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Biocontrol features in an indigenous bacterial strain isolated from agricultural soil of Gujarat, India

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Abstract

The present investigation was carried out to test the biocontrol potential of a phosphate solubilizing bacterial strain, LK11 isolated from mungbean rhizosphere, in Gujarat, India. This strain besides solubilizing the insoluble P also demonstrated inhibition of *Sclerotium rolfsii* growth in agar plate and produced a volatile compound, HCN. During *in vitro* studies LK11 inhibited sclerotia germination by 40%. The most important contributing factor towards increased mungbean growth by reducing fungal attack was the enhanced production of antifungal compounds like PAL (47 mM/ml/mg of tissue), phenolics (90.2 µg/ml/mg) and flavonoids (184.2 mg/ml/g) which is comparable to earlier reports available. Testing the efficiency of this strain in consortium culture alongwith some other PGPR strains and biocontrol microbes at multilocational fields is in offing.

Keywords: PSB, Biocontrol, PAL, phenolics, *S. rolfsii*.

1. Introduction

Biofertilizers are organisms that enrich the nutrient quality of soil. A group of bacterial population closely associated with rhizosphere region of soil is known as plant growth promoting rhizobacteria (PGPR) and is mainly involved in enhancing plant growth by several activities. Such plant growth promotion may be achieved via one or more direct/indirect mechanisms such as phosphate solubilization, nitrogen fixation, biocontrol and production of plant growth regulators etc.

Phosphorus (P) is second essential macronutrient next to nitrogen which is most commonly limiting the growth of crops and is applied to soil in the form of phosphatic fertilizers. Plants acquire P from soil solution as phosphate anions. However, phosphate anions are extremely reactive and may be immobilized through precipitation with cations such as Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+}, depending on the pH of soil. As a result, the amount available to plants is usually a small proportion of this total P (Rodriguez *et al*., 1999; Anamika *et al*., 2007). Phosphate solubilizing bacteria (PSB) can serve as an alternative that can be efficiently developed as biofertilizers, as they support plant growth by solubilizing organic and inorganic phosphates. Several Phosphate solublizing bacteria such as *Pseudomonas putida*, *Pantoea agglomerans, Azotobacter* spp., *Bacillus subtilis*, *Rhizobium* spp., *P. fluorescens*, *P. cepacia* and *Aeromonas vaga* have been studied extensively (Srivastava and Shalini, 2009; Jha *et al*., 2011).

Pathogenic microorganisms affecting plant health are a major and chronic threat to food production and ecosystem stability worldwide. Mung bean (*Vigna radiata*), an important legume, is highly susceptible to the attack of a fungal pathogen, *Sclerotium rolfsii*. It primarily attacks host stems. The first signs of infection, though usually undetectable, are dark-brown lesions on the stem at or just beneath the soil level (Yaqub and Shahzad, 2011). Also, there are number of fastidious diseases for which chemical solutions are few, ineffective, or nonexistent. Biological control is thus being considered as an alternative or a supplemental way of reducing the use of chemicals in agriculture (Compant *et al*., 2005). PGPR strains have been studied for decades for their plant growth-promoting effects through effective suppression of soil borne plant diseases through siderophore-mediated competition for iron, antibiosis, production of lytic enzymes, and induced systemic resistance (ISR) (Mamaghani *et al*., 2009). ISR is mediated through production of several defense compounds in plants such as phenolics and PAL (Phenylalanine Ammonia Lyase) and other secondary metabolites. There has been a large body of literature describing potential uses of plant associated bacteria as agents stimulating plant growth and managing soil and plant health, but an efficient strain in terms of both fertilization and biocontrol potential has been lacking. Further very few reports are available in the direction of PSB inoculant application for the growth of mung bean (Jha *et al*., 2011) and protecting it from the attack of devastating pathogen like *S. rolfsii*. With the aim of developing a phosphate inoculant that would decrease the severity of pathogen attack to mung bean and would promote the overall plant growth, the present investigation was carried out. The bacterial strain has been isolated from the rhizosphere of Mung bean cultivated in agricultural fields located in nearby areas of Vadodara, Gujarat, India. The objective of this study was to evaluate the effect of PSB strain as biocontrol agent for mung bean plants.

