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Elicitation of antioxidants and phenolics in *Vigna radiata* L. Wilczek by twin PGP bacteria against infection of *Macrophomina phaseolina* Tassi (Goid.)

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Abstract

Plant growth promoting *Pseudomonas putida* CRN-09 and *Bacillus subtilis*CRN-16 screened from the rhizosphere of *Vigna radiata* were used to determine antioxidant activity and total phenolic content in germinated seedlings. Seeds of *V. radiata* Pant M5 bacterized with *P. putida* CRN-09 and *B. subtilis* CRN-16 individually as well as consortium showed better effects on *V. radiata* seedlings. Maximum antioxidant level (4.20 mM/g) was determined in consortium-treated seedlings at day 6 of treatment. Consortium of both the isolates also enhanced the maximum level of total phenolic contents (1.20 µg/g) at 6th day. Antagonistic effect of seedling extracts of different treatments was evaluated against *M. phaseolina*. Maximum inhibition area (5 mm) around extract of consortium on first day and 12 mm at 5thday were recorded.

Keywords: *Pseudomonas putida* CRN-09, *Bacillus subtilis* CRN-16, *Macrophomina phaseolina*, antioxidant activity, total phenolic content.

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INTRODUCTION

Mung bean (*Vigna radiata* L. Wilczek) is an important legume of the family Fabaceae which has high source of protein and other nutrients. But its production gets declined due to infection by many pathogens. Adoption of biocontrol mechanism has been given much attention in recent years. Biological control by using PGPR is a better alternative tool to control the infection of several pathogens and to enhance the growth and yield of plant. Several strategies are adopted by PGPR against pathogenic fungi *viz.*, production of siderophore, hydrogen cyanide, antibiotics, chitinase, glucanase, cellulase, etc. (Kumar *et al.*, 2012). Some PGPR induce their host plant cells to synthesis many types of defence proteins which protect plant through the accumulation of phenolic contents, lignin and antioxidants (Singh *et al.*, 2013). Generally, they protect the plants from biotic or/and abiotic stresses. Pentose phosphate, shikimate and phenyl propanoid pathways are main pathways employed for the production of phenolic compounds. Germinating seeds survive by increasing their defence system through phenolic biosynthesis. Earlier experiments have shown a low phenolic and antioxidant activity in dry seeds of *V. radiata* (Randhir *et al.*, 2003). Therefore, the present work was under taken to study *P. putida* CRN-09 and *B. subtilis* CRN-16 mediated alleviation of phenolic contents and antioxidant activity against *Macrophomina phaseolina* infection.

MATERIALS AND METHODS

Screening of potential PGPR

Rhizosphric soil from healthy *V. radiata* plants was collected for isolation of rhisospheric bacteria following serial dilution method (Dubey and Maheshwari, 2012). The rhizobacteria were then screened on the basis of their plant growth promoting attributes like auxin, hydrogen cyanide and siderophore production, phosphate solubilisation (Kumar *et al.*, 2012), and *in vitro* antagonistic effect against *M. phaseolina* (Skidmore and Dickinson, 1976).

Seed bacterization and green house experiment

Both the bacterial isolates were used for seed bacterization individually as well as in their combination following the method of Weller and Cook (1983). Seeds of *V. radiata* Pant M5 were first surface sterilised with 0.5% sodium hypochlorite solution followed by rinsing with sterile distilled water. Cell pellets formed after centrifugation (at 5000 rpm)of 48 hours old cultures of *P. putida* CRN-09 and *B. subtilis* CRN-16 were mixed with 1% carboxy methyl cellulose (CMC) solution to form three different consortia, such as T1 (*P. putida* CRN-09 only), T2 (*B. subtilis* CRN-16), and T3 (consortium of *P. putida* CRN-09 and *B. subtilis* CRN-16). Seeds coated with un-bacterized slurry erved as control.

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Seeds of all the four treatments were sown in four sets of culture tubes containing semisolid agar medium. The tubes were put in the chamber for seed germination up to 6 days to examine the effect of *P. putida* CRN-09 and *B. subtilis* CRN-16 on antioxidant activity, total phenolic contents and effect on the growth of *M. phaseolina*.

