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Potentiality of Native *Pseudomonas* spp. in Promoting Sugarcane Seedling Growth and Red Rot (*Colletotrichum falcatum* went) Management

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ABSTRACT

Eight native strains of *Pseudomonas* spp. were isolated from rhizosphere of various sugarcane clones on *Pseudomonas* agar using standard protocols. These strains were characterized for antagonistic traits on red rot pathogen *in vitro* and Plant growth-promoting (PGP) activities in greenhouse up to 30 days from single node setts in portrays as sett treatment (@10^9 CFU ml^{-1}). The promising isolate was characterized up to species level by PCR amplification of 16S rDNA region using *Pseudomonas* specific primers. Elite strain was identified as *P. putida* strain AKP-1. Our results indicated that, *Pseudomonas* strains were inhibitory to *C. falcatum* (1.4 to 69.2% inhibition). Majority of isolates produced catalase, protease and HCN; however, only few isolates were IAA producers and phosphate solubilizers. In Greenhouse, all the isolates were found to enhance sugarcane seedling growth, however, the *P. putida* strain AKP-1 was found superior over others. Our future studies are directed in establishing the endophytic nature, if any, of these elite strains in sugarcane and in confirming the antagonistic activity on *C. falcatum* in GH and field besides PGP activities.

Introduction

Sugarcane is an important cash crop grown in tropical and sub-tropical regions of the world. India is the second largest producer of sugar after Brazil and accounts for 16 per cent of world production (GOI, 2015). In Andhra Pradesh, sugarcane is grown in 1.39 lakh hectares with a production of 99.8 lakh tones. However, the sugarcane productivity (71 tonnes/ha) is very low compared to states like Tamil Nadu, West Bengal, Kerala and Karnataka (GOI, 2017). Sugarcane productivity in Andhra Pradesh is majorly hampered by several biotic stresses of which red rot caused by the fungus, *Colletotrichum falcatum* Went, is a serious threat to sugarcane production under irrigated conditions. The pathogen infects mature stalks of sugarcane; leaf midribs and cause rot of planting material which results in substantial losses in crop yield and sugar quality (Rao, 2004). This disease is responsible for phasing out of several ruling cultivars previously.

Various approaches like cultural methods, use of chemicals, biocontrol agents and thermotherapy were in vogue for the

management of sugarcane red rot. Some bioagents like *Trichoderma harzianum*, *T. viride* and *Pseudomonas* species were found promising in the management of red rot of sugarcane by reducing soil borne inoculum of *C. falcatum* (Singh *et al.,* 2008; Hassan *et al.,* 2011). Red rot of sugarcane is primarily a sett borne disease that is disseminated through infected setts and irrigation water.

The use of seedlings raised in portrays through single noded setts is gaining popularity among the farmers and the seed rate used per acre is reduced from 4 tonnes to 1 ton enabling sett treatment with fungicides and bioagents. Our present research is aimed at identifying a potential Plant Growth-Promoting Rhizobacterial (PGPR) strain that promotes seedling growth besides managing red rot disease effectively.

Materials and Methods

Isolation of *Pseudomonas* species from sugarcane rhizosphere

Soil samples were collected from the rhizosphere of sugarcane clones, CoA13326, 2001A63, 87A298, CoA13324, Co6907, CoV92102 and CoA13322, during winter 2015. The rhizospheric bacteria were isolated on *Pseudomonas* agar medium (HiMedia) by serial dilution method. The colonies with different morphology were selected, purified and maintained on Luria Bertani Agar medium.

Identification of elite *Pseudomonas* species

The bacterial isolates were multiplied in Luria Bertani broth overnight and the DNA was extracted according to the protocol given by Sambrook and Russell (2001). PCR was performed in a volume of 25 µl containing 1 µl of DNA template, 2.5 µl of 10X PCR buffer, 1 µl of each dNTP, 2mM of $MgCl_2$, 1 µl of each primer and 0.5 U of Taq Polymerase. The 16S rDNA amplification was performed using the primers Psmn 289 (5’-GGTCTGAGAGGATGATCAGT-3’) and Psmn 1258 (5’-TTAGCTCCACCTCGCGGC-3’). PCR was performed on Master Cycler Nexus Gradient (Eppendorf, USA). PCR programme was 5 min at 95^0C; 30 cycles of 30s at 94^0C, 30s at 53^0C, and 1 min at 72^0C; and a final extension for 10 min at 72^0C (Kim *et al.,* 2013).

