Microbiological Research 169 (2014) 40–48

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

Evaluation of *Streptomyces* strains isolated from herbal vermicompost for their plant growth-promotion traits in rice☆

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ARTICLE INFO

Article history:
Received 26 June 2013
Received in revised form
12 September 2013
Accepted 14 September 2013
Available online 19 September 2013

Keywords:
Actinomycetes
Streptomyces spp.
Field evaluation
Plant growth-promotion
Rice

ABSTRACT

Six actinomycetes, CAI-13, CAI-85, CAI-93, CAI-140, CAI-155 and KAI-180, isolated from six different herbal vermi-composts were characterized for *in vitro* plant growth-promoting (PGP) properties and further evaluated in the field for PGP activity in rice. Of the six actinomycetes, CAI-13, CAI-85, CAI-93, CAI-140 and CAI-155 produced siderophores; CAI-13, CAI-93, CAI-155 and KAI-180 produced chitinase; CAI-13, CAI-140, CAI-155 and KAI-180 produced lipase; CAI-13, CAI-93, CAI-155 and KAI-180 produced protease; and CAI-13, CAI-85, CAI-140 and CAI-155 produced β-1-3-glucanase whereas all the six actinomycetes produced cellulase, hydrocyanic acid and indole acetic acid (IAA). The actinomycetes were able to grow in NaCl concentrations of up to 8%, at pH values between 7 and 11, temperatures between 20 and 40 °C and compatible with fungicide bavistin at field application levels. In the rice field, the actinomycetes significantly enhanced tiller numbers, panicle numbers, filled grain numbers and weight, stover yield, grain yield, total dry matter, root length, volume and dry weight over the un-inoculated control. In the rhizosphere, the actinomycetes also significantly enhanced total nitrogen, available phosphorous, % organic carbon, microbial biomass carbon and nitrogen and dehydrogenase activity over the un-inoculated control. Sequences of 16S rDNA gene of the actinomycetes matched with different *Streptomyces* species in BLAST analysis. Of the six actinomycetes, CAI-85 and CAI-93 were found superior over other actinomycetes in terms of PGP properties, root development and crop productivity. qRT-PCR analysis on selected plant growth promoting genes of actinomycetes revealed the up-regulation of IAA genes only in CAI-85 and CAI-93.

1. Introduction

Streptomyces are a group of Gram-positive bacteria, with high G + C content belonging to the order Actinomycetales, found most commonly in soil, compost, fresh and marine water and play an important role in the plant growth promotion (PGP), plant protection, decomposition of organic materials and produce secondary metabolites of commercial interest. PGP potential of *Streptomyces* was reported on tomato (El-Tarabily 2008), wheat (Sadeghi et al. 2012), rice (Gopalakrishnan et al. 2012a), bean (Nassar et al. 2003) and pea (Tokala et al. 2002). *Streptomyces* promote plant growth either by producing siderophores (Tokala et al. 2002) and/or indole-3-acetic acid (Aldesuquy et al. 1998). *Streptomyces* has also been widely used for biocontrol of soil-borne fungal pathogens (Trejo-Estrada et al. 1998; Macagnan et al. 2008; Gopalakrishnan et al. 2011a,b).

The biological degradation and conversion of agricultural wastes or herbals by earthworms and microorganisms, called vermicomposting, is becoming a favored method of recycling wastes (Edwards 1998). One of the advantages of using earthworms in composting is that, it creates aerobic conditions and release coelomic fluids in the decaying biomass, which contains factor(s) that promotes plant growth and kills pathogens such as *Salmonella*, *Serratia marcesens* and *Escherichia coli* (Prabha 2009). Application of vermicompost prepared from the herbals not only benefits crop plants as it contains beneficial microorganisms, that help the plants to mobilize and acquire nutrients, but also promotes plant growth and inhibits many plant pathogenic microorganisms (Postma et al. 2003; Suthar et al. 2005; Perner et al. 2006; Nath and Singh 2009). Efficacy of Jatropha, Annona and Parthenium vermiwash was shown to inhibit *Macrophomina phaseolina*, *Sclerotium rolfsii* and *Fusarium oxysporum* f. sp. *ciceri* (Gopalakrishnan et al. 2010). The objective of this study was to isolate, characterize and evaluate the actinomycetes isolated from vermi-composts for their ability to

☆ This article is part of a Special Issue entitled Plant Growth Promotion.

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http://dx.doi.org/10.1016/j.micres.2013.09.008

promote plant growth in rice grown using the system of rice intensification (SRI; Uphoff 2001; Gopalakrishnan et al. 2013) method.

2. Materials and methods

2.1. Preparation of herbal vermicompost

It was done as per the protocols of Gopalakrishnan et al. (2011b). In brief, foliages of six different botanicals (*Allium sativum*, *Pongamia pinnata*, *Azadirachta indica*, *Melia azedarach*, *Thevetia peruviana* and *Oryza sativa*) were collected from International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) farm and air-dried at room temperature (30 ± 2 °C). The container for vermicomposting was constructed by cutting a 200 L plastic barrel into two halves and the air-dried foliages of herbals were composted with earthworms (*Eisenia foetida*). When the herbal vermicompost was ready in about 2 months, about 100 g of the sample was collected and stored in a refrigerator at 4 °C for further studies.

2.2. Isolation of actinomycetes

Ten grams of vermicompost were suspended in 90 ml of physiological saline (0.85% of NaCl) in a flask and incubated on an orbital shaker (at 100 rpm) for 1 h at room temperature (28 ± 2 °C). At the end of incubation, the vermicompost samples were serially diluted up to 10^6 dilutions with physiological saline. Dilutions 10^4 to 10^6 were plated on actinomycetes isolation agar (AIA) by spread plate technique and incubated at 28 °C for 96 h.

