Enhancing resistance of rice bean to diseases by seed treatment with *Pseudomonas flourescens* and *Bacillus species*

Ankush Gupta*, Deepa Khulbe and P. Srinivas

Department of Seed Science and Technology, G.B.P.U.A & T, Pantnagar-263 145, India.

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**ABSTRACT**

Efficacy of seed bacterization with six rhizobacterial isolates was assessed in laboratory conditions to evaluate their resistance inducing ability in rice bean. *Pseudomonas flourescens* isolates B5, B6, B8, B9, and *Bacillus* species isolate B18, were found effective in reducing the percentage of diseased seedling but showed the plant growth promotion. Increased levels of catalase, phenylalanine ammonia lyase (PAL) and poly phenol oxidase (PPO) were observed in seedlings indicating higher levels of ISR. Maximum induction of defense enzymes was observed with isolate B5 followed by B6 and B18. The utilization of indigenous PGPR strains as inducers of plant defense response may offer a practical solution for eco-friendly management of plant diseases.

**Key words:** ISR- induced systemic resistance, PPO- poly phenol oxidase, PAL- phenylalanine ammonia lyase, PGPR- plant growth promoting rhizobacteria.

**INTRODUCTION**

Rice bean (*Vigna umbellata* (Thunb.) Ohwi & Ohashi) is an underutilized pulse crop having wide utility with good yield potential and is used as food, fodder, green manure and a cover crop. It is largely cultivated under rainfed conditions, and is generally prone to attack of several seed and soil borne diseases. Therefore, protecting the rice bean crop and seed against seed and soil borne pathogens, at initial level becomes inevitable from production point of view and it can be achieved through seed bacterization. Seed priming with biocontrol agents especially plant growth promoting rhizobacteria (PGPR) are known not only to protect the crop plants at the seed and seedling stage but also stimulated their growth. In addition to these benefits, their capability to induce systemically acquirable general resistance in the host system has been well researched and validated in various host-pathogenic interactions. (Van Loon, *et al.*, 1997 and Ramamoorthy, *et al.*, 2002)

Activation of defense genes through pre application with PGPR against a potential pathogen is a novel strategy in plant protection system. Enhanced production of some enzymes and their increased activity are one of the most important processes in plant defense. These enzymes occur frequently in many isoforms and are involved in synthesis of defense substances or have a direct antimicrobial activity. The increased activity of peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL), catalase and esterase in the PGPR treated plant may play either a direct or an indirect role in the suppression of pathogen development in the host ultimately protecting the plants from the pathogenic micro-organisms (Kosuge, 1966). PGPR mediated stimulation of defense related biochemical compounds have been well documented (Albert and Anderson, 1987 and Chen, *et al.*, 1998). However many of these investigations have been carried out in the traditional high value field crops but there lack of such systematic studies in the underutilized pulse crops like rice bean which is traditionally grown worldwide by the small and marginal farmers. Under this backdrop, the present study has been carried out to identify and evaluate the potentiality of the native PGPRs for inducing the disease defense related metabolism in the rice bean plants and also an attempt to reduce the gap in the existing knowledge of disease management.

**MATERIALS AND METHODS**

**Experimental Material:** The freshly harvested seeds of three rice bean varieties viz. PRR-1, PRR-2, RBL-6 were obtained from the Genetics and Plant Breeding Section, G.B. Pant University of Agriculture and Technology, Hill Campus, Ranichauri.

**Isolation and purification of rhizobacteria:** The indigenous rhizobacteria were isolated from the rhizosphere of rice bean crops varieties (PRR-1, PRR-2, RBL-6) grown in the experimental fields of the Genetics and Plant Breeding Section, G.B. Pant University of Agriculture and Technology. For isolation of fluorescent pseudomonads, 1 g rhizosphere soil was added to 10 ml sterile distilled water in a test tube.
After shaking, it was allowed to stand for a few minutes. A 10^4 dilution was made by taking 1.0 ml of the suspension and adding it to 9.0 ml distilled water in another test tube and shaking it. In this way, serial dilutions up to 10^8 dilution were prepared. The soil suspensions thus obtained were used for the isolation of rhizobacteria by the dilution planting method on King’s medium B (KMB) (King et al., 1954)

The colony forming units were counted on plates after 48 hours of growth at 30°C (± 2°C) and the bacterial isolates were examined for their fluorescence under UV light (200-340 nm). The cultures were maintained on KMB agar slants. The fluorescent bacterial isolates were multiplied in KMB broth for 48 hours.

One isolate of Bacillus species was obtained from the Plant Pathology Section of G.B. Pant University of Agriculture and Technology, Pantnagar.