Following amplification 25 µl product were analyzed by electrophoresis at 100V in (1% agarose gel, 0.2 µg of ethidium bromide ml^{-1}) TAE buffer. Ethidium bromide (Ebr) stained agarose gels were observed on UV trans-illuminator and the amplified product was excised from the gel and sent for sequencing to Bioserve technologies, Hyderabad. The nucleotide sequence data obtained was subjected to NCBI BLAST search and the sequences thus identified were submitted to NCBI and accession numbers were obtained.

Antagonistic activity of *Pseudomonas* species against *Colletotrichum falcatum*

The antagonistic properties of *Pseudomonas* species were tested against *C. falcatum* on PDA plates by using dual culture technique. For testing the antagonistic potential of *Pseudomonas* species., an agar block (5 mm diameter) of 5-day-old culture of test pathogen was placed in the centre of Petri plates (90 mm diameter) containing PDA. A loopful of 24-h-old culture of *Pseudomonas* species was streaked on either sides of *C. falcatum* disc at a distance of 2 cm apart. The fungal pathogen culture inoculated centrally on PDA plates, but un-inoculated by bacterial isolates served as control. Each treatment was replicated thrice and the inoculated plates were incubated at 25±10 C for 5 days and per cent inhibition was calculated as per the following formula given by Dennis and Webster (1971).

Determination of catalase activity

The Catalase activity was determined by adding few drops of 3% (v/v) H_2O_2 to 5 ml of 18 hours grown bacterial cultures in Luria-Bertani broth (Kannan, 2002).

Qualitative test for protease activity

All bacterial isolates were screened for protease activity by inoculating on skim milk agar plates. Plates were incubated at 28 + 1^0C for 72 hours and observed for proteolysis, i.e., clear zone production around the inoculated bacterial disc.

Qualitative determination of phosphate solubilisation

Phosphate solubilization ability of bacterial isolates was detected by inoculating them on Pikovaskaya's agar plates (Pikovaskaya, 1948). The inoculated plates were incubated at 28 + 1^0C for 3 days and observed for appearance of clearing zone around the colonies.

Qualitative test for HCN production

HCN production was determined by modified method of Bakker and Schippers (1987). Exponentially grown cultures of bacterial isolates were streaked on to Luria-Bertani agar plates supplemented with 4.4 g glycine L^{-1}. Simultaneously, a filter paper soaked in 0.5% picric acid in 1% Na_2CO_3 was placed in the upper lids of each Petri plate along with uninoculated control. The plates were sealed with parafilm and incubated at 28 + 1^0C for 4 days and observed for colour change from yellow to brown for putative HCN production.

Qualitative test for IAA production

A modified agar plate assay was used for qualitative estimation of IAA production by *Pseudomonas* isolates (Shrivastava and Kumar, 2011). Luria Bertani Agar plate containing 100µg/ml of tryptophan was prepared and poured in Petri plates. After solidification, cavities of 5 mm diameter and 0.2 cm depth were made by a sterile cork borer. Each cavity is filled with 50µl of overnight grown culture and incubated at 30^0C for 24 hours. After overnight growth, the cavities are filled with two drops of Salkowski reagent. The development of pink colour after addition of Salkowski reagent was considered positive for IAA production.

Evaluation of *Pseudomonas* species for their growth promoting activity on sugarcane seedlings

Single node setts of sugarcane variety, 87A 298, were soaked in a solution (10^9cfu/ml) of *Pseudomonas* species for one hour and planted in portrays containing a mixture of cocopit and vermicompost. The setts soaked in water for one hour served as control. Five replications were maintained for each treatment. Germination percentage was recorded 30 days after sowing and the seedling vigor index (SVI) was calculated as per the formula, SVI = Germination percentage X Seedling length (Abdul-Baki and Anderson, 1973).

Results and Discussion

Isolation and identification of rhizosphere bacteria

All the bacterial isolates were found to be Gram negative, rod shaped, catalase positive with polar flagella. Nathan *et al.,* (2013) described plant growth promoting bacteria (PGPB) of the genus *Pseudomonas* as Gram negative rod shaped bacteria with a positive reaction for catalase and oxidase tests. The bacterial isolates were identified as bacteria of the genus, *Pseudomonas* by amplifying the

DNA with *Pseudomonas* specific primers, Psmn 289 and Psmn 1258. The electrophoresis of PCR amplified products had produced an amplicon of 957 bp confirming the bacteria as *Pseudomonas* species. The elite *Pseudomonas* species, based on antagonistic ability against *C. falcatum*, was further identified to species level by partial sequencing of amplified products. The NCBI blast analysis revealed the identity of the elite *Pseudomonas* species as *P. putida* and designated as *P. putida* strain AKP1 (KX758438).

