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Endophytic Bacteria Induced Enzymes Against *M. grisea* in *O. satavia* under Biotic Stress

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Abstract: Two plant growth promoting bacteria, one identified as rhizosphere *Bacillus pumilus* strain and other as endophytic *Pseudomonas pseudoalcaligenes* were isolated from the root surface and from within the roots of paddy variety GJ-17 respectively. Adhesion and invasion of the isolated strains with the paddy root was confirmed by 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) staining. The effects of these two bacteria were tested alone and in combinations on the induction of defence-related enzymes such as phenylalanine ammonia-lyase ((PAL; EC 4.3.1.5) and β-1,3-glucanase (EC 3.2.1.39) in the presence of pathogen *Magnaporthe grisea,* the causative agent of rice blast in paddy variety GJ-17. The isolates also showed differential induction of defence-related proteins in infected rice plants during protein profiling. The results indicate that the endophytic bacteria showed a better response to fight against the fungal infection than the rhizosphere one.

Key words: PGPR · Phenylalanine ammonia-lyase · β-1,3-glucanase · RAPD analysis · TTC staining

INTRODUCTION

Plant growth and yield are influenced by a myriad of abiotic and biotic factors. Among the biotic stresses, pathogenic microorganisms are a major threat to plant health and yield. As agricultural production intensified over past few decades, producers became more and more dependent on agrochemicals as a relatively reliable method for crop protection. However, increasing use of chemicals leads to indiscriminate resistance, environmental pollution, impact on several other non-targeted organisms, high level of chemicals in food chain and harmful effect on economically valuable vegetation [1].

Plants possess various inducible defence mechanisms to protect themselves against pathogen attacks. Some root colonizing non-pathogenic rhizobacteria may also trigger disease resistance in the host plant, a phenomenon that has been termed induced systemic resistance (ISR) [2]. The triggering of disease resistance by non-pathogenic bacteria depends on plant species to a different extent. Biological control is therefore being considered as an alternative or supplemental way of reducing the use of chemicals in agriculture. The potential use of plant associated bacteria as agents stimulating plant growth, managing soil and plant health is well documented [3,4]. The widest groups of such bacteria are PGPR [5], which colonize the root surfaces and closely adhering soil interface of the rhizosphere. These rhizobacteria have immense potential in agriculture for use as biofertilizer, biocontrol agent and in bioremediation due to their plant growth-promoting ability, antagonistic activity and degradation of pollutants [6].

Although plants are naturally exposed to several phytopathogenic microorganisms, they exhibit tolerance to these pathogens, due to their different structures (cuticles, trichomes, stomata and tyloses) and biochemical mechanisms (such as phenols, phytoalexins, cyanogenic glycosides, protease inhibitors and hydrolases) [7-9]. Previous studies have demonstrated that PR proteins like PAL and β-1,3-glucanases are involved in the defence of the plant against viral, bacterial and fungal pathogens [10,11].

PAL catalyzes the deamination of L-phenylalanine to yield trans-cinnamic acid, the common precursor for biosynthesis of phenolic derivatives like flavonoids,

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monolignols and salicylates that are essential for adaptive, vascular and reproductive plant development [12]. Phenylalanine is the starting compound used in the flavonoid biosynthesis. Lignin is derived from phenylalanine and from tyrosine.

β-1,3-Glucanases are involved in defence against pathogen in plants. They are classified as hydrolases [13] and preferentially hydrolyze 1,3-β-D-glycosidic linkages in [6-8]-β-D-and [6-8],[1,6-8,14,15]-β-D glucans in the cell walls of many pathogenic fungi. Apart from their role in plant defence, β-1,3-glucanases are involved in diverse physiological and developmental processes such as endosperm formation [16], microsporogenesis [17], pollen development [18] and seed germination [19].

The widely recognized mechanism of biocontrol mediated by PGPR is competition for an ecological niche or production of inhibitory allelochemicals and induction of induced systemic resistance (ISR) in host. ISR induced by PGPR has not yet been reported for biological control of diseases but has attracted interest because it has led to disease reduction and promotion of plant growth and yield [14]. ISR triggered in the plant by rhizobacteria is referred to, as rhizobacterial medicated ISR was reviewed [2].

It is brought about by PGPRs through fortification of physical and mechanical strength of the cell wall as well as changing the physiological and biochemical reaction of host leading to the synthesis of defence chemicals against the pathogen. PGPRs could act as strong elicitors of plant defence reaction [20]. In the present study, the effect of two bacteria isolated from the roots as well as the rhizosphere soil of paddy variety GJ-17, on the biochemical changes of rice plants infected with *Magnaporthe grisea* is reported.