2. Materials and methods

Phosphate solubilizing bacterial strains were isolated from soil samples (collected from different mung bean fields near Vadodara, Gujarat, India) in a selective media PAMR (Pikovskaya's agar containing 1% Methyl Red). Out of 16 bacterial strains, the strain LK11 showing good solubilization zone was picked up and purified further and stored on PAMR slants at 4°C for further use. It was point inoculated on PAMR plates and incubated at 30±2ºC for 5 days. The size of the halo/clear zones around the colonies showing phosphate solubilization was noted. Solubilization efficiency was calculated according to Nguyen *et al*. (1992) as given below:

$$\text{Solubilization efficiency (E)} = \frac{\text{Diameter of solubilization zone (S)}}{\text{Diameter of the colony}} \times 100$$

Soluble P was also estimated in 100 ml NBRIP broth inoculated with LK11 according to the method of Nautiyal *et al*. (1999).

Biocontrol potential of LK11 was assessed by both *in vitro* and *in planta* studies against the pathogen, *S.rolfsii* in mung bean. *In vitro* antifungal activity was demonstrated through direct inhibition of fungal growth in agar plate, HCN production and inhibition of fungal spore germination by LK11. Dual culture Plate Technique (Ganesan and Gnanamnickam, 1987) was followed where a sclerotium of S. *rolfsii* was placed in the center of a fresh PDA plate. Mid log phase bacterial culture was point inoculated in PDA plate at a distance of 3 cm from the center. A control plate was also prepared which contained the sclerotium of test pathogen only. The plates were incubated at 30±2ºC and were observed after 5 days.

Test for HCN production was carried out by spectrophotometric and inverted plate methods. In spectrophotometric method 18 hrs old bacterial culture was centrifuged at 7000 rpm for 15 min at 4°C. The supernatant was passed through 0.2µ filter; then 2 ml O-phosphoric acid was added and observed for development of pink color. The absorbance was read at 530 nm. Inverted plate technique was performed following the standard method (Fernando *et al*., 2005). The bacterial culture was streaked on NA plate and inverted over the PDA plate containing sclerotium in the centre and then incubated at 30±2ºC for 5 days.

In vitro sclerotia germination was studied by placing a drop of mid log phase bacterial culture on sclerotium of *S. rolfsii* and mixed well. Sclerotia mixed in sterile distilled water only served as control. The slides were placed in moist chambered Petri plates and incubated at 25±2°C for 15 days (Srivastava and Shalini. 2009). All experiments were conducted in triplicate. Sclerotia germination was observed under binocular microscope.

The biocontrol activity of LK11 against *S. rolfsii* was checked in mung bean plants. For this investigation, mung seeds were surface sterilized (in 0.1% $HgCl_2$ for 3 min), coated with 1% CMC (Carboxy Methyl Cellulose) and fresh bacterial culture filtrate and kept for air drying. Then the seeds were challenged with fungal mycelia and kept on 3% water agar tubes for germination at ambient temperature (20-26°C). Seeds coated with CMC and fungal mycelia served as control. Observations were recorded after 7 days.

Plant defense mechanism produces some enzymes, phytoalexins and phenolic compounds during fungal attack. Therefore, the assay of 2 important compounds viz., phenolics and flavonoids (antioxidant) and an enzyme PAL was performed. For PAL assay 0.5 g fresh leaf tissues of mungbean plant were homogenized in 2 ml of 0.1 M sodium phosphate buffer, pH 7.0 at 4ºC. The homogenate was centrifuged for 20 min at 12,000 rpm. Supernatant served as a crude protein extract and was used for the estimation of PAL activity (Anand *et al*., 2007). PAL activity was determined as the rate of

