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Potential role of rhizospheric proteobacteria for plant growth promotion and damping off disease management in cowpea

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Abstract
Damping off disease is one of the major constraints to profitable cowpea production in all agro-ecological zones where the crop is grown. In recent times, the use of rhizospheric proteobacteria is gaining importance for plant growth-promotion and biological control. Rhizospheric proteobacteria can reduce the disease severity in many crops through induced resistance phenomenon. In the present investigation, the effect of plant growth promoting rhizospheric proteobacteria on damping off disease development and growth parameters in cow pea seedlings were studied. The best reduction in pre and post emergence damping off in cowpea seedlings was observed with seven rhizobacterial isolates, i.e., VE1, VE5, VE3, VE2, VE7 VE23 and VE39 which were found to be antagonistic against both the soil-borne pathogens. VE7 was found to be potent antagonistic bacteria against two soil borne pathogen. Highest mycelial inhibition of *R. solani* was recorded 58.89% and 52.78% respectively, when challenged with VE19 and VE7 isolates. The rhizobacterial isolates VE32 and VE7 exhibited maximum mycelial inhibition (62.22% and 60.56%, respectively) against *S. sclerotiorum*. Among the fourteen native rhizobacterial isolates, the isolate VE20 followed by VE4 and VE41 were found to be best plant growth promoter compared to other rhizobacterial isolates. Highest root length were recorded in VE10 and shoot length were recorded in VE7 treated plants. The activation of the defense reactions by these isolates was correlated with an enhanced resistance to damping-off caused by *S. rolfsii* and *R. solani*. This study demonstrated the ability of the rhizobacterial isolates may be in future be used as microbial consortia for sustainable plant health management.

Keywords: Biocontrol, plant growth promoting rhizospheric proteobacteria, vigour index, *Sclerotium rolfsii* and *Rhizoctonia solani*

Introduction
Cowpea is a legume crop of major economic importance globally. Cowpea diseases induced pathogens belonging to various pathogenic groups such as fungi, bacteria, viruses, nematodes, and parasitic flowering plants which constitute one of the major constraints to profitable cowpea production in all agro-ecological zones where the crop is grown (Sendhilvel *et al.*, 2005) [13]. In India, damping off of cowpea has been reported which can provoke 50 to 60% dry yield loss in new alluvial regions of West Bengal. *Sclerotium rolfsii* was the most common species isolated from all the agro-ecological zones and pathogenic on cowpea. Disease management remains one of the most interesting issues to be addressed, which is particularly true for cowpea considering the largely approximate area of cowpea self-defense mechanisms.
Application of chemical fungicides is the conventional approach used for managing damping off for over 50 years. However, fungicides have shown some promising results in controlling damping off, fungicide residues can lead to environmental pollution and human health hazards. Biocontrol methods may help to advance ecofriendly strategies for managing this disease in cowpea seedlings. Biological control signifies both the oldest and youngest technology for the control of plant diseases and pest. Most people approve that agriculture could not have initiated without the benefits of naturally occurring biological controls. However modern biological control achieved with introduced microorganisms is still in its initial stages. The rhizospheric proteobacteria associated with plants include *P. fluorescens, P. putida and P. aeruginosa*. The use of rhizospheric proteobacteria is gaining importance for plant growth-promotion and biological control. Rhizospheric proteobacteria could decrease disease severity in several crop plants through induced resistance phenomenon (Thahir Basha *et al.*, 2012) [12]. Induced systemic resistance in crop plant is considered by the induction of host defense responses including, defense related enzymes synthesis and phenolics accumulation.

In this background, we intended to assess the biocontrol activity of some rhizospheric proteobacteria against *Rhizoctonia solani* and *Sclerotinia sclerotiorum* causing damping off disease in cowpea and to define the mechanisms implicated in this process.

Materials and Methods

Isolation of PGPR strains

Rhizobacteria were isolated from the soil collected from the rhizosphere of different crops like Paddy (*Oryza sativa*),Cucumber (*Cucumis sativus*),Sugarcane (*Saccharum officianarum*), Pointed gourd (*Trichosanthes dioica*), *Colocasia* and some were collected from the uncultivated areas of grassy, pasture lands, fallow lands and forests with King's medium B (KMB Hi-media) (King *et al.*, 1954) [11].