Efficacy of seed bacterization under laboratory conditions: Seeds of three rice bean varieties were surface sterilized with 1% sodium hypochlorite for 30 seconds and then rinsed in sterile distilled water and dried under a sterile air stream. Ten ml of bacterial inoculum was added to Petri-plates. Ten gram seeds of each three varieties were separately soaked in 10 ml of bacterial suspension for 12 hours. Then the bacterial suspension was drained off and the seeds were dried overnight in sterile Petri plates.

Plant growth-promoting activity of fluorescent pseudomonads and Bacillus species was assessed based on the seedling vigour index by the standard roll towel method (ISTA, 2004). The vigour index was calculated by using the formula as described by Abdul Baki and Anderson (1973):

Vigour Index = Standard germination (%) x Seedling length (cm)

Seed health testing: The effect of seed bacterization on seed health of rice bean was assessed through rolled towel method (ISTA, 2004). Fifty seeds from each treatment including control treatment were subjected to standard germination test through rolled towel paper method in three replications in completely randomized design. Ten seedlings were collected at different time intervals (12, 24, 48, 74, 96 hours) and were homogenized with liquid nitrogen in a pre-chilled mortar and pestle. The homogenized tissues were stored in deep freezer until used for biochemical assays for different enzymes.

**Catalase:** The enzyme extract was prepared by homogenizing 1 g of plant tissue in a blender with M/150 phosphate buffer (assay buffer) diluted 10 times at 1-4°C and centrifuge. Stirred the sediment with cold phosphate buffer and allowed to stand in the cold with occasional shaking and the extraction was repeated once or twice. To the 0.04 ml enzyme extract, 3ml hydrogen peroxide-phosphate buffer were added into cuvette. The time required for decreases in absorbance from 0.45 to 0.4 at 240nm was noticed. Cuvette containing the tissue extract and phosphate buffer was used to adjust the absorbance prior to each reading. The enzyme activity was expressed in units/mg protein, where one enzyme unit was defined as the change in absorbance/minute caused by enzyme reaction. (Luck, 1974)

**Phenylalanine ammonia lyase:** The enzyme extract was prepared by homogenizing 1 gram of sample with 2 ml of 0.1 M sodium borate buffer (pH 8.8) at 4°C. The homogenate was centrifuged at 12,000 rpm for 20 minutes at 4°C and the resulting supernatant served as enzyme source. The reaction mixture consisted of 0.4 ml enzyme extract, 0.5 ml of 0.1 M borate buffer and 0.5 ml L-phenylalanine and incubated at 30°C for 30 minutes. The enzyme activity was measured at 290 nm. One unit of enzyme activity was defined as the amount of enzyme forming 1 µmol of trans-cinnamic acid from L-phenylalanine min^-1 g^-1 fresh tissue (Ausable, et al., 1989).

**Polyphenoloxidase (PPO) assay:** Enzyme extract was prepared by homogenizing 1 g seedlings in 2ml 0.1M sodium phosphate buffer (pH 6.5) and centrifuged at 16,000 rpm for 15 minutes at 4°C. The supernatant was used as enzyme source. The reaction mixture consisted of 200 µl enzyme extract and 1.5 ml 0.1M sodium phosphate buffer (pH 6.5). To start the reaction, 200 µl of 0.01M catechol was added. Activity was expressed as changes in absorbance at A_420 minute^-1 g^-1 fresh weight of tissue (Ausable, et al., 1989).

Statistical analysis: The data obtained during the investigation was analyzed by using the two factorial concept of completely randomised block design. Coefficient of Variation (CV %) and Standard Error of Mean (SEMs) were computed in each case and the Critical Difference (CD) at five percentage level of probability was calculated only for significant effects (Panse and Sukhatme, 1995).
RESULT AND DISCUSSION

Isolation and purification of indigenous rhizobacteria:
Five different isolates of indigenous rhizobacteria including fluorescent pseudomonads were isolated from the rhizosphere soils of rice bean crops grown. Isolates of *Pseudomonas* species were observed for fluorescent on King’s B medium. (Table 1)

Effect of seed bacterization on in vitro plant growth promotion: Seed Bacterization with isolated antagonistic rhizobacteria were found to improve significantly the seedling growth of rice bean varieties under laboratory conditions as compared to control. The mean value revealed that treatment with isolate B₅ produced maximum vigour index followed by B₉ isolate over other treatments. The lowest value of vigour index was recorded in the untreated control. (Table 2)

Seed health testing: In the present study, seed bacterization with *P. fluorescens* isolates reduced the incidence of percentage disease seedling. It is evident from data that bacterial inoculation markedly decreased the disease seedling percentage over the uninoculated control. Maximum disease seedling percentage was recorded in RBL-6 (26.7%) by seed treatment with KBM (control) while no disease was occur in PRR-1 by seed treatment with B₅ isolate. (Table 2)

Table 1: Different antagonistic bacterial isolates isolated from rhizosphere soil