conversion of L-phenylalanine to trans-Cinnamic acid at 290 nm as described by Dickerson *et al*. (1984) with minor modifcations. The assay mixture consisted of 200 µl crude protein extract and 1800 µl of 6 µM l-phenylalanine in 1000 µl of 500 mM Tris-HCl buffer (pH 8·5). The mixture was incubated at 37°C for 1 h and absorbance was measured at 290 nm. Enzyme activity was expressed on a fresh weight basis as nmol trans-cinnamic acid min^{-1} mg^{-1}protein (nMol min^{-1} mg^{-1}). Phenolics estimation was done in 1 g root samples that were homogenized in 10 ml of 80% methanol and agitated for 15 min at 70 ºC (Zieslin and Ben–Zaken, 1993). One ml of the methanolic extract was added to 5 ml of distilled water (d.w) and 250 µl of Folin-Ciocalteau reagent (1 N) and the solution was kept at 25 ºC. The absorbance of the developed blue color was measured using a spectrophotometer at 725 nm. Phenol crystal was used as the standard. The amount of phenolics was expressed as µg Phenol crystal mg^{-1}protein. Total flavonoid content was measured by aluminium chloride colorimetric assay (Marinova *et al*., 2005). 1 ml of methanolic extract (as stated above) was added to 10 ml volumetric flask containing 4 ml of d.w. To the above mixture, 0.3 ml of 5% $NaNO_2$ was added. After 5 minutes, 0.3 ml of 10% $AlCl_3$ was added. At 6^{th} min, 2 ml of 1 M NaOH was added and the total volume was made up to 10 ml with d.w. The solution was mixed well and the absorbance was measured against reagent blank at 510 nm. Standard graph was prepared with different concentration of gallic acid and the flavonoid content was expressed as mg/ml/g.

3. Results and discussion

LK11 is a Gram negative, cocci that forms white coloured smooth colony in agar plate. It gave a solubilization zone on PAMR plate (solubilization efficiency-200%). When tested in NBRIP medium it was able to release 25 ppm soluble P.

The results of experiments for testing the biological control ability of LK11 were satisfying and to our surprise, it could fastidiously suppress the growth of *S. rolfsii* up to 38.0% in the dual culture plate after 5 days of incubation. It indicates that the bacteria caused a significant inhibition. Ingle *et al*. (2010) recorded maximum inhibition of *S. rolfsii* (62.6%) by PGPR isolate CORb-9. The bacteria utilize several mechanisms for inhibiting fungal growth. Besides fungal inhibition in direct contact, LK11 also stopped sclerotium germination altogether in comparison to control (5.2 cm mycelia growth) in inverted plate method. The reason might be the production of HCN (a volatile compound) which was confirmed by development of pink color on reaction with o-phosphoric acid. Hayat *et al*. (2010) also reciprocated the same opinion about involvement of HCN in antifungal mechanisms. During *in vitro* studies it was found that in presence of LK11, the *S. rolfsii* spores exhibited 2 different patterns of germination i.e. hyphal and eruptive, which are characteristics of the sclerotial germination (Figure 1). Eruptive type of germination dominated the hyphal type, whereas, one sclerotium just broke without production of any mycelia. It is inferred from the results that there was remarkable 40% inhibition of sclerotia germination even after 15 days of incubation (Table 1).

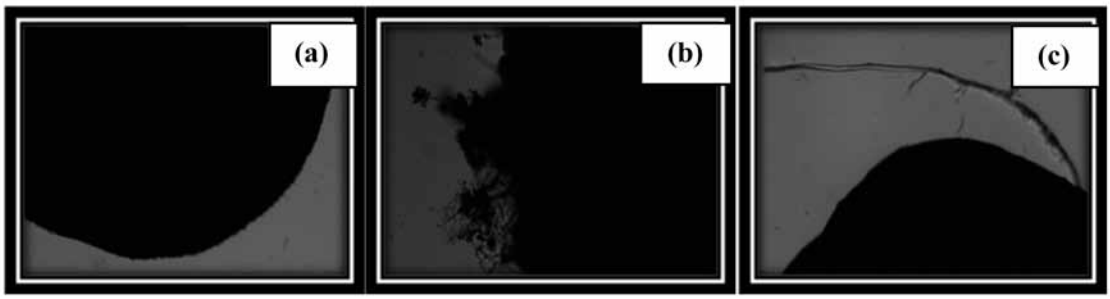

Figure 1. Sclerotia germination in presence of LK11 bacterial strain (a) Control sclerotia (b) eruptive germination and (c) hyphal germination of sclerotia.