3% KOH Test

3% KOH solution was taken and drop of this solution was placed on microscopic slide using Pasteur pipette. Part of single colony was taken from the medium using cool sterile loop. Bacteria was mixed into KOH solution until an even suspension is obtained. Loop was lift upside from the slide. If mucoid thread was obtained with the loop then the bacterium is gram negative and if water suspension is produced instead of mucoid thread, then the bacterium is gram positive. Through KOH test, potent gram negative bacterial isolates were selected and these isolates were used for further experimentation.

The gram negative bacterial isolates thus selected were characterized on the basis of their morphological (cell shape, cell arrangement, gram reaction), cultural (colony type, pigment production) and biochemical identification keys of Bossis (1995) [4].

Antifungal efficacy of rhizospheric proteobacteria

Isolated rhizobacteria were tested for their *in vitro* anti-fungal bio-control potentiality by following standard protocol of dual culture assay proposed by Shivakumar *et al*., (2000) [14]. Soil borne fungal phytopathogens *viz*., Rhizoctonia solani and Sclerotinia sclerotiorum was used in the evaluation of bacterial antagonistic activity through dual culture method. The bacterial isolates were streaked by a thin line along both the opposite end of the plates containing sterile potato dextrose agar (PDA) media and a 5mm disc of the freshly cultured pathogen was placed exactly in the centre of the plates. Three replications of each isolate including a control i.e. without inoculation of the antagonist were maintained at 28±1 °C for 96 hr. The mycelial inhibition percentages of the pathogens were calculated by the following equation.

$$\%\ Mycelial\ inhibition = \frac{C - T}{C} \times 100$$

Where
C= mycelial growth of the pathogen in control
T= mycelial growth of the pathogen in treatments.

In vitro seed germination and seedling vigour test

The potent rhizobacterial gram negative isolates were screened for their ability to augment the germination and vigour of cowpea seeds by *in vitro* seed bacterization and germination test. All the potent rhizobacterial gram negative isolates were grown in 20 ml nutrient broth medium for 48 hr at 28 ± 1 °C in a rotary shaker at 150 rpm. Culture suspension ($2X10^{10}$ cfu/ ml) of 1 ml was centrifuged at 10, 000 rpm for 6 min. The supernatant was discarded and the cell pellet was washed and re-suspended in 1 ml sterile distilled water. Pinch of sodium carboxy methyl cellulose (Na-CMC) was added into the cell suspension and mixed well. Cowpea (variety: Kasi Kanchan) seeds were surface sterilized with 70% ethanol for 1 min followed by 1% sodium hypochlorite for 5 min and after each step seeds should be washed with sterilized distilled water. Surface sterilized seeds were soaked into the culture suspension for 30 min. The bacterized seeds were then air dried and placed on water agar (0.6% agar in distilled water) medium with a sterile forceps and incubated for 7 days at 28±0.5°C for seed germination, root and shoot length and vigour index. Vigour index of the seedlings were calculated by the following equation:

$$VI = (RL + SL) * G(\%)$$

Where, *VI* is vigor index
RL, root length
SL, shoot length
G (%) is germination percentage

Results and discussion

In vitro antifungal efficacy of the native rhizospheric proteobacteria

All the rhizobacterial isolates obtained in the present study, were tested for their *in vitro* antagonistic potentiality by dual culture plate assay against two different soil-borne phytopathogens *viz*. *R. solani* (leaf & sheath blight, root rot), and *S. sclerotiorum* (root and stem rot). Mycelial inhibition percentages of the fungal pathogens were calculated as per the formula mentioned in materials and methods. The maximum mycelial inhibition of *R. solani* was recorded 58.89% and 52.78% respectively, when challenged with VE19 and VE17 isolates. The maximum mycelial inhibition of *S. sclerotiorum* was observed to be 62.22%, 60.56% and 56.11% respectively, when challenged withVE32, VE7, and VE11 isolates. The average mycelial inhibition against all the tested fungal pathogen was exhibited by the isolates VE5, VE2, VE7 and VE4. Experimental data on mycelial inhibition percentage are shown in Table 1. Two dendrogram based on hierarchical cluster analysis of the antagonistic rhizobacteria was constructed based on their performance of mycelial inhibition of the tested two fungal pathogens. The forty six rhizobacterial isolates used in this study showed different degree of mycelial inhibition suggesting functional variability as a consequence of different hosts and edaphic factors from where these isolates were recovered.

