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PLANT GROWTH PROMOTING AND ANTAGONISTIC PROPERTIES OF *Pseudomonas putida* CRN-09 AGAINST *Macrophomina phaseolina* (TASSI) GOID

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ABSTRACT

A good plant growth promoting (PGPR) isolate, *Pseudomonas putida* CRN-09 was screened from the rhizosphere of *Vigna radiata* on the basis of several attributes like production of phytohormone (indole acetic acid), siderophore, hydrogen cyanide (HCN), and solubilisation of phosphate. Dual culture technique and scanning electron microscopy revealed the inhibitory effect of *P. putida* CRN-09 against *Macrophomina phaseolina in vitro. P. putida* CRN-09 inhibited 65% growth of *M. phaseolina* on dual culture plates. It caused several deformities, such as vacuolation, lysis and hyphal fragmentation in pathogen. Furthermore, this strain also produced extracellular cell wall degrading chitinase and β-1,3 glucanase.Seeds of *V. radiata* var. Pant M5 bacterized with *P. putida* CRN-09 significantly increased seed germination, seedling height, fresh weight and vigour index. Inhibitory effect of *P. putida* against *M. phaseolina* was also evinced by reduction of disease incidence up to 66.66%.This study shows an eco-friendly approach for sustainable agriculture.

KEYWORDS: *Vigna radiata, Macrophomina phaseolina,* PGPR, *Pseudomonas putida* CRN-09

Vigna radiata L. Wilczek, commonly known as green gram, is one of the important legumes which is rich in protein, vitamins and minerals. These biomolecules provide vital energy to animals and human beings. Hence, green gram is one of a good nutritive food for human and animal healthbut its yield is negatively affected by several pathogenic microorganisms including fungi. *Macrophomina phaseolina* is one of such fungi which causes charcoal rot disease in more than 500 plants. It survives for several years in soil through resting structures called slerotia, and infects many crops for a long time (Sinclair, 1984). It also infects green gram and causes economic loss in its production. Several bacteria are associated with rhizosphere of plants; some of them are beneficial for their host by producing bio-chemicals like phytophormones, siderophore, HCN, and solubilise phosphate, zink, potassium etc. Such bacteria are called as plant growth promoting rhizobacteria (PGPR). Therefore, they offer more significant potential against chemical fungicides. Antibiosis, competition and plant growth promotion are specific qualities of these bacteria. They produce chitinase, β-1,3 glucanase, β-1,4 glucanase, cellulase etc. which are known to degrade fungal cell wall and protect their host plant from the pathogen invasion.

However chemical fertilizers and fungicides are used vigoursly by farmers for enhanced crop yield but they impart some deleterious effects on crop quality and soil fertility. To overcome this dilemma, application of PGPR has proven as a good biofertilizer (Dubey et al., 2014). Biofertilizers enhance plant growth and improve yield, protecting them from pathogenic fungi. Moreover, use of several bioinoculants has been suggested by many workers for better crop productivity and biological control of diseases (Sharma *et* al., 2015; Rathi *et al*., 2015).The presence of *Pseudomonas* spp. in the rhizosphere has been recorded to protect host from pathogen attack (Kumar *et al*., 2005). *Pseudomonas* species assist in plant growth promotion and disease suppression.Hence, they are important microorganisms to be used for sustainable agriculture. The present work is focused to study the growth enhancement and disease suppression of *V. radiata* by using *P. putida* CRN-09.

MATERIALS AND METHODS

Isolation of Rhizospheric Bacteria

Healthy green gram plants were gently uprooted from the farmer's field and transported to the laboratory. Soil particles adhered to plant roots were gently brushed off and serially diluted. An aliquot of 100 µl from each dilution was transferred aseptically on the surface of nutrient agar plates. The colonies were purified on separate culture plates after 24 hours of incubation at 37±1°C and pure cultures were stored in slants at 4°C for further use (Dubey and Maheshwari, 2012).

Screening of Plant Growth Promoting Rhizobacteria (PGPR)

Isolated bacteria were primarily screened for their PGP properties, such as indole acetic acid production, solubilisation of inorganic phosphate, production of volatile HCN and secretion of siderophore (Kumar et *al*., 2012).

Quantitative Measurement of IAA

For quantitative measurement of IAA production, absorbance of colour was measured spectrophotometrically (UV-Visible Spectrophotometer, Shimadzu, Japan) at 530 nm. IAA concentration was

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determined in respect of standard curve of IAA (Gordon and Weber, 1951).