Antioxidant activity

The antioxidant activity of *V. radiata* seedlings was measured following the method of Prieto *et al.* (1999). Seedling (1g) were homogenised in 28 mM sodium phosphate buffer. The homogenate was centrifuged at 4000 rpm for 15 minutes. The supernatant was used as the source of enzyme. 3 ml supernatant was added in 3 ml of reagent solution in a glass tube. The reagent solution contained 28 mM sodium phosphate buffer (1ml), 4 mM ammonium molybdate (1ml), and 0.6 M sulphuric acid (10 ml). The reaction was incubated at 90 °C for 90 minutes in a water bath. After incubation the absorbance of the reaction was recorded at 695 nm using a spectrophotometer (UV-vis spectrophotometer, Shimadzu, Japan). The optical density of the reaction mixture was compared with the standard calibration curve of ascorbic acid.

Total phenolics analysis

Seedlings (250 mg) of *V. radiata* were homogenised with 80% methanol (2.5 ml). The homogenate was centrifuged at 4000 rpm for 5 minutes to collect supernatant. 1N-Folin-ciocalteua reagent (250 µl) was added in a glass tube containing distilled water (5 ml) and supernatant (1 ml). The reaction mixture was kept at room temperature under dark for 10 minutes. After incubation the absorbance was determined under a spectrophotometer at 725 nm. The absorbance was compared with the standard calibration curve of catechol solution (Saikia and Upadhyaya, 2011).

Antagonistic effect against *M. phaseolina*

Inhibitory property of *V. radiata* seedling extract was studied against *M. phaseolina*. Seedlings (25 mg) of *V. radiata* from each treatment were collected up to 5 days. The seedlings were suspended in sterile distilled water and kept at 4 °C. The samples were homogenised and centrifuged at 5000 rpm for about 15 minutes. Supernatant was used as the source of inhibitory agent. *M. phaseolina* was procured from the culture collection unit of the Department of Botany and Microbiology, Gurukul Kangri Vishwavidyalaya, Haridwar (India) and was grown in potato dextrose agar (PDA). Four holes were made with a borer (5 mm) at 2 cm apart from the centre. Supernatant from each of the treatment of different days were loaded in each hole on different PDA plate. The plates were incubated at 28±1 °C for 5 days and zone of inhibition (mm)of each treatmentwas measured (Skidmore and Dikinson, 1976).

The fungal colonies were picked up from the zone of interaction with the help of borer. These discs (5 mm diameter) were then prepared for their scanning electron microscopy (SEM)(Kumar *et al.*, 2011). Samples were sent to Wadia Institute of Himalayan Geology, Dehradun (India) for the analysis of inhibition under SEM.

RESULTS

Screening of potential PGPR

Pseudomonas putida CRN-09 (KY580134) and *Bacillus subtilis* CRN-16 (KY580132) were screened from the rhizospheric isolates of healthy *V. radiata* on the basis of their plant growth promoting attributes such as production of auxin, hydrogen cyanide and siderophore, and phosphate solubilisation. Both the bacterial isolates were found as good antagonists against *M. phaseolina*.

Antioxidant activity

P. putida CRN-09 and *B. subtilis* CRN-16 individually as well as in the form of consortium enhanced antioxidant activity. In a six day study combinational effect of both the isolates was observed as highest (4.20 $mMmg^{-1}$) at fifth day of experiment. T3 showed the highest level of antioxidant activity in each day except day 1 in comparison of individual treatment and control. A reduction in antioxidant activity from 3.44 to 1.44 mM/mg of fresh wt of seedlings in control seedlings was observed (Fig. 1).

Total phenolics analysis

Total phenolic content was measured in the seedlings of *V. radiata* for five days after seed germination. Seeds

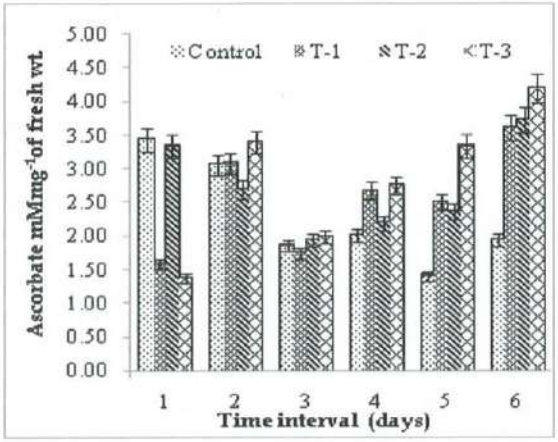

Fig. 1. Effect of bacterial treatments on the antioxidant activity of seedlings.