It has been reported previously that the plant species or cultivars, age and especially the composition of root exudates plays a key role in the diversity of rhizobacterial populations and can influence the frequency of antagonistic bacteria (Kremer *et al.*, 1990; Siciliano *et al.*, 1998) [9, 10]. Besides stimulating plant growth by direct mechanisms, PGPR can also indirectly induce plant growth by protecting plants against soil-borne pathogens (Bloemberg and Lugtenberg, 2001; Mercado- Blanco and Bakker, 2007) [7, 8]. Prasada *et al.*, (2013) found antagonistic activity of PATPT1, PATPT2 isolates against *S. rolfsii*. Wael *et al*., (2014) [3] also done *in vitro* screening of some PGPR isolates based on their

antagonistic activity against *S. Sclerotiorum* and *F. oxysporum* and they obtained 50% of the rhizosphere isolates active against *F. oxysporum* and 71.43% against *S. sclerotiorum*.

Vigour index *in vitro*

Cowpea seeds were treated with native rhizobacterial isolates and after seven days of seed bacterization, the germination percentage, root length and shoot length of seedlings were recorded.

Table 1: Antagonistic activity of rhizobacterial isolates against *R. solani* and *S. Sclerotium*

Isolate	*Antagonistic activity against R. solani*	*Antagonistic activity against S sclerotiorum*
VE-1	38.33 (38.24) d	58.33 (49.81) bc
VE-2	41.11 (39.87) cd	18.89 (25.72) h
VE-3	14.44 (22.30) g	33.89 (35.60) f
VE-4	0.00 (0.57) h	0.00 (0.57) i
VE-5	38.89 (38.52) d	21.11 (27.35) g
VE-6	44.44 (41.81) c	0.00 (0.57) i
VE-7	52.78 (46.61) b	60.56 (51.10) ab
VE-8	32.22 (34.57) e	0.00 (0.57) i
VE-9	41.67 (40.16) cd	0.00 (0.57) i
VE-10	0.00 (0.57) h	0.00 (0.57) i
VE-11	0.00 (0.57) h	56.11 (48.52) c
VE-12	0.00 (0.57) h	18.89 (25.74) h
VE-14	25.56 (30.37) f	0.00 (0.57) i
VE-15	0.00 (0.57) h	0.00 (0.57) i
VE-16	27.78 (31.77) f	0.00 (0.57) i
VE-17	0.00 (0.57) h	37.78 (37.92) de
VE-18	0.00 (0.57) h	0.00 (0.57) i
VE-19	58.89 (50.13) a	0.00 (0.57) i
VE-20	0.00 (0.57) h	0.00 (0.57) i
VE-21	0.00 (0.57) h	0.00 (0.57) i
VE-22	0.00 (0.57) h	0.00 (0.57) i
VE-23	51.67 (45.97) b	37.78 (37.92) de
VE-24	0.00 (0.57) h	40.00 (39.23) d
VE-25	0.00 (0.57) h	0.00 (0.57) i
VE-26	0.00 (0.57) h	0.00 (0.57) i
VE-27	0.00 (0.57) h	20.56 (26.91) gh
VE-28	0.00 (0.57) h	0.00 (0.57) i
VE-29	0.00 (0.57) h	37.78 (37.93) de
VE-30	0.00 (0.57) h	0.00 (0.57) i
VE-31	0.00 (0.57) h	0.00 (0.57) i
VE-32	0.00 (0.57) h	62.22 (52.09) a
VE-33	0.00 (0.57) h	0.00 (0.57) i
VE-34	0.00 (0.57) h	36.67 (37.25) e
VE-35	0.00 (0.57) h	36.11 (36.93) e
VE-36	0.00 (0.57) h	0.00 (0.57) i
VE-37	52.22 (46.30) b	0.00 (0.57) i
VE-38	0.00 (0.57) h	0.00 (0.57) i
VE-39	40.00 (39.23) cd	33.33 (35.26) f
VE-40	0.00 (0.57) h	0.00 (0.57) i
VE-41	0.00 (0.57) h	33.33 (35.26) f
VE-42	0.00 (0.57) h	0.00 (0.57) i
VE-43	0.00 (0.57) h	0.00 (0.57) i
VE-44	32.22 (34.56) e	0.00 (0.57) i
VE-45	0.00 (0.57) h	0.00 (0.57) i
VE-46	0.00 (0.57) h	0.00 (0.57) i
S.E(m)±	0.879	0.472
CD	2.459	1.319