2.3. In vitro PGP attributes of the actinomycete isolates

Actinomycetes (CAI-13, CAI-85, CAI-93, CAI-140, CAI-155 and KAI-180) were evaluated for production of PGP traits including siderophore, chitinase, cellulase, lipase, protease, hydrocyanic acid (HCN) and indole acetic acid (IAA) and β-1,3-glucanase. Siderophore production was estimated as per the protocol of Schwyn and Neilands (1987). Chitinase production was determined as per the protocols of Hirano and Nagao (1988) and Gopalakrishnan et al. (2011a). The standardized protocols of Hendricks et al. (1995) were used to evaluate the cellulase production. The lipase and protease production was estimated as per the protocols of Bhattacharya et al. (2009). All the treatments were replicated three times and the experiment was conducted three times. Observations of the selected actinomycetes to siderophore, cellulase, lipase and protease were recorded on a 0–4 rating scale as follows: 0, no halo zone; 1, halo zone of <1 mm; 2, halo zone of 1–3 mm; 3, halo zone of 4–6 mm; 4, halo zone of 7–9 mm and above. Chitinase production was recorded on a 0–5 rating scale as follows: 0, no halo zone; 1, halo zone of 1–5 mm; 2, halo zone of 6–10 mm; 3, halo zone of 11–15 mm; 4, halo zone of 16–20 mm and 5, halo zone of 21 mm and above.

HCN was qualitatively assessed by sulfocyanate colorimetric method (Lorck 1948). In brief, the actinomycetes were grown in Bennett agar amended with glycine (4.4 g l^{-1}). One sheet of Whatman filter paper no. 1 (8 cm diameter) was soaked in 1% picric acid (in 10% sodium carbonate; filter paper and picric acid were sterilized separately) for a minute and stuck underneath the Petri dish lids. The plates were sealed with Parafilm and incubated at 28 °C for 4 days. All the treatments were replicated three times and the experiment was conducted three times. Development of reddish brown color on the filter paper indicated positive for HCN production. Observations were recorded on a 0–3 rating scale (based on the intensity of the reddish brown color) as follows: 0, no color; 1, light reddish brown; 2, medium reddish brown and 3, dark reddish brown.

IAA was estimated as per the protocols of Patten and Glick (1996). The actinomycetes grown in starch casein broth supplemented with L-tryptophan (1 μg ml^{-1}) for 4 days were centrifuged at 10,000 *g* for 10 min and the supernatants collected. One ml of this culture filtrate was allowed to react with 2 ml of Salkowsky reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35% $HClO_4$) at 28 °C for 30 min. The development of pink color indicates IAA production. Quantification of IAA was done my measuring the absorbance in a spectrophotometer at 530 nm. A standard curve was plotted to quantify the IAA (μg ml^{-1}) present in the culture filtrate.

β-1,3-glucanase was done as per the protocols of Singh et al. (1999). In brief, the actinomycetes were cultured in Tryptic soy broth, supplemented with 1% colloidal chitin (weight/volume), at 28 °C for four days. At the end of incubation, the cultures were centrifuged at 10,000 *g* for 12 min and the supernatants collected. One ml of the culture filtrate was allowed to react with 0.1 ml of laminarin solution (2%, w/v) in 0.2 M acetate buffer (pH 5.4) at 40 °C for 1 h. The reaction was arrested by adding 3 ml of dinitrosalicylic acid to the mixture and kept at boiling for 10 min. The development of dark red color indicated the presence of reducing sugar, and the concentration of the reducing sugar was determined by measuring the absorbance at 530 nm in a spectrophotometer. Calibration standards were prepared using glucose at 0–1 mg mL^{-1} at the interval of 0.2 mg mL^{-1}. 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. Treatments were replicated three times and the experiment was conducted three times.

2.4. Evaluation of actinomycetes for their physiological traits

2.4.1. Salinity, pH, temperature and resistance to fungicides

The selected actinomycetes (CAI-13, CAI-85, CAI-93, CAI-140, CAI-155 and KAI-180) were streaked on Bennett's agar with various concentrations of NaCl ranging from 0 to 14% at an interval of 2%. The plates were incubated at 28 °C for five days and the intensity of growth was measured at the end of incubation. For pH, the selected actinomycetes were streaked on Bennett's agar, adjusted to pH 5, 7, 9, 11 and 13, and incubated for five days at 28 °C. For temperature, the actinomycetes were streaked on Bennett's agar and incubated at 20, 30 and 40 °C for five days, while for 50 °C, the Bennett's broth was inoculated, and at the end of the five-day incubation, the intensity of growth was measured at 600 nm in a spectrophotometer. Treatments were replicated 3 times and the experiment was conducted 3 times.

The six strains of actinomycetes were also evaluated for their tolerance to fungicides at field application level. The fungicides studied include Bavistin (carbendazim 50%; methyl benzimidazol-2-ylcarbamate), Captan (captan 50%; *N*-trichloromethylthio-4-cyclohexene-1,2-dicarboximide), Radonil (*N*-(2,6-dimethylphenyl)-*N*-(methoxyacetyl) alanine methyl ester), Thiram (dimethylcarbamothioylsulfanyl *N*,*N*-dimethylcarbamodithioate), Benomyl (methyl [1-[(butylamino)carbonyl]-1H-benzimidazol-2-yl] carbamate) and Benlate (benomyl 50%; methyl [1-[(butylamino) carbonyl]-1H-benzimidazol-2-yl] carbamate) at field application levels of 2500, 3000, 3000, 3000, 3000 and 4000 ppm concentrations, respectively. The required quantities of fungicides were dissolved in sterilized Milli-Q water and mixed into Bennett's agar just before pouring into the Petri plates. The plates were incubated at 28 °C for five days and the intensity of growth was measured. There were three replications for each test and the experiment was done thrice.