Table 1. *In vitro* sclerotia germination in presence of LK11 bacterial strain

Sclerotium Germination	Incubation time		
	5 days	10 days	15 days
Hyphal	0	1	1
Eruptive	1	3	4
Broken sclerotia	0	1	1
Total	1/10	5/10	6/10
% Germination	10	50	60

In planta studies in agar tubes showed magnificent results and limited the fungal invasion in mung bean plants. All the LK11 treated plants were able to germinate successfully in comparison to control where seeds could not germinate at all. After 7 days of growth LK11 treated plants attained a height of 14 cm with root length of 7cm at 2 leaf stage. Though the plant resisted pathogen attack but few brown patches could be observed on shoot. Highest root colonization by *S. rolfsii* was observed in mung bean plants growing in soil artificially infested with sclerotia of *S. rolfsii* (Yaqub and Shahzad, 2011). Several changes are induced in plants during pathogen invasion which are manifested morphologically and physiologically. The treatment of mung bean with LK11 led to enhanced production of antifungal compounds that boosted up the plant defense against *S. rolfsii.* PAL is the first enzyme in phenylpropanoid metabolism and is involved in the synthesis of plant defense molecules phytoalexins and phenolics. In the present investigation, high induction of PAL activity (Figure 2) was noted in LK11 treated plants (47 nM/ml/mg of tissue) in comparsion to control (5 nM/ml/mg of tissue). Ramamoorthy *et al*. (2002) noted maximum induction of PAL activity in *P. fluorescens* treated tomato roots after 4 days of inoculation with pathogen, *F. oxysporum* (45 nM/ml/mg of tissue). Similarly, elevated levels of phenolics in plants are

also observed during fungal attack. Accumulation of 90.2 µg/ml/mg phenolics was observed in mung bean plants inoculated with LK11 strain in comparsion to control (15 µg/ml/mg). Ramamoorthy *et al*. (2002) noted accumulation of 150 µg phenolics/mg of tissue in tomato roots treated with *P. fluorescens* and pathogen *F. oxysporum* after 5 days. Flavonoids are the antioxidant compounds that indirectly act as antifungal agents. They are naturally present in low concentration in plants, but during pathogen invasion the elevated levels are seen. Plants treated with LK11 showed 2 folds increase in flavonoids concentration (184.2 mg/ml/g) when compared with control (90.3 mg/ml/g). It can be inferred that PSB helped the plant to produce flavonoids in sufficient amount to resist the pathogen attack.

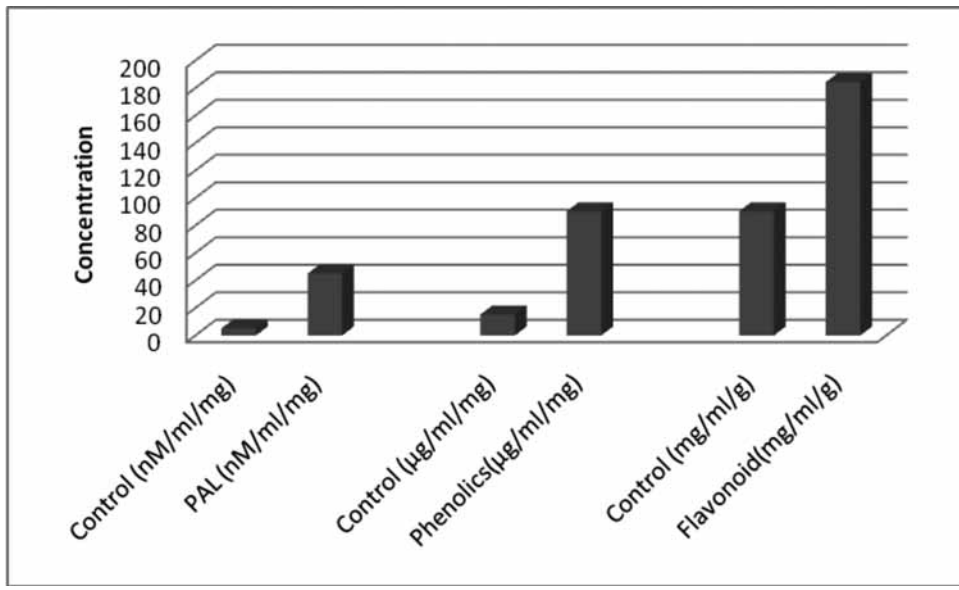

Figure 2. Production of antifungal compounds by mung bean during infection with *S. rolfsii* in presence of LK11.

4. Conclusions

The present investigation has revealed that the phosphate solubilising bacterial strain, LK11 has the potential to be applied in fields as a biocontrol agent for mungbean against its fungal pathogen *S. rolfsii*. Further, detail investigations of this strain are needed to present more conclusive results.

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