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Biochemical characterization of effective PGPRs from rhizosphere of banana against tip over disease caused by *Erwinia carotovora* subsp. *carotovora*

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Abstract
In recent years, there has been a reverse interest in the search of plant growth promoting rhizobacterias (PGPRs) for sustainable crop production. Banana is an economically important tropical fruit crop, which is subjected to infection by fungai, bacteria, virus and nematodes. A total of 64 *Bacillus* spp. were isolated from rhizosphere of banana. Among 64 isolates 12 isolates were found to be effective against the *Erwinia carotovora* subsp. *carotovora in vitro.* Among them most effective isolates were further subjected for biochemical characterization. The biochemical studies confirmed them to be as *Bacillus pumilis*, *Bacillus subtilis* and *Bacillus cereus*.

Keywords: Banana, *Erwinia carotovora* subsp. *carotovora*, *in vitro*, *Bacillus pumilis*, *Bacillus subtilis*, *Bacillus cereus*

Introduction
Biological control of plant pathogens by antagonistic microorganisms is a potential non-chemical means (Harman, 1991) [6] and is known to be a cheap, effective and eco-friendly method for the management of crop diseases (Cook and Baker, 1983) [4]. The use of biological control agents as an alternative to fungicides and bactericides is increasing rapidly in the present day agriculture due to the deleterious effects of chemical pesticides. Efforts to control plant diseases with antagonistic bacterial agents have been made successfully (Chen *et al*., 1995) [3]. *Bacillus* spp. have special characteristics that make them good candidates as biological control agents. First, they are well known as antibiotic producers with antagonistic activity against fungal and some bacterial pathogens (Krebs *et al*., 1998) [8]. This ability also appears to contribute to the establishment and persistence of the antagonist in the plant (Krebs *et al*., 1993) [7]. Second, they form spores that can be easily formulated, and have high viability compared with vegetative cells (Bochow, 1995) [2]. Third, they are commonly found in soils (Stabb *et al*., 1994) [10]. The soil bacteria that aggressively colonize the root zone and promote plant growth are generally termed as Plant Growth Promoting Rhizobacterias (PGPRs). The tropical banana rhizosphere harbor's a wide diversity of antagonastic bacteria that may not only aid in beneficial symbiotic relationships but also stimulate the plant growth by suppressing pathogenic organisms. Tip over is one of the important disease of banana caused by *Erwinia carotovora* subsp. *carotovora* causing yield losses upto 65.28 per cent (Totagi, 2012) [13] and the disease is transferred through tissue cultured materials, infected seedlings, soil and water.

Material and methods
Isolation of PGPRs from the rhizosphere of banana plant
Rhizospheric soil samples were collected from the neighbouring healthy plants of banana in the field. The collected soil were transferred to sample collection bags, antagonistic bacterium was isolated by following serial dilution and Pour plate method by using Hicrome Bacillus agar and Nutrient agar media.

Isolation of *Bacillus* spp.
Different species of *Bacillus* were isolated from soil using a specific media *viz.*, Hichrome bacillus agar medium following serial dilution and plating technique was done. Then the plates

Were incubated at 28±1ºC for 48 h. Colonies formed were picked up and purified by repeated streaking on the Nutrient agar medium. Well isolated colonies were transferred to 20 per cent glycerol stock for preservation.

In vitro* evaluation for efficacy of isolated PGPRs against *Erwinia carotovora* subsp. *carotovora

Isolated PGPRs were evaluated for their efficacy against the growth of *Erwinia carotovora* subsp. *carotovora* by well diffusion method. A heavy suspension of *Erwinia carotovora* subsp. *carotovora* was multiplied in nutrient broth (20 ml) was mixed with lukewarm nutrient agar medium in flask. The inoculated flasks were incubated at 28 ± 1 ºC for 48 h. The bacterial suspension was then seeded to the lukewarm nutrient agar medium. The seeded medium was poured into the sterilized Petri plates and was allowed to solidify. Then, a well with a diameter of 6 to 8 mm was punched aseptically with a sterile cork borer and a volume (20-100 µL) of the isolated PGPRs cultured in the nutrient broth was introduced into the well. The inoculated plates were incubated at 28 ± 1 ºC for 48 h. The observations for the production of inhibition zone around the PGPRs was measured by taking mean diameter of the zone formed and then were analysed statistically.