Observations of the six actinomycete strains to salinity, pH, temperature and fungicide tolerance were recorded as follows: 0, no growth; 1, little growth; 2, medium growth and 3, good growth.

2.5. Evaluation of actinomycetes for PGP potential on rice under field conditions

The field experiment was conducted as described previously (Gopalakrishnan et al. 2012a) with a medium duration (130–135 days) rice variety, Sampada, which normally yields 6.5–7.0 t ha^{-1} in 2011–2012 (post rainy season) at ICRISAT, Patancheru, Andhra Pradesh, India. Soils at the experimental station are classified as sandy loam in texture (55% sand, 17% silt and 28% clay) with organic carbon content of 0.76–1.27% and alkaline pH of 8.5–9.4. The mineral content of the top 15 cm rhizosphere was as follows: available phosphorus 26.8 kg ha^{-1}, available nitrogen 292 kg ha^{-1} and available potassium 527 kg ha^{-1}. The experiment was conducted in a randomized complete block design (RCBD) with three replications and subplot sizes of 10 m × 7.5 m. Rice was grown by the system of rice intensification (SRI) method proposed by the Central Rice Research Institute (http://crri.nic.in), Cuttack, Orissa, India. The six strains of actinomycetes (CAI-13, CAI-85, CAI-93, CAI-140, CAI-155 and KAI-180) were grown on a starch casein broth at 28 °C for six days. The 12-day-old single rice seedlings were uprooted from the nursery and the roots were dipped in the respective actinomycete broth (containing 10^8 CFU ml^{-1}) for 45 min and transplanted on 28th December 2011 at a row-to row and a plant-to-plant spacing of 25 cm. The actinomycetes (1000 ml; 10^8 CFU mL^{-1}) were applied once in two weeks until the flowering stage along with the irrigation. A consortium of the six actinomycetes was also included as one of the treatments. Control contained no actinomycete strains.

Weeding was done four times by cono-weeder to incorporate weeds into the soil at 10, 20, 30 and 40 days after transplanting. Irrigation was done as required for the SRI method, i.e. the alternate wetting and drying method. The crop was harvested manually on 24th May 2012 and observed for plant height (cm), total tillers (m^{-2}), total panicles ($plant^{-1}$), panicle length (cm), filled grain number and weight (g), SPAD reading, stover yield (g m^{-2}), grain yield (g m^{-2}), total dry matter (g m^{-2}) and 1000 grain weight. Root samples were also collected from 0–15 and 15–30 cm soil profile and analyzed for root length (mm^{-2}; EPSON expression 1640×, Japan), volume (cm^3 m^{-2}) and dry weight (gm^{-2}) dried in an oven at 60 °C for 48 h. Soil samples were collected from 0–15 cm soil profile, at harvest, and analyzed for soil chemistry (total nitrogen [ppm], available phosphorous [ppm] and % organic carbon as per the protocols of Novozamsky et al. (1983), Olsen and Sommers (1982) and Nelson and Sommers (1982), respectively) and biological analysis (microbial biomass carbon [μg g^{-1} soil] by fumigation method, microbial biomass nitrogen [μg g^{-1} soil] by Kjeldahl distillation method and dehydrogenase activity [μg TPF g^{-1} soil 24 h^{-1}] by Triphenylformazan production method as per the protocols of Anderson and Domsch (1989), Brooks et al. (1985) and Casida (1977), respectively).

2.6. Molecular identification of the actinomycete isolates

Pure cultures of the selected actinomycetes were grown in starch casein broth until log phase (4 days) and genomic DNA was isolated according to Bazzicalupo and Fani (1995). The amplification of 16S rDNA gene was done by using universal primer 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1492R (5′-TAC GGY TAC CTT GTT ACG ACT T-3′) as per the protocols described by Pandey et al. (2005). The PCR product was sequenced at Macrogen Inc., Seoul, Korea. The sequences obtained were compared with those from the GenBank using the BLAST program (Alschul et al. 1990), aligned using the Clustal W software (Thompson et al. 1997), and phylogenetic trees inferred using the neighbor-joining method (Saitou and Nei 1987) in the MEGA version 4 program (Tamura et al. 2007).

2.7. Gene expression profile

2.7.1. RNA extraction

The six selected actinomycete isolates were grown in Bennett's broth at 28 °C for 72 h. Total RNA was extracted from all the actinomycetes using PureLink RNA mini kit (Ambion, USA) according to the manufacturer's protocol. The RNA samples were quantified using Qubit fluorometer (Invitrogen, USA). The quality was also checked using Bioanalyzer (Agilent, USA).

2.7.2. Quantitative real time-PCR (qRT-PCR)

Quantitative real-time polymerized chain reaction (qRT-PCR) was performed using Applied Biosystems 7500 Real Time PCR System with the SYBR green chemistry (Applied Biosystems, USA) according to the manufacturer's instructions. Gene-specific primers for qRT-PCR were designed using primer 3 software (Rosen and Skaletsky 2000). Well characterized genes (Spaepen et al. 2007) relating to IAA production from IAM/IPyA/TAM were collected from UniprotKB database (http://www.uniprot.org/uniprot). The siderophore related genes were manually collected from the MetaCyc pathway database (http://metacyc.org) considering siderophore biosynthesis pathways excluding the plant related siderophore biosynthesis pathways. RNA polymerase principal sigma factor *HrdB* (SCO5820) was used as the endogenous control. Specific primer sequences of *HrdB* (F: GGTCGAGGTCATCAACAAGC; R: CTCGATGAGGTCACCGAACT), siderophore (F: ATCCTCAACACC-CTGGTCTG; R: TCCTTGTACTGGTACGGGACTT) and IAA (F: GTCAC-CGGGATCTTCTTCAAC; R: GATGTCGGTGTTCTTGTCCAG) has been used for the analysis. PCR reactions were carried out in 10 μl reaction containing 30 ng of first strand cDNA, 1X PCR buffer, 125 mM dNTPs, 1.5 mM MgCl2, 0.2 mM primers and 1U Taq polymerase. PCR program is as follows: 50 °C for 2 min and denaturation at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. The data from different PCR runs or cDNA samples was compared by using the mean of the CT values of the three biological replicates that were normalized to the mean CT values of the endogenous gene. The expression ratios were calculated using the $2^{-\Delta\Delta Ct}$ method. Relative transcription levels are presented graphically.