MATERIALS AND METHODS

Isolation of Bacteria from Rice Field of GJ-17: Certified seeds of rice variety GJ-17 were obtained from Main Rice Research Center, Navagam, Anand, Gujarat. These seeds were planted in pots and maintained in greenhouse for forty days. Microorganisms were isolated from the root tissue as well as rhizosphere soil. For isolation of endophytic bacteria from roots, fresh roots of paddy were surface sterilized with 70% alcohol and $HgCl_2$ for 5 min each, followed by washing with sterile distilled water. The root tissues were then homogenized in a sterile mortar and pestle and the extract was used for isolation of bacteria. For isolation of rhizosphere bacteria soil samples collected from the rhizosphere of paddy plants were subjected to serial dilution and both sample were plated on YMA (Yeast Mannitol Agar) medium.

Identification of Isolates: Various biochemical tests were performed followed by 16S rDNA ribo-typing to identify the isolates. The 16S rDNA universal primers 8 F and 1510 R were used for PCR amplification of the DNA having followed PCR cycle-initial denaturation at 95°C for 2 min, denaturation at 95°C for 30 sec, annealing at 60°C for 1 min and extension at 72°C for 90 sec followed by sequencing. PCR amplificons of 16S rDNA of about 1500 bp was observed as discreet bands in agarose gel. The sequences were subjected to BLAST analysis and submitted to NCBI data Bank which has accession nos. EU921258 and EU921259. The culture showed 99.9% homology with for *Pseudomonas pseudoalcaligenes* and *Bacillus pumilus* respectively in distance matrix.

Rice Cultivation and Inoculation: Seeds of rice variety GJ-17 were washed thoroughly with distilled water followed by surface sterilization with 0.1% $HgCl_2$ solution for 4 min and 70% ethanol for 10 min. The seeds were washed thoroughly with sterile distilled water and kept in sterile water on a rotary shaker for 5-6 h. Later the seeds were transferred to Petri dishes containing tryptone glucose yeast extract agar medium and incubated in dark at 30°C to test for possible contamination. The germinated seedlings devoid of any contamination were used for inoculation experiments.

To study the effect of the isolated bacteria on the biochemical parameters selected, rice seedlings were transferred to culture tubes containing 400 µl Hoagland's nutrient medium, 400 µl micronutrients and 1% agar in 40 ml distilled water. Before the transfer, bacterial inoculum of the isolated bacteria was added with the medium at a concentration of 6×10^8 cfu ml^{-1}. To obtain a mixture of both bacterial cultures, an equal volume of both the cultures were mixed in the medium to give a concentration of 6×10^8 cfu ml^{-1}. All experiments were carried out in five replicates. The tubes were incubated at 27 °C in a 12-hr light-dark cycle in a growth chamber.

Visualization of Association of Bacteria in the Paddy Root: Association of PGPR within the root was confirmed by TTC staining (2,3,5-triphenyl-2H-tetrazolium chloride) which consisted of maleic acid and 1.5 gm of TTC in sterile potassium phosphate buffer (pH 7). Paddy roots inoculated with isolates were surface sterilized with

sodium hypochlorite and were incubated overnight in the TTC stain and cross sections of root were examined under image analyzer microscope (Carl Zeiss).

Compatibility Between Bacterial Strain and Fungus: Bacterial strains were tested for their compatibility with each other. Bacterial strains were streaked parallel to each other and incubated at 28°C in Yeast Extract Glucose Agar medium. Compatibility was tested by the overgrowth or inhibition of growth and observations were made for a period of 72 hrs. Antagonism between bacteria and fungus was also determined by inoculating at 28°C both organisms on the same Yeast Extract Glucose Agar plate and antagonistic effects were tested by inhibition of fungus growth by bacteria.

Biocontrol under Greenhouse Conditions: The bacterial strains, either singly or as mixture, were assessed for their efficiency in suppressing rice blast under glasshouse conditions. The spore suspension of *Magnaprothe grisea* with a spore load of 10^4 conidia ml^{-1} was sprayed on the plants, which caused more than 75% infection under glasshouse conditions. Observations on the percent disease incidence of rice blast were recorded. In addition, growth parameters like plant height, root length, tiller number and biomass production were recorded on 45 days after planting (maximum tillering) as grades 0 to 5 [21].

Disease index = Total grade x 100/ No. of sheaths observed x maximum grade.