Bio-primed cowpea seeds were kept in water agar plates to evaluate the seed germination and seedling vigor enhancing efficacy of the native rhizobacterial isolates. A total of five bacterized cowpea seeds were placed in each plate and three such replicates were kept for each isolate. Among the fourteen native rhizobacterial isolates, the isolate VE20 followed by VE4 and VE 41were found to be best plant growth promoter compared to other rhizobacterial isolates. Perusal of the data presented in the Table 1, it was found that seeds bio-primed with VE1, VE5, VE7, VE14, VE16, VE20, VE37 gave highest per cent (100%) of seed germination as compared to 60% seed germination in control. Root length was found maximum for the VE4 (4.48 cm) followed by VE41 (4.38 cm) and shoot length of the seedlings was found maximum for the both VE4 and VE41 (6.25 cm) bacterized seeds. The calculated vigour index based on germination percentage, root length and shoot length was also recorded maximum for the isolate VE 20 (974) followed by VE4 (858) and VE41 (850).

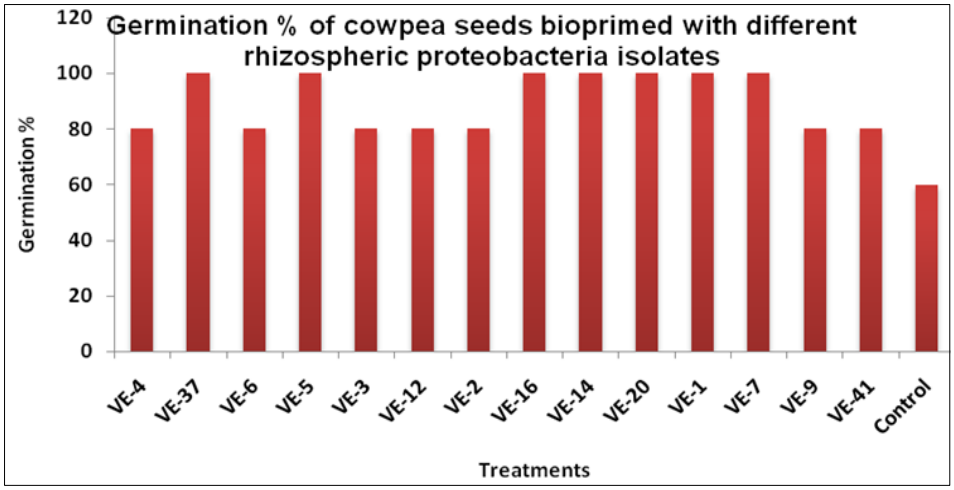

Fig 1: Germination% of cowpea seeds bioprimed with different rhizospheric proteobacterial isolates