2.8. Statistical analysis

Data were analyzed by using Analysis of Variance (ANOVA) technique, by SAS GLM (General Linear Model) procedure (SAS Inst. 2002-08, SAS V9.3) considering isolates and replication as fixed in RCBD. Depth-wise ANOVA was performed for the traits root length, volume and dry weight. Isolate means were tested for significance and compared using Fisher's protected least significant difference (LSD).

3. Results

3.1. Isolation of actinomycetes

The most prominent actinomycete colonies (the ones which were found abundantly in the AIA plate, inhibited the adjacent colonies and produced pigments) were isolated and maintained on AIA slants at 4 °C. A total of six actinomycetes, CAI-13, CAI-85, CAI-93, CAI-140, CAI-155 and KAI-180, from herbal vermicomposts of *A. sativum*, *P. pinnata*, *A. indica*, *M. azedarach*, *T. peruviana* and *O. sativa* respectively, were selected for further studies.

3.2. In vitro PGP attributes of the actinomycete isolates

When the six actinomycetes were evaluated for their PGP attributes, all strains produced cellulase, HCN, IAA, siderophore

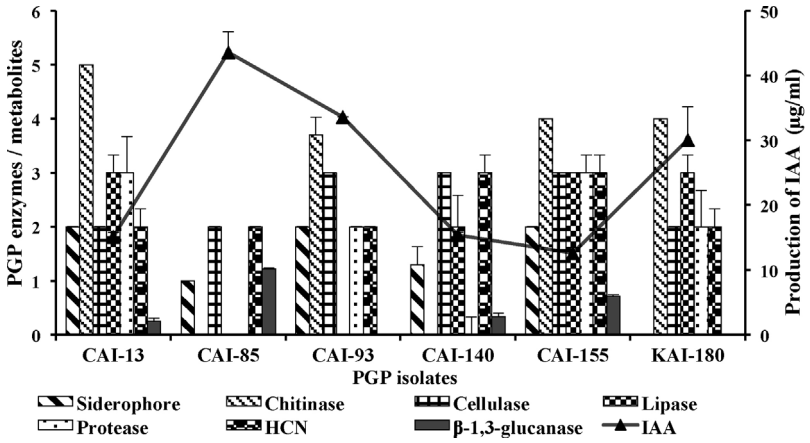

Fig. 1. *In vitro* PGP traits of selected actinomycete isolates. HCN, hydrocyanic acid; IAA, indole acetic acid; Values on primary (left) axis are rating scales for *in vitro* PGP traits of (i) siderophore, cellulase, lipase and protease production as: 0, no halo zone; 1, halo zone of <1 mm; 2, halo zone of 1–3 mm; 3, halo zone of 4–6 mm and 4, halo zone of 7 mm and above; (ii) chitinase production as: 0, no halo zone; 1, halo zone of 1–5 mm; 2, halo zone of 6–10 mm, 3, halo zone of 11–15 mm; 4, halo zone of 16–20 mm and 5, halo zone of 21 mm and above; (iii) HCN production as: 0, no color change; 1, light reddish brown; 2, medium reddish brown and 3, dark reddish brown; (iv) β-1,3-glucanase (U) – one unit is an amount of enzyme that liberated 1 μmol of glucose hour^{-1} at defined conditions. Values on secondary (right) axis indicate IAA production.

(except KAI-180), chitinase (except CAI-85 and CAI-140), lipase (except CAI-85 and CAI-93), protease (except CAI-85 and CAI-140) and β-1,3-glucanase (except CAI-93 and KAI-180). Isolate CAI-85 produced the maximum IAA (43.6 μg ml^{-1}of culture filtrate) and β-1,3-glucanase (1.22 units) when compared to other actinomycetes. Though CAI-155 registered higher enzyme production on siderophore, chitinase, cellulase, lipase, protease, HCN and β-1,3-glucanase, it showed lowest production of IAA when compared to other isolates (Fig. 1).

3.3. Evaluation of actinomycetes for their physiological traits

All the actinomycete isolates were able to grow up to 8% NaCl and none grew at 14% of NaCl conditions. NaCl concentration of 10% was discriminatory for the isolates, CAI-13 and CAI-93 showed medium to good growth, while others exhibited no growth. Isolate CAI-93 also registered little growth on 12% NaCl. Actinomycetes grown under a gradient of pH (5–13) indicated that none of the isolates grew in pH 5 and all of them grew well from pH 7–11. A pH of 13 was discriminatory for the isolates, where the isolates CAI-85 and CAI-155 showed medium to good growth, while others exhibited no growth. Temperatures between 20 and 40 °C were found optimum for growth of all actinomycete isolates, whereas none of them grew at 50 °C. The actinomycetes were found highly tolerant to fungicide bavistin, slightly tolerant to thiram (except CAI-85 and KAI-180) and captan (except CAI-85, CAI-140 and KAI-180) but highly sensitive to radonil, benomyl and benlate (Table 1).