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Fluorescent pseudomonas species</th>
<th>Bacillus species</th>
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</thead>
<tbody>
<tr>
<td>1. Isolate B₅</td>
<td>-</td>
<td></td>
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<tr>
<td>2. Isolate B₆</td>
<td>Isolate B₁₈</td>
<td></td>
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<tr>
<td>3. Isolate B₇</td>
<td>-</td>
<td></td>
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<tr>
<td>4. Isolate B₉</td>
<td>-</td>
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<tr>
<td>5. Isolate B₁₈</td>
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Table 2: Effect of seed bacterization on vigour index and diseased seedling percentage in rice bean varieties

<table>
<thead>
<tr>
<th>Treatments</th>
<th>PRR-1</th>
<th>PRR-2</th>
<th>RBL-6</th>
<th>Mean</th>
<th>PRR-1</th>
<th>PRR-2</th>
<th>RBL-6</th>
<th>Mean</th>
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<tr>
<td>B₅</td>
<td>3237</td>
<td>3264</td>
<td>2835</td>
<td>3112</td>
<td>0.0</td>
<td>4.0</td>
<td>6.7</td>
<td>3.6</td>
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<tr>
<td>B₆</td>
<td>2437</td>
<td>2658</td>
<td>2120</td>
<td>2405</td>
<td>8.0</td>
<td>9.3</td>
<td>10.7</td>
<td>9.3</td>
</tr>
<tr>
<td>B₇</td>
<td>3023</td>
<td>2766</td>
<td>2720</td>
<td>2836</td>
<td>6.7</td>
<td>6.7</td>
<td>12.0</td>
<td>8.4</td>
</tr>
<tr>
<td>B₉</td>
<td>2729</td>
<td>3244</td>
<td>2904</td>
<td>2959</td>
<td>10.7</td>
<td>10.7</td>
<td>13.3</td>
<td>11.6</td>
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<tr>
<td>B₁₇</td>
<td>2595</td>
<td>2701</td>
<td>2630</td>
<td>2642</td>
<td>8.0</td>
<td>9.3</td>
<td>14.7</td>
<td>10.7</td>
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<tr>
<td>B₁₈</td>
<td>2895</td>
<td>3305</td>
<td>2471</td>
<td>2890</td>
<td>10.7</td>
<td>9.3</td>
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<tr>
<td>C (D)</td>
<td>2198</td>
<td>2233</td>
<td>2020</td>
<td>2150</td>
<td>14.7</td>
<td>13.3</td>
<td>18.7</td>
<td>15.6</td>
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<tr>
<td>C (KB)</td>
<td>2039</td>
<td>2052</td>
<td>1703</td>
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<td>20.0</td>
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<tr>
<td>Mean</td>
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<td>2615</td>
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<td>14.7</td>
<td>11.5</td>
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<tr>
<th></th>
<th>CD at 5% (Bacteria)</th>
<th>CD at 5% (Variety)</th>
<th>CD at 5% (Bacteria X Variety)</th>
<th>CV (%)</th>
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<tr>
<td></td>
<td>50</td>
<td>47</td>
<td>132</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>0.95</td>
<td>0.89</td>
<td>2.53</td>
<td>25.49</td>
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Estimation of defense related proteins upon seed bacterization: All the isolates increased the amount of catalase over time due to seed bacterization. In variety PRR-1 isolate B₅ induced the maximum catalase activity at 48 hours, followed by B₆ and B₇ at 48 and 72 hours while in case of PRR-2 the isolate B₁₈ showed maximum activity at 24 and 72 hours followed by B₉ at 48 hours. No appreciable change in PO activity was observed in the untreated control. In case of variety RBL -6, maximum activity was induced by isolate B₁₈ at 96 hour which was followed by B₅ and B₁₇ (Figure 1).

In all the three rice bean varieties, B₅ isolate is found to induce the maximum PPO activity followed by B₉ isolate as compared to untreated control. In all the three varieties the induced activity was maximum at 72 hours whereas untreated seedlings (control) did not induced much PPO activity (Figure 2).

All the bacterial isolates induced considerable quantities of PAL in the treated seedling over the period of time, whereas the untreated seedling did not show any change in the pattern of PAL production. In variety PRR-1, the isolate B₅ induced the maximum activity which was followed by isolate B₉ whereas in case of PRR-2 and RBL-6 varieties, maximum PAL activity was induced at 12 hours by isolate B₁₇, B₈ and isolate B₁₈ (Figure 3).