Biochemical characterization of the effective PGPRs

Biochemical characterization was done by using Hi-Bacillus Identification Kit. This kit is used to differentiate the *Bacillus* at the species level based on biochemical tests. This kit includes around 12 wells to study 12 biochemical tests such as Malonate, Voges proskauer's, Citrate, ONPG, Nitrate reduction, Catalase, Arginine, Sucrose, Mannitol, Glucose, Arabinose, Trehalose. For these wells a loopfull of bacterial culture was added under aseptic condition in laminar air flow chamber. To perform Voges proskauer's (well no.2) test 1-2 drops of Baritt reagent A and 1-2 drops of Baritt reagent B was added. To perform Nitrate reduction test (well no.5) 1-2 drops of N, N-Dimethyl-1-Napthylamine reagent was added. To perform catalase test (well no.6) a drop of 3 per cent H_2O_2 was added. These reagents were added after the inoculation of bacterial cultures in the wells of kit for 48 h at 35 ºC. After 48 h, wells were observed for the change in colour and the *Bacillus* spp. were differentiated by referring the colour change as described in the Hi-Bacillus identification kit description.

Results and discussion

Isolation of PGPRs from rhizosphere of banana

Number of isolates collected from rhizosphere of banana varied from one place to other place. A total of 64 isolates were collected from surveyed area and were identified as *Bacillus* spp. Different species of *Baciilus* formed different colony colour on Hicrome bacillus agar medium(Plate 1). Gechemba *et al.* (2016) [5] reported that the tropical banana rhizosphere harbor's a wide diversity of antagonastic bacteria that may not only aid in beneficial symbiotic relationships but may stimulate the plant growth by suppressing pathogenic organisms.

In vitro* evaluation of isolated PGPRs against *Erwinia carotovora* subsp. *carotovora

Among 64 isolates of isolated *Bacillus* spp. 12 isolates were found to be effective compared to other isolates (Table 1 & Fig 1). Among 12 effective isolates of *Bacillus* spp. maximum inhibition (16.67 mm) was observed by Belagavi isolate 13 and minimum inhibition (12.00 mm) was observed by Haveri isolate 5. Similarly Apastambh *et al.* (2016) [1] isolated and studied 8 strains of fluorescent pseudomonas and 4 strains of Bacillus from Banana rhizosphere. The highest zone of inhibition was given by Yps8 against *alternaria solani* and highest zone of inhibition was given by Yps1 against *Fusarium oxysporum*.

Biochemical characterization of the effective PGPRs

Out of 12 effective isolates of *Bacillus* spp. 8 most effective isolates were characterized biochemically. The results observed such as Belagavi isolate 12, Vijayapur isolate 7 and Belagavi isolate 13 were characterized as *Bacillus cereus*, Vijayapur isolate 2, Vijayapur isolate 4, Bagalkote isolate 15 and Haveri isolate 5 were characterized as *Bacillus subtilis*, Vijayapur isolate 8 was characterized as *Bacillus pumilis* biochemically (Table 2 and Plate 2). Similarly, Malleswari and Bagyaarayana (2013) they identified isolated PGPRs on the basis of colony morphology, gram staining and biochemical tests and further characterized through 16S rRNA gene sequencing which led to their identification as *Pantoea* sp. (Cf 7), *Bacillus* sp. (Cf 60) and *Pseudomonas* sp. (Te1, Av 30).

Table 1: *In vitro* evaluation of *Bacillus* spp. against the growth of *Erwinia carotovora* subsp. *Carotovora*

Treatments	Isolates	Mean diameter of inhibition zone (mm)
T_1	Vijayapur isolate 2	15.33 (4.04) *
T_2	Vijayapur isolate 4	14.33 (3.92)
T_3	Vijayapur isolate 5	14.00 (3.87)
T_4	Vijayapur isolate 7	16.00 (4.12)
T_5	Vijayapur isolate 8	14.67 (3.96)
T_6	Haveri isolate 5	12.00 (3.61)
T_7	Dharwad isolate 1	14.00 (3.87)
T_8	Belagavi isolate 12	15.00 (4.00)
T_9	Belagavi isolate 13	16.67 (4.20)
T_{10}	Bagalkote isolate 15	13.00 (3.74)
T_{11}	Belagavi isolate 16	14.00 (3.87)
T_{12}	Belagavi isolate 17	13.67 (3.83)
S. Em.±		0.059
C.D. at 1%		0.235

* - $\sqrt{x+1}$ transformed values

Table 2: Biochemical studies of effective isolates of *Bacillus* spp.