3.4. Evaluation of actinomycetes for PGP potential on rice under field conditions

When the six actinomycetes were evaluated for their PGP potential in the rice field, the actinomycetes-treated plots (including consortia) significantly enhanced the agronomic performance by influencing the plant height (up to 7%), total tillers (up to 18%), total panicles (up to 26%), panicle length (up to 12%), filled grain numbers (up to 42%) and weight (up to 42%), SPAD (up to 15%), stover yield (up to 39%), grain yield (up to 17%) and total dry matter (up to 38%) over the un-inoculated control (Table 2 and Fig. 2). The plots treated with actinomycetes and their consortia also significantly enhanced the root development, at both 0–15 and 15–30 cm depths, including the root length (up to 35%), the root volume (up to 35%) and the root dry weight (up to 33%) over the un-inoculated control (Table 3 and Fig. 3). Of the six actinomycetes studied, CAI-85 and CAI-93 enhanced both the yield parameters (including stover yield, grain yield and total dry matter) and root development (including root length, root volume and root dry weight) the most than the other isolates.

In the actinomycetes-inoculated plots, in the top 15 cm rhizosphere at harvest, the soil mineral nutrients such as total N, available P and % organic carbon were also found significantly higher (up to 42%, 39% and 35%, respectively) than the un-inoculated control (Table 4). Further, the soil biological activities such as microbial biomass carbon, microbial biomass nitrogen and dehydrogenase activity were found significantly enhanced (up to 55%, 160% and 36%, respectively) in the actinomycetes-inoculated plots over the un-inoculated control. The actinomycetes consortium was also found to significantly enhance both soil mineral nutrients and biological activities (Table 4). The high magnitude of soil mineral properties and biological activities was shown by CAI-13 followed by CAI-140, CAI-85, CAI-93, CAI-155 and KAI-180.

3.5. Molecular identification of the actinomycete isolates

In order to determine the identity of the six PGP potential actinomycetes, its 16S rDNA was sequenced and analyzed. A neighbor-joining dendrogram was generated using the sequence from the six PGP potential actinomycetes (1400 bp) and representative sequences from the databases. Phylogenetic analysis of 16S rDNA sequences of the six actinomycetes matched with genus *Streptomyces* but with different species. The isolate CAI-13, CAI-85 and CAI-155 had maximum sequence similarity (99%) with *S. caviscabies*, CAI-93 and KAI-180 showed maximum sequence similarity (99%) with *S. globisporus* sub sp. *caucasicus* whereas CAI-140 showed maximum similarity (98%) with *S. griseorubens* (Fig. 4).

3.6. qRT-PCR of plant growth promoting genes

RNA was isolated from all the actinomycete isolates but good quality RNA was obtained only from CAI-155, CAI-85, CAI-193 and CAI-140. The quality of RNA from actinomycetes CAI-13 and KAI-180 were poor and hence are excluded from the study. qRT-PCR validation of IAA genes revealed that gene IAA showed high up-regulation in the CAI-85 isolate (24 folds) followed by CAI-93 (12 folds) while gene siderophore was highly up-regulated in CAI-155 (25 folds) followed by CAI-85 (4.54) and CAI-93 (1.4). No change

Table 1
Effect of salinity, pH, temperature, and fungicides on the growth of six actinomycetes with PGP activity.

Traits	CAI-13	CAI-85	CAI-93	CAI-140	CAI-155	KAI-180	Mean	SE±	CV%
Salinity[a]									
0	3	3	3	3	3	3	3	0.001*	0.1
2	3	3	3	3	3	3	3	0.001*	0.1
4	3	3	3	3	3	3	3	0.001*	0.1
6	3	2	3	3	3	3	3	0.00	0
8	3	1	3	1	1	1	2	0.00	0
10	2	0	3	0	0	0	1	0.02***	4
12	0	0	1	0	0	0	0	0.01***	7
14	0	0	0	0	0	0	0	0.00	0
pH[a]									
5	0	0	0	0	0	0	0	0	0
7	3	3	3	3	3	3	3	0.01*	1
9	3	3	3	3	3	3	3	0.01*	1
11	2	3	3	3	3	3	3	0.01***	1
13	0	3	0	0	2	0	1	0.01***	1
Temperature, °C[a]									
20	3	3	3	3	3	3	3	0.01*	1
30	3	3	3	3	3	3	3	0.01*	1
40	3	3	3	3	3	3	3	0.01*	1
50	0	0	0	0	0	0	0	0	0
Fungicide tolerance[a]									
Bavistin @ 2500 (ppm)	3	3	3	3	3	3	3	0.01*	1
Captan @ 3000 (ppm)	1	0	1	0	1	0	1	0.02***	8
Radonil @ 3000 (ppm)	0	0	0	0	0	0	0	0	0
Thiram @ 3000 (ppm)	2	0	1	1	1	0	1	0.01***	3
Benemyl @ 3000 (ppm)	0	0	0	0	0	0	0	0	0
Benlate @ 4000 (ppm)	0	0	0	0	0	0	0	0	0

* Statistically significant at 0.05.
*** Statistically significant at 0.001.
[a] Responses of the six actinomycetes to salinity, pH, temperature and fungicide tolerance were recorded as follows: 0, no growth; 1, little growth; 2, medium growth; 3, good growth.

in expression profiling of both the genes, siderophore and IAA, was observed in isolate CAI-140 (Fig. 5).

4. Discussion

Vermicompost have been extensively used in organic agriculture not only for its beneficial effects on soil biota and soil structure, but also for its ability to promote plant growth and inhibit plant pathogens. Vermicompost at 25% enhanced plant growth and controlled *Rhizoctonia solani* that causes damping off in Patience-plant (*Impatiens walleriana*) (Asciutto et al. 2006). In the present investigation, herbal vermicomposts of *A. sativum*, *P. pinnata*, *A. indica*, *M. azedarach*, *T. peruviana* and *O. sativa* were used for isolating actinomycetes (such as CAI-13, CAI-85, CAI-93, CAI-140, CAI-155 and KAI-180) based on their production of pigments and inhibition of adjacent colonies on AIA plate.