were treated with the inoculum of *P. putida* CRN-09 and *B. subtilis* CRN-16 alone (T1 and T2) and in their combination (T3) as compared to control. An enhanced level of total phenolic content was measured in T3 treated seeds up to day 6.The highest level (1.20 µg/g)of total phenolic content was recorded at day 6 in T3. *P. putida* CRN-09 showed a steady increase in

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catechol production from 0.38 to1.08 µg/g and *B. subtilis* CRN-16 was enhanced catechol production from 0.61 to 0.93 µgg^{-1} (Fig. 2).

Inhibitory effect against *M. phaseolina*

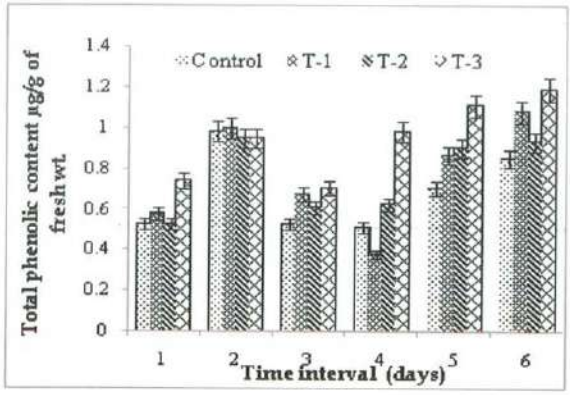

Fig. 2. Effect of bacterial treatments on total phenolic content of seedlings.

Seedlings treated with *P. putida* CRN-09 and *B. subtilis* CRN-16 individually as well as in their combination imparted antagonistic effect on growth of *M. phaseolina*. No inhibition was recorded in the control against *M. phaseolina*up to 3 days after germination. At the day 4th and 5th days seedling extract of control seeds showed non-significant inhibition. Seeds treated with *P. putida* CRN-09 and *B. subtilis* CRN-16 individually showed increasing inhibitory effect against *M. phaseolina* with time. Both the isolates in combination showed cumulative effect on growth inhibition of *M. phaseolina*. T3 resulted in the maximum zone of inhibition (5 mm) at first day (Fig. 3). Scanning electron micrograph showed the deformities viz., fragmentation,

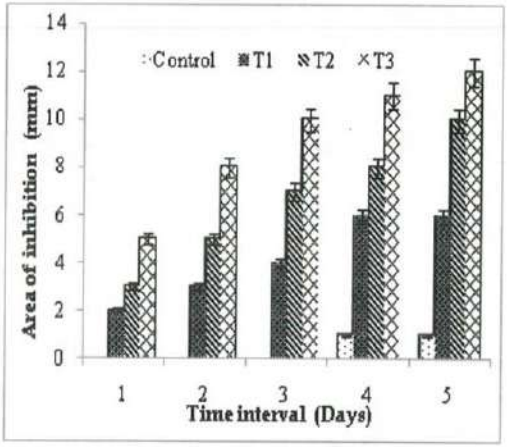

Fig. 3. Inhibitory effect of seedling extract on the growth of *M. phaseolina*.

lysis, sclerotial degradation, formed in the hyphae of *M. phaseolina* by T3 seedling extract (Fig. 4).

DISCUSSION

In this study *P. putida* CRN-09 and *B. subtilis* CRN-16 were used as PGPR for seed bacterization purpose.