Antagonistic Properties

M.phaseolina was procured from the Department of Botany and Microbiology, Gurukul Kangri University, Haridwar (India). Bacterial isolates having PGP properties were demonstrated for their antagonistic behaviour over *M. Phaseolina* by using dual culture technique (Skidmore and Dickinson, 1976). Growth inhibition (%) of *M. phaseolina* was calculated using the formula: 100×(C-T)/C, where C= radial growth of fungal colony in control, T= radial growth of fungal colony in treatment.

Post-interaction Events in *M. phaseolina* Hyphae

Agar discs (5 mm in diameter) was cut from the zone of interaction and prepared for scanning electron microscopy (SEM) following the method of Kumar *et al*. (2011). Prepared samples were transported to Wadia Institute of Himalayan Geology, Dehradun (India), coated with gold and observed for the presence of deformities in fungal hyphae under scanning electron microscope.

Production of Fungal Cell Wall Degrading Enzymes

For examination of the production of chitinase, bacterial isolates were streaked on the agar plates containing chitin minimal medium and incubated at $37\pm1^{\circ}$C for 7 days (Dunne *et al*., 1997). To examine the activity of β-1,3-glucanase the isolates were again streaked on minimal medium having laminarin azure (Sigma–Aldrich Co., USA) as a carbon source and incubated for 7 days at $37\pm1^{\circ}$C (Rangel-Castro *et al*., 2002).

Green House Experiment

P.putida CRN-09 was used for seed bacterization following Weller and Cook (1983). *P.putida* CRN-09 was grown in nutrient broth at 37±1°C for 48 hours. The culture was centrifuged at 7100 g for 15 min at 4°C. Supernatant was discarded and pellets were washed and re-suspended in sterile distilled water to obtain the final bacterial cfu 10^8 cells/ml. The bacterial suspension was mixed with 1% carboxy methyl cellulose (CMC) solution. The certified seeds of *V. radiata* var. Pant M5 surface sterilized with 0.5% sodium hypochlorite (NaOCl) solution for 3 minutes followed by thrice rinsing with sterile distilled water. The seeds were imbibed in the CMC slurry for about 12 hours. These seeds acted as bacterized seeds (T1). Similarly, the seeds coated with un-bacterized slurry served as control.

Mycelial discs (5 mm diameter) of *M. phaseolina* were grown in potato dextrose broth at 28±1°C for seven days. The mycelial culture was centrifuged at 5000 rpm for ten minutes. Supernatant was decanted and pellets were washed with sterile distilled water and re-suspended in distilled water. Bacterized as well as un-bacterized seeds were dried in shade and again inoculated with the fungal suspension and driedin shade (T2). Seeds of all the treatments were sown in four sets of culture tubes having semi solid agar medium and put in seed germination chamber to observe the effect of *P.putida* CRN-09 on seed germination and disease incidence in *V. radiata* under challenged and un-challenged conditions. Seedlings from four replicates were chosen randomly to measure seed germination, seedling length, seedling weight, disease incidence and vigour index.

RESULTS

Isolation of PGPR

Pseudomonas putida CRN-09 was screened and identified as potential isolate (accession No. KY580134) from a total of 74 bacteria isolated from the rhizosphere of healthy *V. radiata* plants. It developed pink colour in reaction mixture having Salkovaki reagent proved the production of IAA by *P.putida* CRN-09. Colony of *P.putida* CRN-09 on Pikovskaya's agar medium also showed the solubilisation of inorganic phosphate. Formation of orange halo zone on chrome-azurol S agar medium confirmed the production of siderophore. HCN production was also observed by the change of filter paper colour from yellow to brown.

Quantitative Measurement of IAA

Developed pink coloured reaction mixture was further evaluated for its quantitative analysis. IAA production gradually increased with time reaching the maximum (88.24 μg/ml) at168 h of inoculation with gradually decline (Figure 1).

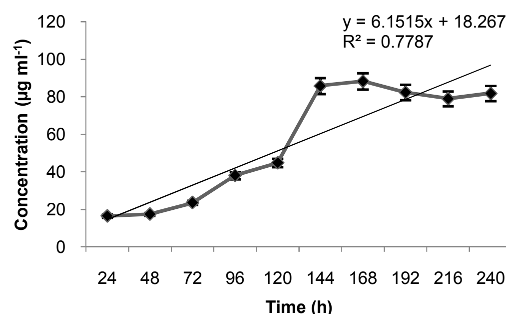

Figure 1: IAA production by *Pseudomonas putida* CRN-09 at different intervals

Antagonism Assay

In dual culture bioassay *P. putida* CRN-09 inhibited the growth of *M. phaseolina* by 65%. The fungal hyphae revealed the loss of structural integrity, lysis, fragmentation and perforations of hyphae, and sclerotial degradation (Figure2).