PGP bacteria stimulate plant growth directly by solubilization of nutrients (Rodriguez and Fraga 1999), nitrogen fixation (Han et al. 2005), production of growth hormones such as IAA (Correa et al. 2004) and indirectly by antagonizing plant pathogens through production of siderophores, chitinase, β-1,3-glucanase, antibiotics, fluorescent pigments and cyanide (Pal et al. 2001). In the present investigation, it was noticed that all the isolates produced cellulase, protease (except CAI-85 and CAI-140), chitinase (except CAI-85 and CAI-140) and lipase (except CAI-85 and CAI-93). Cellulose and lipids are present abundantly in the plant biomass which can be degraded by microbial enzymes such as cellulase and lipase, respectively (Lynd et al. 2002). Protease, chitinase, lipase and cellulase-producing microbes not only play an important role in the

Table 2
Effect of the six actinomycetes with PGP activity on agronomic performance and yield potential of rice.

Isolates	Plant height (cm)	Tiller numbers (m^{-2})	Panicle numbers ($plant^{-1}$)	Panicle length (cm)	SPAD reading	Stover yield (g m^{-2})	Grain yield (g m^{-2})	Total dry matter (g m^{-2})	1000 grain weight (g)
CAI-13	75.6	526	44	22.8	36.6	1540	802	2343	16.16
CAI-85	78.1	550	44	22.5	35.2	1617	901	2518	15.34
CAI-93	73.7	601	42	23.0	37.2	1851	865	2716	15.15
CAI-140	74.2	502	45	21.6	40.3	1354	785	2139	15.92
CAI-155	74.3	623	42	22.5	35.0	1739	852	2592	15.85
KAI-180	73.8	492	41	21.0	37.4	1210	780	1990	15.98
Consortia	73.0	517	40	21.5	35.3	1312	795	2107	16.47
Control	73.0	487	36	20.5	35.0	1112	773	1885	16.14
Mean	74.5	537	42	21.9	36.5	1467	819	2286	15.88
SE±	1.00*	27.4*	0.8***	0.22***	0.68***	67.6***	29.7*	91.4***	0.210*
LSD (5%)	3.05	80.9	2.7	0.74	2.06	201.6	88.7	272.6	0.703
CV%	2	10	3	1	3	9	7	8	2

SE, standard error; LSD, least significant difference; CV, coefficient of variance.
* Statistically significant at 0.05.
*** Statistically significant at 0.001.

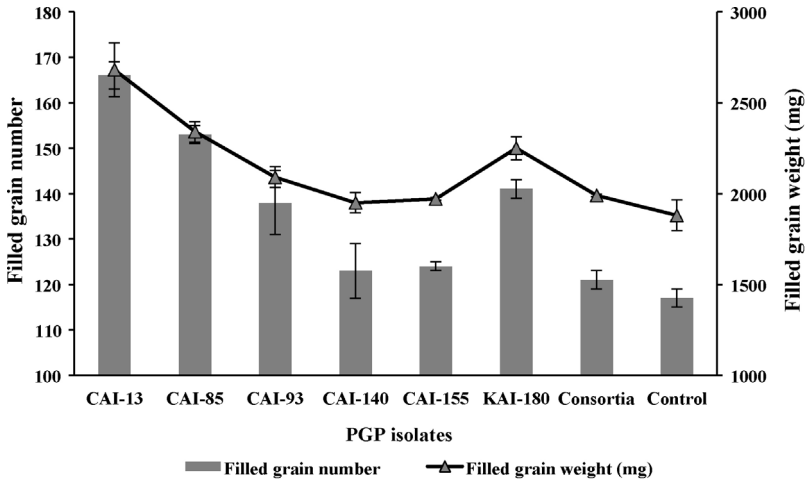

Fig. 2. Effect of the six actinomycetes on filled grain number and filled grain weight of rice. Each bar on right axis and points on left axis are the mean of triplicate analysis. Error bar indicates the standard deviation.

Fig. 3. Effect of the actinomycetes CAI-85 and CAI-93 on root development of rice. (a) Un-inoculated control; (b) CAI-85; and (c) CAI-93.

nutrient mineralization, organic matter decomposition and PGP, but also act as biocontrol agents, on cellulose, chitin, lipid and protein cell wall-bearing pathogens such as *Phytophthora* and *Pythium* spp. (Lima et al. 1998).

In the present studies, all six actinomycetes produced HCN, IAA, siderophore (except KAI-180) and β-1,3-glucanase (except CAI-93 and KAI-180). HCN production play a role in disease suppression, for instance, strains of *Pseudomonas fluorescens* is reported to

Table 3
Effect of the six actinomycetes with PGP on the root development in rice.

Isolates	Root length ($m\,m^{-2}$)			Root volume ($cm^3\,m^{-2}$)			Root dry weight ($g\,m^{-2}$)		
	0–15 cm	15–30 cm	Mean	0–15 cm	15–30 cm	Mean	0–15 cm	15–30 cm	Mean
CAI-13	8012	1112	4562	2106	173	1140	131.3	9.3	70.3
CAI-85	8186	1016	4601	2342	178	1260	138.0	9.3	73.7
CAI-93	7766	947	4357	2019	149	1084	130.6	7.3	69.0
CAI-140	6989	420	3705	2394	66	1230	117.4	3.4	60.4
CAI-155	6610	606	3608	1889	73	981	109.0	3.2	56.1
KAI-180	6447	595	3521	1965	93	1029	107.6	3.6	55.6
Consortia	6960	457	3709	1842	71	957	109.3	3.5	56.4
Control	6385	375	3380	1829	65	947	105.7	3.3	54.5
SE±	183.4(127.3)**		159.5***	51.9(54.7)**		34.6***	4.73(4.60)*		3.43**
Mean	7169	691		2048	109		118.6	5.4	
SE±		45.0***			19.3***			1.63***	
CV%		9			9			13	

SE, standard error; CV, coefficient of variance.
* Statistically significant at 0.05.
** Statistically significant at 0.01.
*** Statistically significant at 0.001.
SE in parentheses is to compare means within same treatment.