Preparation of Soil for Pot Experiment: Soil samples were collected from wet rice fields and various physio-chemical properties; as pH, electrical conductivity, CEC, organic carbon, available nitrogen per square decimeter, available Ca, available P per square decimeter, available K per kg, Fe per kg, Zn per kg, Mn per kg, Cu per kg and salinity were analyzed by Multi Parameter Analyzer Eutech (SICART). Soil samples were dried, passed through 4 mm sieve and mixed with fungal (*Magnaporthe grisea)* conidial suspension in a concentration of about 500 conidia per mm^2 determined by microscopic observation at a magnification of 150 × by adding distilled water. Seven-day old rice plants were carefully removed from different test tubes inoculated with the strain of bacterium, cultured together and planted in a pot. Similarly, the control plants (uninoculated) were also transferred to a fresh pot. The quantity of the soil was maintained at 5 kg per pot. Rice seedlings were planted at the rate of 4 plants per transplant and 6 transplantations per pot. Pots were watered at the time of transplantation of the rice seedlings. Five days after transplantation, they were watered again and a 2 cm water level was maintained in pots throughout the experiment.

Extraction of enzymes for PAL and β-1,3-glucanase assays: Paddy leaves (1 g) were homogenized in 5 ml of 0.1 M borate buffer, pH 7.0 containing 0.1 g poly-vinylpolypyrrolidone (PVP). The homogenate was centrifuged at 10,000 × *g* for 35 min at 4 °C. The supernatant was collected and used in the enzyme assays.

Phenylalanine Ammonia-lyase Assay: Phenylalanine ammonia-lyase (PAL) activity was measured following the method of [22]. The assay mixture containing 100 µl of plant extract, 500 µl of 50 mM Tris HCl (pH 8.8) and 600 µl of 1 mM L-phenylalanine was incubated for 60 min at room temperature and the reaction was arrested by adding 0.5 ml 2N HCl. The assay mixture was extracted with 1.5 ml of toluene by vortexing for 30 sec. Toluene was recovered after centrifuging at 1,000 × g for 5 min and the absorbance of the toluene phase containing trans-cinnamic acid was measured at 290 nm against the blank of toluene. Enzyme activity was expressed as nmol trans-cinnamic acid released min^{-1} g^{-1} fresh weight.

β-1,3-glucanase assay: Enzyme activity was assayed by the laminarin-dinitrosalicylic acid method [23]. The reaction mixture was prepared by mixing 62.5 µl of 4% laminarin and 62.5 µl of plant extract and incubating at 40°C for 10 min. The reaction was stopped by adding 375 µl of dinitrosalicylic reagent (DNS, prepared by adding 300 ml of 4.5% NaOH to 880 ml containing 8.8 g of dinitrosalicyclic acid and 22.5 g potassium sodium tartrate) with subsequent heating for 5 min in a boiling water bath. The resulting coloured solution (0.5 ml) was diluted with 4.5 ml of distilled water and vortexed. Products released were estimated for reducing groups at 500 nm. The enzyme activity was expressed as 1 nmol of reducing substances min^{-1} g^{-1} of fresh weight.

Field Trails Assay: Field experiments were conducted to determine whether selected PGPR treatments could elicit ISR activity against target pathogen *Magnaporthe grisea*. Field trails were conducted in constitutively three rainy seasons July to October 2007-2009 at a local sick agricultural field. The experimental design was a randomized complete block with three different treatments

and three replications. Plots with 6 m^2 of area were separated by 1 m and having plants in 4 rows within were spaced at 40 cm. Plots were periodically watered and were covered with white polyethylene plastic film. Weeds were periodically eradicated either mechanically or by hand. Treatments consisted of a non-bacterized control, individual PGPR strain rhizosphere *Bacillus pumilus,* endophytic *Pseudomonas pseudoalcaligenes* and mixtures of PGPR.

RAPD analysis of GJ-17: The RAPD analysis of GJ-17 were done with the known susceptible (GAUR-100) and resistant (GR-8) variety of paddy. The total genomic DNA of all the three variety were isolated [24]. The isolated DNA was qualified and quantified by agarose gel electrophoresis and UV spectrophotometric methods. RAPD profiles were generated using 5 decameric primer (AH1, AH2, AH3, AH4 and AH5) from MWG Bangalore India. The reaction mixture contained 2 µl of primer (0.3 µM), 1 unit Taq DNA polymerase, 0.5 µl $MgCl_2$, 2 µl 4dNTPs, 5 µl 10x PCR assay buffer, 2 µl DNA sample (100 ng) and adjusted to a final volume of 25 µl with nuclease free water.

RAPD-PCR reaction was performed in an Eppendorf thermocycler. The standard conditions used 35 cycles were as follows: initial denaturation temperature 95°C for 1 min, denaturation temperature 95°C for 30 sec, annealing temperature 36°C for 1 min, extension temperature 72°C for 1 min and final extension at 72°C for 5 min. Amplification product were analyzed on 2% agarose gel electrophoresis.