Antagonistic activity against *C. falcatum*

The *Pseudomonas* isolates were tested for their antagonistic activity against *C. falcatum* by dual culture method. Most of the *Pseudomonas* isolates tested (Table 1) were found inhibitory to the mycelial growth of *C. falcatum* under *in vitro* conditions. Highest inhibition (69.23%) of the test pathogen was recorded with *Pseudomonas putida* strain AKP1 isolated from the rhizosphere of sugarcane clone, CoV 92102 (Fig. 1). This was followed by *Pseudomonas* species isolated from CoA13326 and 2001A 63 with an inhibition of 54.23 and 48.58 per cent, respectively. Least inhibition of mycelial growth of *C. falcatum* was recorded with the *Pseudomonas* isolated from the rhizosphere of 87A 298.

Hassan *et al.,* (2011) evaluated bacterial strains isolated from sugarcane rhizosphere against *C. falcatum* and reported that *Pseudomonas putida* strain NH-50 (EU627168) reduced red rot severity by 44-60% in different field conditions and attributed the pathogen suppressing ability of the isolates to the production of various extracellular metabolites and diffusible antibiotics. The biocontrol abilities of *Pseudomonas* spp. depend essentially on aggressive root colonization, induction of systemic resistance in the plant, the production of diffusible or volatile antifungal antibiotics (Haas and Keel, 2003) and enzymes that degrade pathogen cell walls and hydrogen cyanide production (Ahmad and Kibret, 2014).

Screening for growth promoting traits

Biochemical characterization of *Pseudomonas* isolates for the production of enzymes revealed that all the isolates could produce catalase as evidenced by the production of effervescence on addition of hydrogen peroxide. Most of the isolates produced protease except the isolates retrieved from the rhizosphere of the sugarcane clones, CoA13324 and CoA13326. All the isolates tested have produced hydrogen cyanide (Fig. 2) as evidenced by the change in color of the filter paper from yellow to brown (Table 2).

HCN production was attributed as one of the factors for biological control of plant diseases by fluorescent Pseudomonads due to the induction of plant resistance against certain pathogens. Stutz *et al.,* (1986) highlighted that cyanide secreted by *Pseudomonas fluorescens* strain CHAO played a role in the suppression of black root rot of tobacco (*Thielaviopsis basicola*).

Though, all the *Pseudomonas* isolates produced HCN, only some of the isolates could inhibit the mycelial growth of *C. falcatum*. The efficacy of bacterial strains against *C. falcatum* depends upon the pathotype of *C. falcatum*. Malathi *et al.,* (2008) reported that the *Pseudomonas fluorescens* isolates, ARR1G and VPT4, were found effective against tropical pathotypes of *C. falcatum* and the isolate FP7 showed moderate effect against all the pathotypes. The antagonistic potential of *Pseudomonas* spp. was found to be dependent on the virulence of the pathotypes.

Table.1 *In vitro* evaluation of rhizospheric bacteria (*Pseudomonas* sp.) of sugarcane in inhibiting mycelial growth of *Colletotrichum falcatum*

Sl. No.	Treatment	Per cent inhibition of mycelial growth
1	*Pseudomonas* species (CoA13326)	54.23 (47.41) *
2	*Pseudomonas* species (2001A63)	48.58 (44.16)
3	*Pseudomonas* species (87A298)	1.41 (6.82)
4	*Pseudomonas* species (CoA13324)	35.68 (36.66)
5	*Pseudomonas* species (Co6907)	46.00 (42.68)
6	*Pseudomonas* species (CoA13326)	46.71 (43.10)
7	*Pseudomonas* species (Co V92102)	69.23 (56.30)
8	*Pseudomonas* species (CoA13322)	43.67 (41.35)
	CD (P=0.05)	**1.934**
	SEm (±)	**0.646**
	CV	**3.161**

*Figures in the parenthesis are angular transformed values

Table.2 Evaluation of native *Pseudomonas* spp. for plant growth promoting traits

Sl. No.	Treatment	Catalase production	Protease activity	HCN production	IAA production	Phosphate solubilizing activity
1	*Pseudomonas* sp. (CoA13326)	+	+	+	+	-
2	*Pseudomonas* sp. (2001A63)	+	+	+	-	-
3	*Pseudomonas* sp. (87A298)	+	+	+	-	+
4	*Pseudomonas* sp. (CoA13324)	+	-	+	-	-
5	*Pseudomonas* sp. (Co6907)	+	+	+	+	-
6	*Pseudomonas* sp. (CoA13326)	+	-	+	+	-
7	*Pseudomonas* putida AKP1 (Co V92102)	+	+	+	+	+
8	*Pseudomonas* sp. (CoA13322)	+	+	+	-	-