Table 4
Effect of the six actinomycetes with PGP on soil properties and biological activities at the harvesting stage in rice.

Isolates	Total N (ppm)	Available P (ppm)	Organic Carbon (%)	Microbial biomass C ($\mu g\,g^{-1}$ soil)	Microbial biomass N ($\mu g\,g^{-1}$ soil)	Dehydrogenase activity ($\mu g\,TPF\,g^{-1}$ soil $24\,h^{-1}$)
CAI-13	2026	101	1.45	3559	31.3	81.3
CAI-85	1820	94	1.28	3010	34.7	63.3
CAI-93	1657	93	1.28	2572	20.7	63.7
CAI-140	1913	111	1.45	2400	43.0	72.7
CAI-155	1828	92	1.46	2437	24.4	69.3
KAI-180	1863	102	1.36	2785	28.8	84.0
Consortia	1789	93	1.29	2562	46.1	68.7
Control	1426	80	1.08	2300	17.7	62.0
Mean	1790	96	1.33	2703	30.8	70.6
SE±	25.3***	2.8***	0.06**	96.3***	3.79***	3.12***
LSD (5%)	77.4	8.5	0.20	292.2	11.50	9.46
CV%	3	5	8	6	21	8

SE, standard error; LSD, least significant difference; CV, coefficient of variance.
** Statistically significant at 0.01.
*** Statistically significant at 0.001.

suppress black root rot of tobacco (Haas et al. 1991; Wei et al. 1991). It is reported that 80% of microorganisms isolated from the rhizosphere of crops possess the ability to synthesize and release auxins as secondary metabolites which are known to promote root elongation and plant growth (Patten and Glick 2002). By producing the plant hormones, microorganisms stimulate plant growth in order to increase the production of plant metabolites which can be beneficial for their growth.

Iron an essential element of life is unavailable to plants and microbes in an aerobic environment due to its principal insoluble form as Fe^{3+}. Siderophores, low molecular iron chelators, produced by various soil microorganisms bind Fe^{3+} and make it available for its own growth and also for plants (Wang et al. 1993). This has been demonstrated in *Arabidopsis thaliana* and *Vigna radiata* by *Pseudomonas* strains (Sharma et al. 2003; Vansuyt et al. 2007). Actinomycetes found in the rhizosphere compete with other rhizosphere plant pathogens for iron, hence competition for iron is also a possible mechanism to control the phytopathogens (Tokala et al. 2002). The cell wall of higher pathogenic fungi, such as *Fusarium oxysporum*, is composed of layers of β-1,3-glucan and lysis

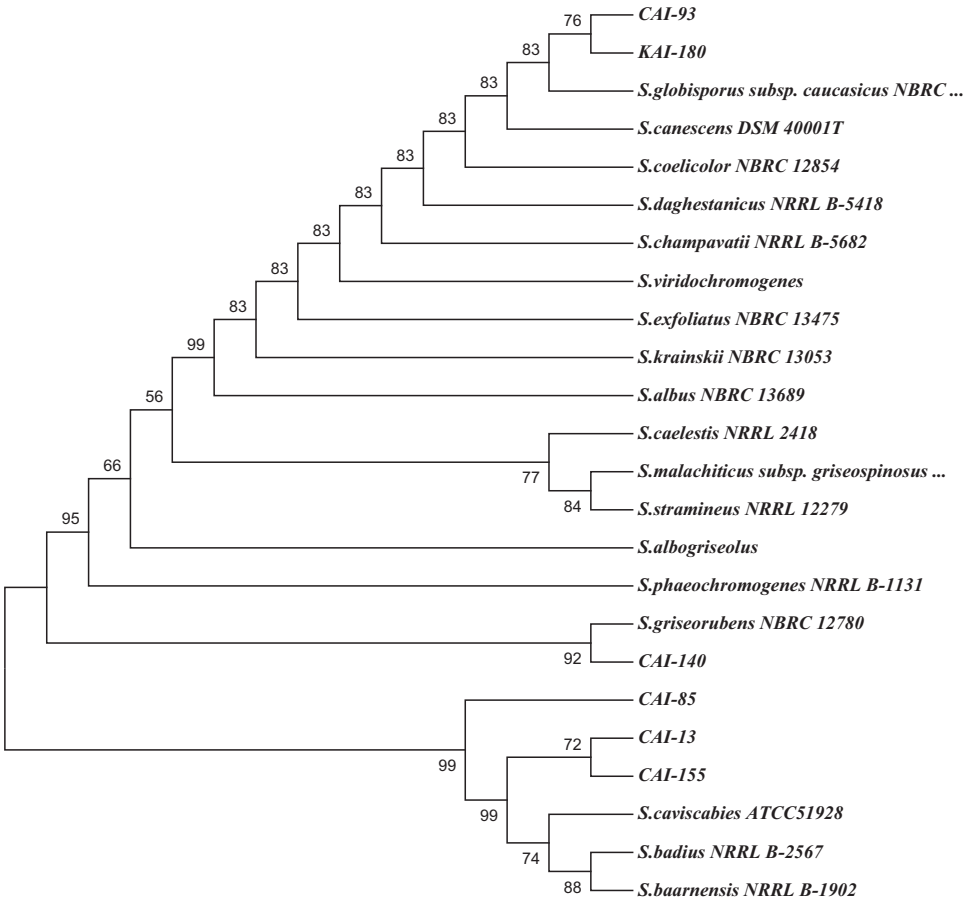

Fig. 4. Phylogenetic relationship between the six PGP potential actinomycetes and representative species based on full-length 16S rDNA sequences constructed using the neighbor-joining method. The number at each branch is the percentages of times the group of strains in that branch occurred, based on 1000 cycles in bootstrap analysis.