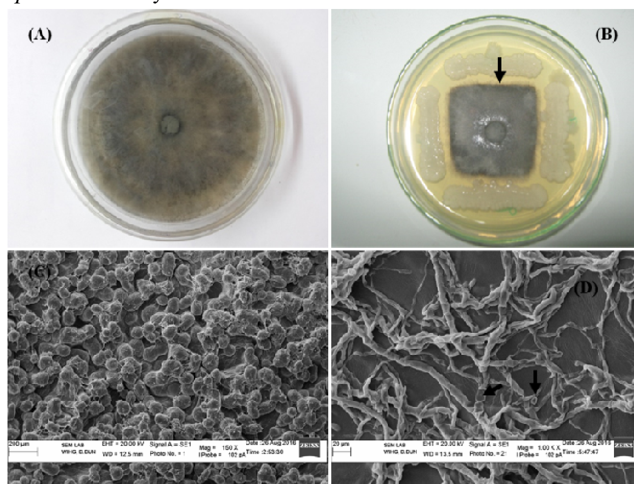

Figure 2: Antagonistic effect of *Pseudomonas putida* CRN-09 against *M. phaseolina*; A- control, B- growth of *P. putida* CRN-09 and *M. phaseolina* on dual culture showing zone of inhibition, C- Scanning electron micrograph of sclerotia in control, D- hyphal deformities caused by *P. putida* CRN-09

Production of Fungal Cell Wall Degrading Enzymes

Chitin and laminarin were utilised by *P.putida* CRN-09 on different agar plates having chitin and laminarin as source of sole carbon. Formation of clear zones around the bacterial colony on chitin and laminarin containing media confirmed the production of fungal cell wall degrading enzymes, chitinase and β-1,3 glucanase (Figure 3).

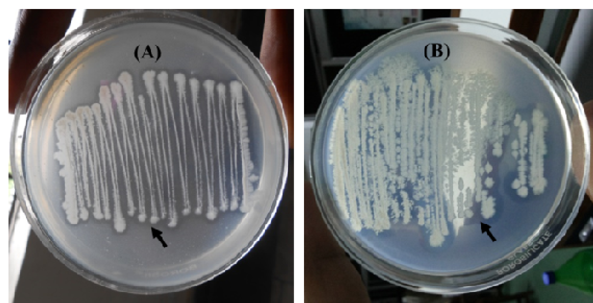

Figure 3: Production of chitinase (A) and β-1,3 glucanase by *Pseudomonas putida* CRN-09

Seed Germination and Plant Growth Parameters

P. putida CRN-09 was tested for its ability to promote plant growth and ability to protect the host against infection by *M. phaseolina*. *P. putida* CRN-09 with or without fungus promoted seedling growth (Figure 4) and increased vigour index (Table 1). No seed germination was observed upto 3rd day after sowing in T3. On 4th day only 25% seed germination was recorded. *P. putida* CRN-09 coated seeds showed enhanced seedling growth of *V. radiata* with reduced disease severity. Pre-emergence of seedlings was observed in the seeds bacterized with *P.putida* CRN-09. Bacterial treatment also reduced the infection of *M. phaseolina* as evinced by reduction in disease incidence (Table 1) .100% seed germination was recorded in T1, wherein disease incidence was decreased by 66.66 % in comparison to T3, and 60.42% more seeds were protected from the pathogen. The seedlings (T2) were protected and strengthened by *P. putida* CRN-09, showing 88.09% more vigour index than infected seeds.The seeds coated with *P. putida* CRN-09 also showed better seedling weight than the control. Seeds coated with *P. putida* CRN-09 challenge with *M. phaseolina* showed comparatively more seedling length, germination and fresh weight than the seeds challenged with *M. phaseolina* alone. Hence, *P. putida* CRN-09 showed the protective effect on host plant (*V. radiata*) against the infection of *M. phaseolina.*

Table 1: Effect of *Pseudomonas putida* CRN-09 on disease suppression and growth of *V. radiata*