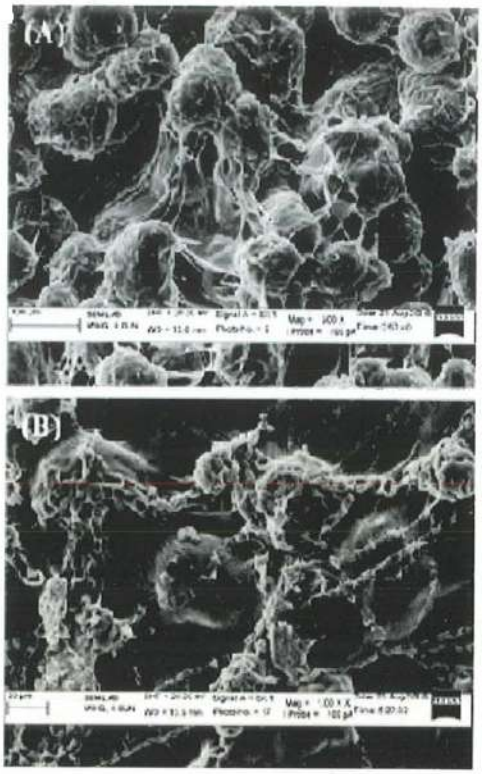

Fig. 4. Healthy mycelia and sclerotia of *M.phaseolina* (A), inhibitory effect of seedling extract on *M. phaseolina* hyphae and sclerotial loss of viability (B).

P. putida and *B. subtilis* have been reported as good PGPR for several crops (Yao *et al.*, 2010; Yu *et al.*, 2011). Good PGPR produce PGP compounds to solubilize phosphate and to produce organic acids, phytohormone, siderophore and ACC deaminase in rhizosphere to impart sufficient availability of nutrients for plant growth enhancement. The PGPR also produce metabolites like hydrogen cyanide, chitinase, glucanase, cellulase to inhibit the growth of various phytopathogens (Kumar *et al.*, 2012). Production of these primary and secondary metabolites in the vicinity of plant root benefit the plant by inhibited the growth of their pathogens. Moreover, PGPR induce the physiology of host plant for synthesis of several defence enzymes, phenolic contents, antioxidants and antimicrobial substances (Randhir *et al.*, 2003).

Antioxidants are the molecules which limit oxidation reactions and production of free radicals that are responsible for damage cells. They cause oxidative damage to biomolecules, which causes degenerative diseases. Several antioxidants *viz.*, thiols and ascorbate are known to stop such reactions and protect from cell lysis. In this study the higher level (4.20 mM/mg) of antioxidant was recorded after treatment with PGPR. Islam *et al.* (2014) also reported *P. aeruginosa* as an ideal bio-inoculant for the protection of wheat from abiotic oxidative stress by triggering antioxidant defence system. An increasing level of antioxidant activity was also reported in *V. radiata*

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sprouts after the treatment of natural food grade elicitors to improve its nutritional and health-relevant functional value (Randhir *et al.*, 2003).

Production of phenolic compounds by host is also a strategy of defence system. In the present study an enhanced level of total phenolic content has also been found which relates with the products of protective pathways like shikimate and phenylpropanoid (Michalak, 2006).The total phenolic content of mung bean seedlings was estimated continuously for 5 days. Bacterial treatment stimulated the total phenolic content in mung bean seedlings. Combined effect of both the isolates, *P. putida* CRN-09 and *B. subtilis* CRN-16, enhanced the level of total phenolic contents up to 1.20 µg/g in *V. radiata*. Most of the phenolic components have antimicrobial activity (Michalak, 2006).Increasing concentration of soluble phenolics in host plant causes endurance of cell wall preventing the cells from pathogen invasion (Underwood, 2001).Enhanced level of phenolics as well as antioxidant activity correlates with the antimicrobial activity of host metabolites against *M. phaseolina*. The maximum antifungal activity was recorded after the treatment of *P. putida* CRN-09 and *B. subtilis* CRN-16 as consortia followed by *B. subtilis* CRN-16 and *P. putida* CRN-09 alone. The inhibitory effect of seedling extract was observed against *M. phaseolinain vitro*. Consortium of both the bacterial isolates mostly affected the growth of *M. phaseolina*. The highest phenolic contents were also measured in the consortium treated seedlings. The enhancement of phenolic content is directly related with the development of lignin and several other antimicrobial products (Michalak, 2006). *M. phaseolina* causes charcoal rot disease in *V. radiate* resulting in high loss in yield. In this study extracts of treated seedlings with *P. putida* CRN-09 and *B. subtilis* CRN-16 individually as well as in the form of consortia significantly inhibited fungal hyphae, causing lysis as it was confirmed by scanning electron microscopy. It may be concluded that seeds of *V. radiata* treated with consortia of *P. putida* CRN-09 and *B. subtilis* CRN-16 protected the host from infection by *M. phaseolina* through enhanced level of antioxidants and phenolic contents.

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