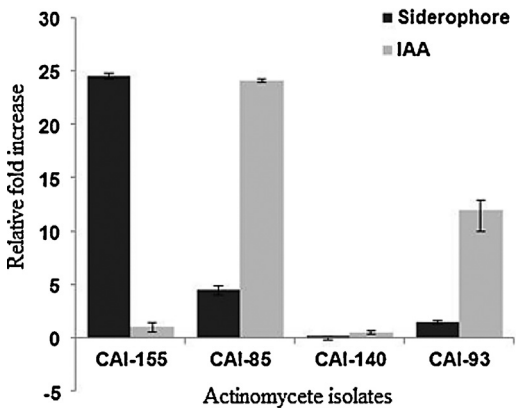

Fig. 5. Expression profile of plant growth promoting genes.

of this by β-1,3-glucanase-producing microbe leads to leakage of cell contents and collapse of the pathogenic fungi (Singh et al. 1999; Macagnan et al. 2008). Therefore, actinomycetes having these traits can be exploited for PGP, degradation of organic residues and/or biological control of plant pathogens.

The six PGP potential actinomycetes were able to grow in NaCl up to 8% (CAI-13 and CAI-93 showed good growth up to 10% NaCl), pH values between 7 and 13, temperatures between 20 and 40 °C and highly tolerant to fungicide bavistin. The ability of actinomycetes to tolerate abiotic stresses including pH, salinity, temperature and fungicide are widely reported (Waksman 1959; Gopalakrishnan et al. 2012a; Sadeghi et al. 2012). Therefore, it can be concluded that these strains not only have the capability to survive in harsh environments but also can be used in the integrated disease management programs.

In the present study, in field, all six isolates of actinomycetes significantly enhanced morphological and yield traits of rice including plant height, total tillers, total panicles, panicle length, filled grain numbers and weight, stover yield, grain yield, 1000 grain weight, total dry matter and root length, volume and dry weight over the un-inoculated control. The efficacy of actinomycete isolates for PGP is extensively reported in the literature (Nassar et al. 2003; El-Tarabily 2008; Gopalakrishnan et al. 2012a). The mineral nutrients and biological activities (including total N, available P, % organic carbon, microbial biomass carbon, microbial biomass nitrogen and dehydrogenase) of the rhizosphere (0–15 cm) soil in the actinomycetes-inoculated rice plots, at harvest, were also found significantly higher over the un-inoculated control plots. The mechanism by which the actinomycetes enhanced morphological and yield traits of rice could be attributed not only to their enzymatic activities such as siderophore and IAA (direct stimulation of PGP) and/or chitinase, cellulase, lipase, protease, hydrocyanic acid and β-1,3-glucanase production capabilities (indirect stimulation of PGP) but also to their ability to survive under harsh environments. The influence of bacteria on PGP has been reported by Birkhofer et al. (2008), Uphoff et al. (2009) and Gopalakrishnan et al. (2011a, 2012a,b). It is a well-known fact that the SRI method of rice cultivation supports the growth of PGP microbes and microbial enzyme activities (Turner and Haygarth 2001; Gayathry 2002; Gopalakrishnan et al. 2013). However, in the present investigation, only such enhanced activities were found in the actinomycetes-inoculated treatments. In the present study, although rice roots were not inspected for colonization, the data on root morphology (including root volume, length and dry weight), soil biological (microbial biomass carbon, nitrogen and dehydrogenase) and chemical activities (total N, available P and % organic carbon) strongly suggest that the six actinomycetes had multiplied and colonized the roots of rice plants. It was also observed that the consortium of actinomycetes not only enhanced PGP activities in rice but also improved rhizosphere soil health. Hence, it can be hypothesized that the six actinomycetes were able to survive in the rice rhizosphere and promote PGP and enhance soil health.

Phylogenetic analysis of 16S rDNA sequences of the six PGP actinomycetes showed that all the isolates belong to genus *Streptomyces* but different species. *Streptomyces* spp. has been widely reported to have PGP potential (Tokala et al. 2002; El-Tarabily 2008; Gopalakrishnan et al. 2012a; Nassar et al. 2003; Sadeghi et al. 2012) and protect many crop plants against pathogenic fungi (Liu et al. 1996; Getha et al. 2005; Nonoh et al. 2010; Gopalakrishnan et al. 2011a,b).

Validation of the genes IAA and siderophore confirmed the results of *in vitro* PGP attributes of actinomycete isolates. The actinomycetes CAI-85 and CAI-93 produced higher level of IAA when compared to other actinomycetes in the *in vitro* PGP traits studies and high level of expression profiling of the gene IAA confirmed these results. Furthermore, high level of expression profile of the gene, siderophore was observed in CAI-155 which confirmed the results that CAI-155 had high siderophore production.

The present study was successful in selecting effective isolates of actinomycetes, from herbal vermicompost, that can be a useful component of integrated nutrient management. Though, all the six actinomycetes have been demonstrated for their PGP potential in rice CAI-85 and CAI-93 were found to have superiority over other isolates in terms of crop productivity and root development. Demonstration of PGP activity by the up-regulation of IAA genes provided further evidence for these potential PGP actinomycetes CAI-85 and CAI-93. So, these two isolates can be exploited as PGP agents for rice. However, further experiments are needed to determine the effectiveness of these isolates under different field conditions and to understand the nature of interaction with other soil native microflora and fauna and the host plant.

Acknowledgements

We thank the National Bureau of Agriculturally Important Microorganisms (NBAIM) for providing financial support. We also thank all the staff of the biocontrol unit of ICRISAT including M/s PVS Prasad, P Manohar, B Nagappa, D Barath, A Jabbar and S Rohini for their significant inputs in the laboratory and field studies.

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