Sl. No.	Tests	Effective Isolates of *Bacillus* spp.							
		Belagavi isolate 13	**Vijayapur isolate 2**	**Vijayapur isolate 7**	**Belagavi isolate 12**	**Vijayapur isolate 8**	**Vijayapur isolate 4**	**Bagalkote isolate 15**	**Haveri isolate 5**
1.	Malonate	-	-	-	-	-	-	-	-
2.	Vogesproskauer's	+	+	+	+	+	+	+	+
3.	Citrate	+	+	+	+	+	+	+	+
4.	ONPG	-	+	-	-	+	+	+	+
5.	Nitrate reduction	+	+	+	+	-	+	+	+
6.	Catalase	+	+	+	+	+	+	+	+
7.	Arginine	+	-	+	+	-	-	-	-
8.	Sucrose	+	+	+	+	+	+	+	+
9.	Mannitol	-	+	-	-	+	+	+	+
10.	Glucose	+	+	+	+	+	+	+	+
11.	Arabinose	-	+	-	-	+	+	+	+
12.	Trehalose	+	+	+	+	+	+	+	+

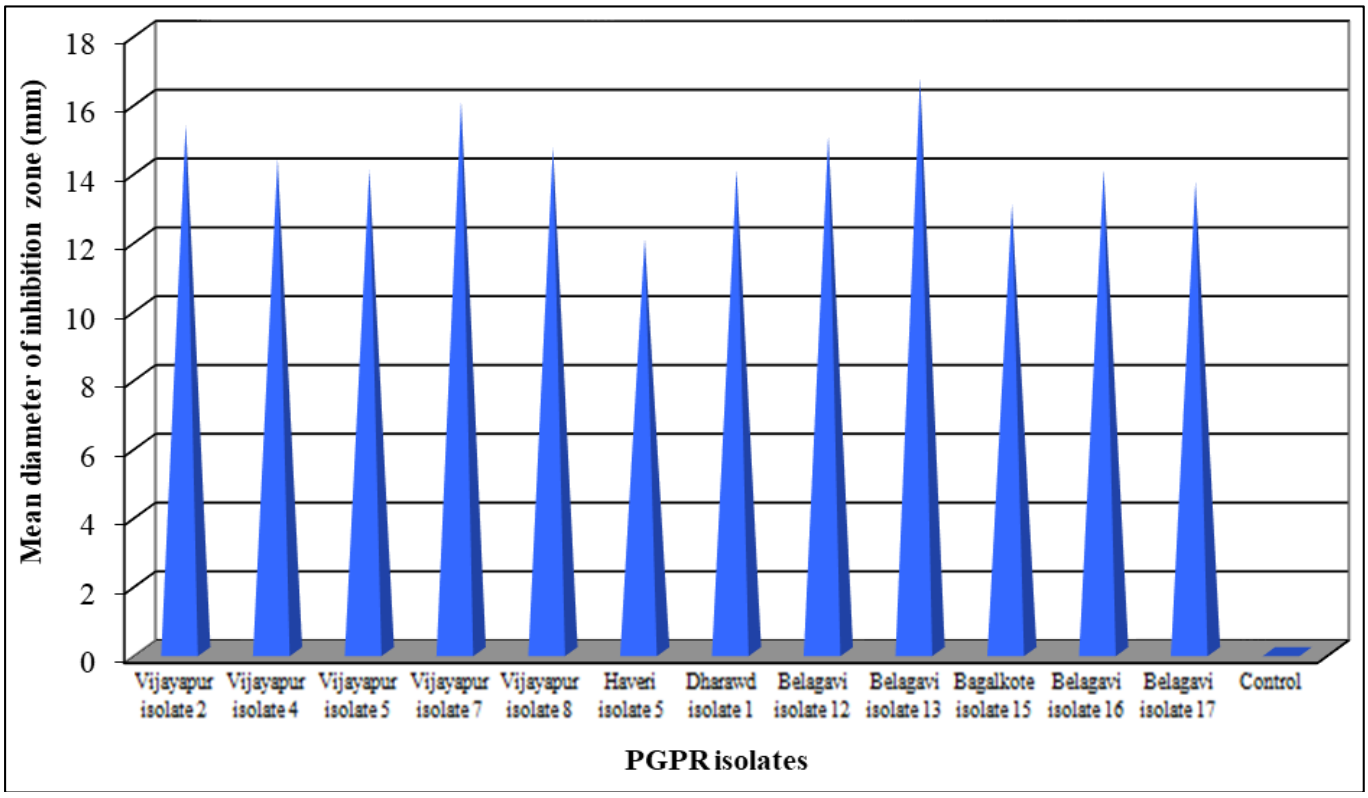

Fig 1: *In vitro* evaluation of *Bacillus* spp. against the growth of *Erwinia carotovora* subsp. *carotovora*