*Pseudomonas. fluorescens* and *Bacillus* species are the biological control agents that are known to show the inhibitory effects against considerable pathogens and the antibiotics that they produce are generally assumed to be responsible for the control activity (Helbig, et al., 1998 and Krebs, et al., 1998). In this investigation, the multiple isolates of *P. fluorescens* and *Bacillus* species were used with the objective to study the expression of ISR in rice bean through seed treatment and to investigation there plant growth promoting activity. Some authors have suggested that the...
Fig1: Changes in catalase activity in the seedlings of ricebean varieties due to seed bacterization with various rhizobacteria

use of antimicrobially active species and strains of the genus *Bacillus* and *Pseudomonads* or the use of their metabolites, may be an alternative or supplementary method to chemical plant protection (Handelsman, et al., 1990 and Berger, et al., 1996). Ramamoorthy (2002) suggested that instead of using single strain, it would be more effective to apply a mixture of strains showing synergistic action for broad spectrum activity against multiple pathogens and pests. Recent studies have shown that prior application of *P. fluorescens* and *Bacillus* species strengthen host cell wall structures resulting in restriction of pathogen invasion in plant tissue (Benhamou, et al., 1996 and Chen, et al., 2000). *P.*
fluorescens isolate B, increased the vigour index and decrease disease incidence. Such growth response by PGPR either directly by assisting in resource acquisition (nitrogen, phosphorus and essential minerals) or modulating plant hormone levels, or indirectly by decreasing the inhibitory effects of various pathogens on plant growth and development in the forms of biocontrol agents. The plant-beneficial rhizobacteria may decrease the global dependence on hazardous agricultural chemicals which would destabilize the agro-ecosystems. (Ahemad and Kibret, 2014). The present study clearly indicates that seed treated with P. fluorescens and Bacillus species showed the increased
Fig 3: Changes in PAL activity in the seedlings of ricebean varieties due to seed bacterization with various rhizobacteria

activities of various defense-related enzymes which may lead to subsequent resistance to various diseases in plants.

Catalases (EC 1.11.1.6) are a second superfamily of peroxidases found in peroxisomes of nearly all aerobic cells that protect the cell from the toxic effects of hydrogen peroxide (H$_2$O$_2$). Catalase is a major enzymatic system for removal of H$_2$O$_2$ from plant cells. Catalases catalyze the dismutation of H$_2$O$_2$ into O$_2$ and H$_2$O which prevents the damaging effects of H$_2$O$_2$ accumulation and protects cells from oxidative stress (Chelikani, et al., 2004). In the present study, all the rice bean varieties showed increased catalase activity upon application, of the various rhizobacterial
isolates, generally 24 hours after application, which corresponded to the period prior to the establishment of the pathogen. No appreciable change in catalase activity was observed in the untreated control. Several authors have observed the increase in the catalase activity in plant tissues in response to infection and presumed the participation of the enzyme in forming defense barriers. (Blilou, et al., 2000 and Dorey, 1998).

PPO catalyze the last step in the biosynthesis of lignin and other oxidative phenols. It is generally assumed that PPO in plant cells is mainly compartmentalized in vesicles or plastids (Butt, 1980) and in cell walls. In the present study, seed treatment with P. fluorescence isolates and Bacillus species isolates induced the PPO activities as compared to control. The increased activity of PPO has been reported due to activation of latent polyphenol oxidase (Robb, et al., 1964). Tomiyama (1963) suggested that increase in PPO activity may be directly or indirectly involved in resistance and may contribute to defense through the production of oxidized forms of quinines, which can inactivate pectinolytic enzymes produced by pathogens (Leatham, 1980). Peroxidase and polyphenol oxidase are capable of oxidizing phenolic compounds (Kosuge, 1966). These enzymes catalyse the oxidation of phenolics to quinines or free radicals, which can react with innumerable biological molecules, creating an environment that may be unfavourable for growth of potential pathogen in the host plant.

Similar to other enzymes, seed treatment with various rhizobacterial isolates resulted in increased PAL activity. The product of PAL, is directly linked to cell lignification processes and the highest levels of PAL activity usually occur about 24 hours after initial infection (Poldie and Laxmi, 1998). All the varieties responded very well for B isolates. The increase in PAL activity indicated the activation of the phenyl propanoid pathway. De Meyer et al., (1999) reported that rhizosphere colonization by P. aeruginosa (7NSK2) activated PAL in bean roots and increased salicylic acid levels in leaves. Seed bacterization with B. subtilis AF1 resulted in a distinct increase in the activity of PAL in pigeon pea (Poldie and Laxmi, 1998).

In conclusion seed bacterization with six selected rhizobacterial isolates resulted in increased plant growth response as indicated by the increase in seedling vigour index. Seed bacterization also resulted in increased activity of three defense related enzymes i.e., Catalase, PPO and PAL studied which was correlated with reduced number of diseased seedlings in rice bean. This improved plant growth response under laboratory conditions advocated the applicability of seed bacterization with indigenous rhizobacteria under field condition too.

REFERENCES