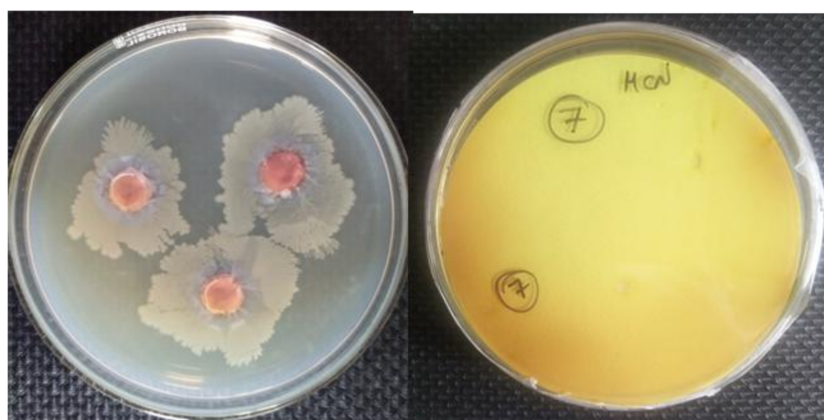
(+) Positive, (-) negative

Table.3 Seedling vigour index of sugarcane seedlings as influenced by sett bacterization with *Pseudomonas* species

Sl. No.	Treatment	Seedling vigour index
1	*Pseudomonas* species (CoA13326)	1860
2	*Pseudomonas* species (2001A63)	1260
3	*Pseudomonas* species (87A298)	776
4	*Pseudomonas* species (CoA13324)	800
5	*Pseudomonas* species (Co6907)	1400
6	*Pseudomonas* species (CoA13326)	1480
7	*Pseudomonas putida* AKP1 (Co V92102)	1880
8	*Pseudomonas* species (CoA13322)	1100
9	Control	608

Fig.1 *In vitro* inhibition of mycelial growth of *Colletotrichum falcatum* by *Psudomonas putida* isolate AKP 1

Fig.2 IAA and HCN production by *Psudomonas putida* isolate AKP 1



In IAA agar plate assay, pink zone was observed around the cavities in which the *Pseudomonas* spp. isolated from the rhizosphere of the sugarcane clones, CoA13326, Co6907, CoA13326 and CoV92102 (Fig. 2), were inoculated indicating the ability to produce IAA in the presence of tryptophan. Only few isolates could solubilize phosphate as shown by the formation of clear halos around the point of inoculation. IAA biosynthesis has been correlated with stimulation of root proliferation by rhizosphere bacteria, which enhanced uptake of nutrients by the associated plants (Lifishitz *et al.,* 1987).

Effect of sett bacterization on seedling vigour index

Soaking the single node setts in bacterial suspension for one hour prior to planting in portrays enhanced sett germination and seedling vigour (Table 3) compared to control. Seedling vigour index was higher when the setts were treated with *P. putida* strain AKP1 (1880) and the *Pseudomonas* species isolated from the rhizosphere of CoA13326 (1860) compared to all other treatments. The seedling growth increase caused by sett bacterization with *Pseudomonas* species qualifies them as PGPR. Plant growth promotion by *Pseudomonas* species was related to the production of phytohormones like auxins (Bharucha *et al.,* 2013), gibberellins (Kapoor *et al.,* 2016) and cytokinins (Grobkinsky *et al.,* 2016). Microorganisms inhabiting the rhizosphere of plants utilize the rich source of substrates from the roots and are expected to synthesize and release phytohormones as secondary metabolites (Berendsen *et al.,* 2012) that controls many important physiological processes like cell enlargement and division, tissue differentiation and responses to light and gravity (Lambrecht *et al.,* 2000). Plant growth promoting rhizobacteria (PGPR) have beneficial effects on plants which have been variously attributed to their ability to produce various compounds including phytohormones, organic acids and siderophores, to fix atmospheric nitrogen, to solubilize soil phosphate, to produce antibiotics that suppress deleterious microorganisms (Glick, 1995).

In our study, *P. putida* strain AKP1 isolated from the rhizosphere of sugarcane clone, CoV 92102 was found superior to all other isolates in having high inhibitory effect against *C. falcatum* besides its ability to solubilize inorganic phosphate and to produce catalase, protease, HCN, and IAA. Overall, our results suggested the scope and potentiality of *P. putida* strain AKP1 in inhibiting mycelial growth of *C. falcatum* besides plant growth promotion. Our future studies are directed in establishing the endophytic nature, if any, of *P. putida* strain AKP1 in sugarcane and in confirming the antagonistic activity on *C. falcatum* in greenhouse and field besides plant growth promoting activities.

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