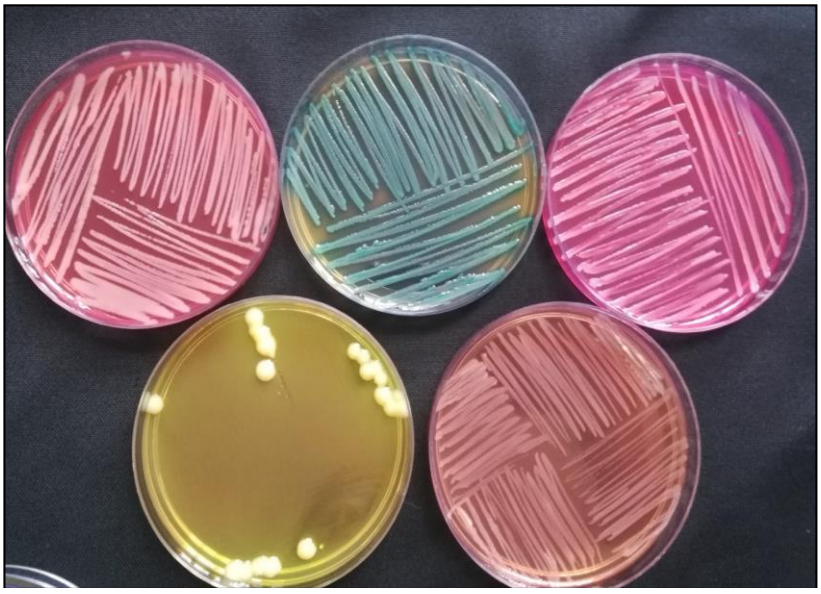

Plate 1: Different isolates of *Bacillus* spp. From rhizosphere of banana on Hicrome bacillus agar medium

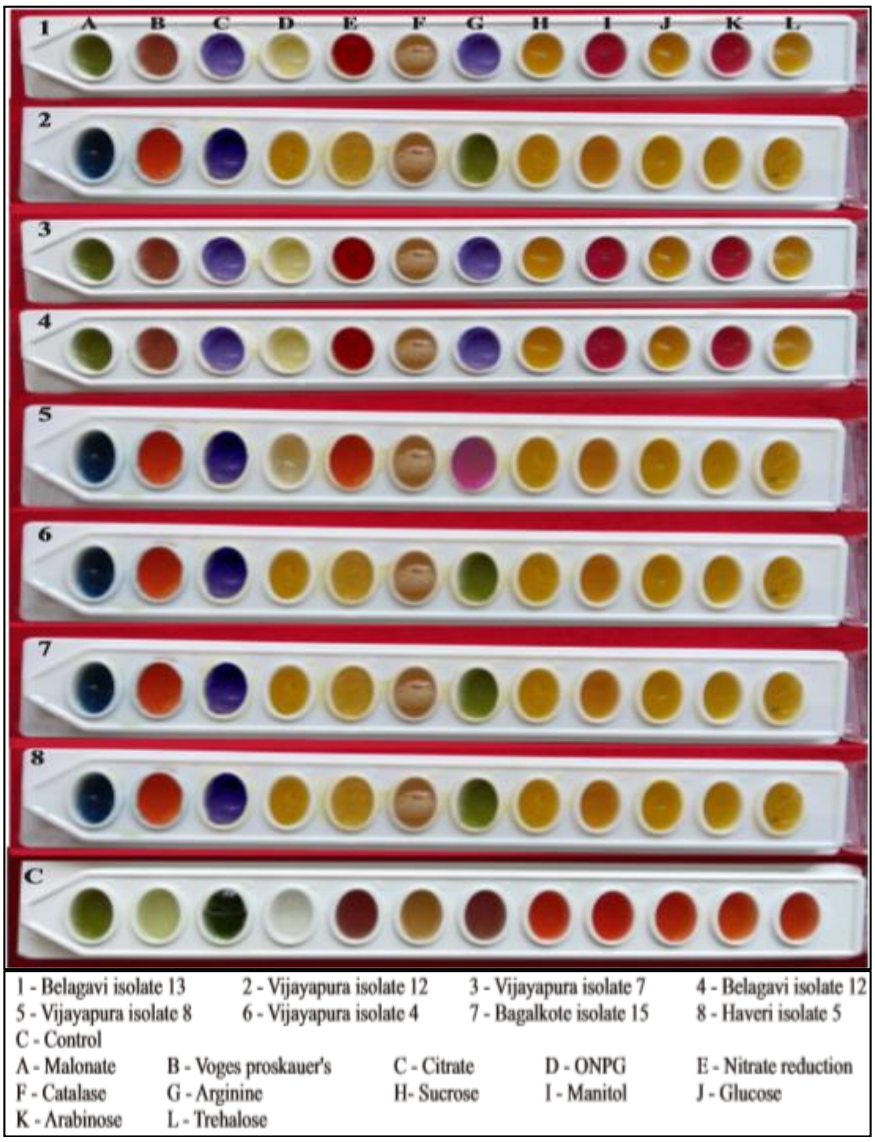

Plate 2: Biochemical studies of effective *Bacillus* spp. Using HiBacillus identification kits

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