] reported 22.5% of chickpea yield increase during the co-inoculation of endophytic *Bacillus subtilis* and *Mesorhizobium* sp. Similarly *Pseudomonas brassicacearum* Zy-2-1, a nodule endophyte of leguminous weed *Sphaerophysa salsula* enhanced the plant height, nodule number, nodule weight and nitrogen content of *Medicago lupulina* during its co-inoculation with *Sinorhizobium meliloti* than the single inoculation of *S. meliloti* [45].

Generally, plants have an inherent strategy for protecting themselves from any external factors. However, priming of plants with PGP bacteria increased the intensity of defense response and stress tolerance. In the present study, the selected root endophytes were found to reduce the severity of *B. cinerea* both as single inoculation and consortium irrespective of the chickpea genotypes used. Similarly potato



**Fig. 5.** Principal component analysis of antioxidant parameters in three chickpea genotypes against *B. cinerea* by selected endophytic actinobacteria. 1, 8, 15 – Control; 2, 9, 16 – Disease Control (challenged with *B. cinerea*); 3, 10, 17 – *Streptomyces* sp. AUR2 treated; 4, 11, 18 – *Streptomyces* sp. AUR4 treated; 5, 12, 19 – *Streptomyces* sp. ARR4 treated; 6, 13, 20 – Consortium consists of AUR + AUR4 + ARR4 treated; 7, 14, 21 – *Trichoderma harzianum* treated.

endophytes *Pseudomonas* sp. IMBG294 and *Methylobacterium* sp. IMBG290 showed significant disease reduction towards the necrotroph *Pectobacterium atrosepticum* over the un-treated potato plants [46]. Singh et al. [47] reported antagonistic activity against *Rhizoctonia solani* on tomato plant mortality (%) by *Streptomyces* species such as *S. coelicolor* (31%), *S. girseus* (33%), *S. albus* (26%), *S. antibiotics* (43%) and *S. champavatii* (37%) over the control plants (78%).

Besides the toxin production, virulence of *B. cinerea* positively correlates with the intensity of oxidative burst [48] and hence we analyzed key antioxidants parameters. In the current study, role of antioxidant in normal cellular processes is indirectly indicated by their consistent basal level expression in control plants. MDA, a final product of lipid peroxidation is an indicator for oxidative stress and cell membrane damage. In the present study, endophytic *Streptomyces* treatment



**Table 3**Pearson correlation coefficients of antioxidant parameters of chickpea genotypes against *B. cinerea* by endophyte treatment.

Antioxidant parameters	MDA	SOD	CAT	APX	GPX	GR	PAL	PPO	TPC
MDA	1								
SOD	−0.028	1							
CAT	0.112	.954**	1						
APX	−0.131	.857**	.854**	1					
GPX	−0.153	.868**	.820**	.926**	1				
GR	0.053	.848**	.826**	.743**	.843**	1			
PAL	−.501*	.671**	.607**	.761**	.816**	.687**	1		
PPO	−0.293	.493*	.449*	.758**	.743**	.457*	.553**	1	
TPC	0.225	.927**	.962**	.812**	.819**	.871**	.575**	.438*	1

MDA – Malondialdehyde; SOD - Superoxide Dismutase; CAT – Catalase; APX - Ascorbate Peroxidase; GPX - Guaiacol Peroxidase; GR - Glutathione Reductase; PAL - Phenylalanine Ammonia-Lyase; PPO - Polyphenol Oxidase; TPC - Total Phenolic Content; \* Correlation is significant at  $p < 0.05$ ; \*\* Correlation is significant at  $p < 0.01$ .

**Table 4**Analysis of variance on antioxidant parameters of chickpea genotypes against *B. cinerea* by selected endophytes.

Source of variation	Df	Mean square								
		MDA	SOD	CAT	APX	GPX	GR	PAL	PPO	TPC
Genotype	2	122.94*	237.04**	13492 <sup>NS</sup>	3222.9***	1162.23***	140.95 <sup>NS</sup>	4.716*	2.29029***	152.4 <sup>NS</sup>
Treatment	6	356.334***	461.73***	48668.4***	1412.3***	856.27***	645.45***	8.8218***	0.47595***	1277.191***
Time	4	5421.67***	2129.98***	167211***	6263.6***	2557.0***	2418.4***	5.254**	3.1005***	5577.4***
Genotype × Treatment	12	3.692***	7.0590***	155.83***	42.2***	25.9074***	29.570***	0.1647 <sup>NS</sup>	0.049821***	8.912***
Genotype × Time	8	78.25 <sup>NS</sup>	39.75 <sup>NS</sup>	3244 <sup>NS</sup>	105.8 <sup>NS</sup>	89.7 <sup>NS</sup>	371.2***	0.175 <sup>NS</sup>	0.3743***	268.9 <sup>NS</sup>
Treatment × Time	24	190.63***	148.46***	8977.9***	488.7***	388.29***	170.63***	0.5731 <sup>NS</sup>	0.3549***	324.84***