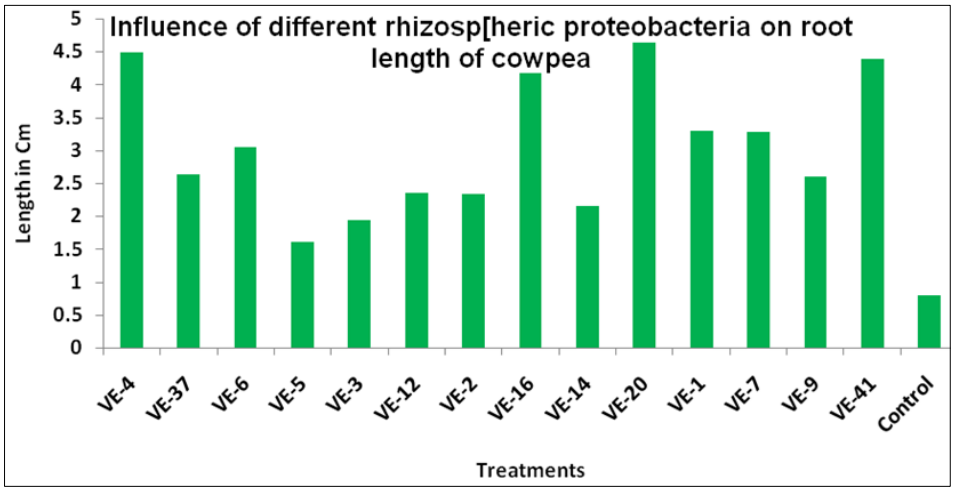

Fig 2: Influence of different rhizosperic proteobacteria on root length of cowpea

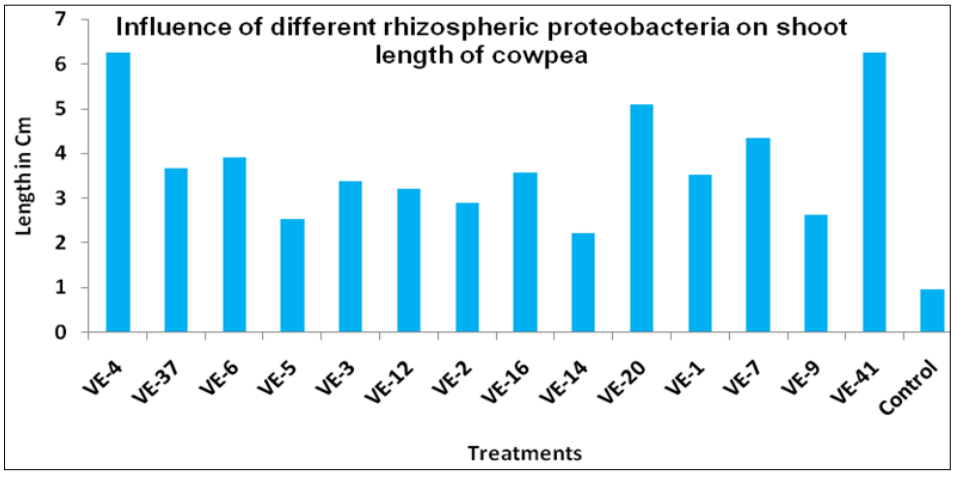

Fig 3: Influence of different rhizospheric proteobacteria on shoot length of cowpea

All the isolates were grouped into low efficacy, moderate efficacy and high efficacy group based on their vigour index augmentation efficacy. The data ranges of the groups were observed to be 414-438.4 (low efficacy), 632-776 (moderate efficacy) and 850-974 (high efficacy) respectively (Table 2). Significant increases in growth and yield of agronomical important crops in response to inoculation with PGPR have been reported by (Asghar *et al.*, 2002; Bashan *et al.*, 2004) [5, 6]. Similar findings like us were also reported where seed bacterization with rhizospheric *Pseudomonas* isolates was found to increase germination percentage, root length and shoot length of cotton, groundnut, chilli and soybean. Findings of Lugtenberg *et al.*, (2002) [2] also support that bacterial inoculants are able to increase plant growth and germination rate, improve seedling emergence, responses to external stress factors and protect plants from disease.

Table 2: Distribution and range of vigor index (VI) of bio-primed cowpea seedling

	High PGPR Efficiency	Moderate PGPR Efficiency	Low PGPR Efficiency
Range of VI of cowpea seedlings	850-974	632-776	414-438.4
Average	828.5	694	426.06
± SD (Standard deviation	142.72	100.88	12.27
Member Isolates	VE-4, VE-20, VE-41,	VE-1, VE-6, VE7, VE-16, VE-37	VE-5, VE-3, VE-12, VE-14, VE-9

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