Treatment	Germination %	Disease incidence (%)	Seedling length (cm)	Seedling weight (g)	Vigour index
Control	79.17	2.08	15.93	0.43	1261.67
T-1	100.00**	0.00ns	22.43**	0.39**	2242.50**
T-2	85.42**	16.67**	24.05**	0.54**	2053.96**
T-3	25.00**	83.33**	9.80**	0.08**	244.58**
CD at 1%	12.10	13.61	1.75	0.07	240.57
CD at 5%	22.21	25.00	3.22	0.13	441.81
SEM±	14.19	17.00	2.83	0.08	393.81

Note: CD = Critical Differences, SEM = Standard error of means;Values are mean of four randomly selected plants by ANOVA from each group; ns – non-significant, ** – significant at 5%, * – significant at 1% level of LSD compared to control; Control (un-bacterized seeds).

Figure 4: Effect of *P. putida* CRN-09 on the growth of *V. radiata*; T1- seeds coated with *P. putida* CRN-09, T2- Coated seeds with *P. putida* CRN-09 challenged by *M. phaseolina.* T3- Challenged seeds with *M. phaseolina.* Control- Un-bacterized seeds

DISCUSSION

Pseudomonas belongs tothe group aerobic, Gram-negative, non-spore forming, Gamma proteo bacteria. Several *Pseudomonas* species reside in plant rhizosphere, among which some are good plant growth promoting rhizobacteria viz, *P. fluorescens, P. putida, P. aeruginosa* etc. PGPR scolonize the plant root and enhance their growth. The progressive effects of *Pseudomonas* sp. were reported in a variety of hosts like *Cajanus cajan, Trigonella foenum, Zea mays, Lycopersicon esculentum* and *Oryza sativa* (Kumar *et al*., 2011; Sharma *et al*., 2015).Gupta *et al*. (2002) reported the growth enhancement of peanut by *Pseudomonas* sp.

In this study *Pseudomonas putida* CRN-09 was found as a good PGPR which solubilised inorganic phosphate and produced IAA, HCN and siderophore.

Many species of *Pseudomonas* are known as plant growth-promoting rhizobacteria. Moreover, they also have inhibitory properties against a number of phytopathogenic fungi, and are often applied as biocontrol agents. Gupta *et al.* (2002) reported 74% growth inhibition of *M. phaseolina* by fluorescent *Pseudomonas.* In the present study, 65% growth inhibition of *M. phaseolina* by *P. putida* CRN-09 was recorded. Negil *et al*. (2005) also emphasized the role of *Pseudomonas* spp. as biocontrol agent against *Fusarium, Sclerotium, Erwinia, Macrophomina, Rhizoctonia* and *Pythium*. *P. putida* CRN-09 hindered the growth of *M. phaseolina* by hyphal and sclerotiallysis as confirmed by SEM analysis. The antagonist-mediated hyphal lysis would have been due to competition for nutrients, production of cell wall lytic enzymes as well as secondary metabolites like volatile HCN, siderophores and antibiotics. Morphological deformities of fungal hyphae viz., fragmentation, shrinkage, cytoplasm leakage, vacuolation, lysis, breakage, sclerotial degradation and swelling etc.by *Pseudomonas* spp. have also been reported in earlier studies (Gupta *et al.,* 2002; Kumar *et al*., 2011).

P. putida CRN-09 showed an orange-yellow coloured zone on iron-deficient medium that evinced the production of siderophore. Such results have also been observed by many other workers (Kumar et *al*., 2012) .*P.putida* CRN-09 coated seeds showed better plant growth and suppression of charcoal rot disease in *V. radiata* seedlings. Hence, this evidence proved *P. putida* CRN-09 as a potential PGPR and biocontrol agent against *M. phaseolina.* Similar results of *Pseudomonas* for *in vivo* plant growth promotion have been reported by Khare and Arora (2010). Root colonization property of *Pseudomonas* and increased seedling emergence and its establishment in the rhizosphere of peanut was studied earlier by Gupta *et al*. (2002). Enhancement in seedling height, seedling

weight, vigour index, seed germination, early seed germination would have been due to IAA production and phosphate solubilisation. Reduction in disease incidence may be due to the inhibitory effects of HCN, chitinase, beta-1,3glucanase and antibiotics (Gupta et *al*., 2002) .Conclusively,*P. putida* CRN-09 bears plant growth promoting properties of *V. radiata* and antagonistic properties against *M. phaseolina.* Hence, *P. putida* CRN-09 may be recommended for useas a good PGPR and biocontrol agent against *M. phaseolina.*

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