MDA – Malondialdehyde; SOD - Superoxide Dismutase; CAT – Catalase; APX - Ascorbate Peroxidase; GPX - Guaiacol Peroxidase; GR - Glutathione Reductase; PAL - Phenylalanine Ammonia-Lyase; PPO - Polyphenol Oxidase; TPC - Total Phenolic Content; \* Significant at  $p < 0.05$ ; \*\* Significant at  $p < 0.01$ ; \*\*\* Significant at  $p < 0.001$ ; <sup>NS</sup> – Non significant.

revealed its efficacy by low MDA content rather than the disease control groups. Similarly, Singh et al. [47] reported 2.3 fold MDA reduction in tomato plants by *S. coelicolor*, *S. girseus*, *S. albus*, *S. antibiotics* and *S. champavatii* treatment, during *R. solani* infection.

During BGM development, superoxide radical ( $O_2^-$ ) is generated as a virulence factor, which was dismutated by SOD. It's continuous expression to overcome the initial radical load, further lead to the generation of another radical hydrogen peroxide ( $H_2O_2$ ) and hence initiates the cascade of oxidative burst [49]. Results of the present study document the induction of both SOD and CAT in the hierarchy of consortium and *T. harzianum* treatments followed by single inoculations and *B. cinerea* challenged treatment. Su et al. [50] reported increased SOD and CAT by the endophyte *Harpophora oryzae* against rice blast pathogen *Magnaporthe oryzae*. Singh et al. [47] also documented the induction of CAT in tomato plants primed with *S. coelicolor*, *S. girseus*, *S. albus*, *S. antibiotics* and *S. champavatii* than the *R. solani* challenged untreated plants and normal control plants.

APX, GPX and GR are the vital components after SOD and CAT, and they works through cascade and neutralizes the  $H_2O_2$  at different proportions and also involved in scavenging of other reactive oxygen intermediates [51]. In the current study, their interactive effect is emphasized by the significant positive correlations (APX vs. GPX, 0.926,  $p < 0.01$ ; APX vs. GR, 0.743,  $p < 0.01$ ; GPX vs. GR, 0.843,  $p < 0.01$ ). Singh et al. [47] observed significant induction of APX, GPX and GR in *S. coelicolor*, *S. girseus*, *S. albus*, *S. antibiotics* and *S. champavatii* treated tomato plants than the *R. solani* challenged and normal control plants. Similarly, increased APX and GPX were noticed on potato by endophytic treatment with *Pseudomonas* sp. and *Methylobacterium* sp. against *P. atrosepticum* infection [46].

Phenyl propanoid pathway, a key pathway in plant defense system, synthesizes wide class of phenolic substances. PAL the first enzyme in this pathway, forms trans-cinnamic acid which is a precursor for structural barrier lignin and many other phenolics [52]. In this study, simultaneous increase of PAL activity and TPC indirectly indicates the

induction of phenyl propanoid pathway by endophytic treatment which was further supported by significant positive correlations (PAL vs. TPC, 0.575,  $p < 0.01$ ). PPO inhibits the pathogens by their toxic quinone derivatives and confers plant protection by creation of lignin-like physical barriers [53]. Induction of four isoforms of PPO in endophytic *B. subtilis* treated rice seedlings and one isoform in *R. solani* alone inoculated rice seedlings demonstrates the role of PPO and endophyte priming in defense induction [54]. Singh and Gaur [11] reported induction of PAL, PPO and TPC in chickpea against *S. rolfii* by endophytic *Streptomyces* spp.

## 5. Conclusions

Priming of plants with beneficial microbes is evidenced with many benefits to the plants. Some key points were better understood through this study and the foremost is that, single inoculations and consortium would have not been behaved same in all the context, as this study observed better growth performance and nodulation by single inoculations, whereas host-plant resistance induction by consortium. Though, the strains were compatible, they showed varied effects which indicate that the degree of endophyte establishment and expression of beneficial traits is linked to the host plant and the growing conditions. The moderate and constant activation of antioxidant enzymes and phenolics might be a key mechanism for host-plant resistance and they might be the first barrier to challenge the radical load generated by *B. cinerea*. With this initial information, future studies on endophyte colonization behavior and their role on other defense related substances might paves a way for the development of ideal growth-promoting and biocontrol agents for chickpea.

## Conflicts of interest

Authors declare that there is no conflict of interest.

## Acknowledgements

This study is supported by Young Scientist scheme of SERB, DST, India through SB/YS/LS-345/2013 and author RV is grateful to this. This work was undertaken as part of the CGIAR Research Program on Grain Legumes. ICRISAT is a member of CGIAR Consortium. We thank Mr K Sivasakthi, Crop Physiology, ICRISAT for statistical assistance, Dr G Senthilraja Centre of Excellence in Millets, Tamil Nadu Agricultural University, Tiruvannamalai in AUDPC calculation. Technical assistance of Mr Rameshwar for BGM experiments is also acknowledged. We also thank our lab members M/s PVS Prasad and A Jabbar for technical assistance.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.micpath.2018.06.019>.

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