Statistical Analysis: All the data were analyzed for significance using analysis of variance ANOVA followed by Fisher's least significant difference test (P= 0.05), using SAS software (SAS Institute, Cary, NC, USA).

RESULTS

***In vitro* Analysis of Antagonistic Effects of Isolates on Fungus and Localization in the Paddy Root:** Both *B.pumilus* and *P. pseudoalcaligenes* suppressed the growth of the pathogenic fungus *Magnaporthe grisea* (Fig.1) near their vicinity in the plate. A zone of inhibition could be observed around the isolates in the upper quadrant of the plate (Figs. 2). Bacterial cells could be observed as red coloured cells under the microscope after TTC staining. The presence of bacterial colony in the root cortex region could be clearly visualized, as a red spot because it stains living cell (respiring) only the dead cell of root cortex remain colourless (Fig. 3).

Fig. 1: The plate *Magnaporthe grisea* on Yeast Extract Glucose Agar medium

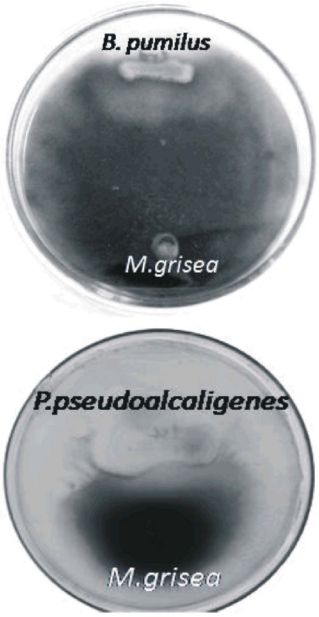

Fig. 2: Photograph of plates showing the zone of inhibition due to antagonistic effect of *B. pumilus* (right) and *P. pseudoalcaligenes* (left) on the growth of *Magnaporthe grisea* on Yeast Extract Glucose Agar plate.

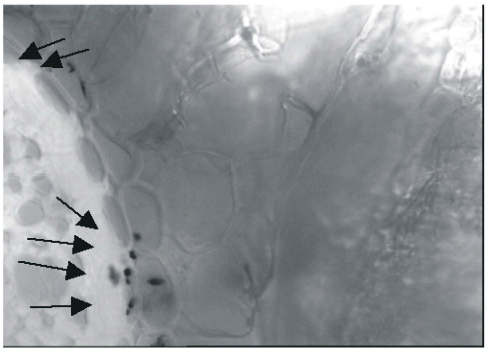

Fig. 3: Photomicrograph of a section of paddy root showing the association of bacteria in root cortex as red spots due to TTC staining.

Table 1: Effect of biocontrol agent against rice blast disease under greenhouse conditions

Bacterial Isolate Co-inoculated	% Disease Index	Plant Height (cm)	Root Length (cm)	Number of tillers/ hill	Dry Weight (g plant $^{-1}$)
Control	Nil	68.8^{cd}	11.2^{cd}	6.9^{cd}	2.3^{d}
Control+*B. pumilus*+Pathogen	43.2	70.6^{c}	12.5 ^{bc}	7.2 ^{c}	2.74^{b}
Control+*P. pseudoalcaligenes* +Pathogen	38.2	77.4^{a}	17.1 ^{a}	8.2 ^{a}	3.85^{a}
Control + *B. pumilus*+ *P. pseudoalcaligenes*+ Pathogen	49.6	74.6^{b}	13.3^{b}	7.8^{b}	2.63^{bc}
Control + Pathogen	72.4	65.5^{de}	10.2^{de}	6.4 ^{e}	2.14^{de}

Values are mean of three replications. Means within columns sharing the same letters are not significantly different (P= 0.05; LSD test).

Table 2: Effect of biocontrol agent against rice blast disease and yield in field study

		Plant dry weight/plant				
Treatment	Disease Incidence (%)	45 days	70 days	90 days	Yield Kg/ha	Grain Straw
Control	100	0.115^{a}	0.628^{a}	0.989^{a}	704^{a}	1213^{a}
Control+ *B.pumulis*	64	0.121^{ab}	0.697^{b}	1.147^{b}	841^{b}	1450^{b}
Control + *P.pseudoalcaligenes*	77	0.157^{d}	0.788^{cd}	1.574^{d}	973^{d}	1573^{cd}
Control+*B.pumulis*+ *P.pseudoalcaligenes*	58	0.142^{c}	0.780^{c}	1.233^{c}	892^{c}	1476^{bc}

Values are mean of three replications. Means within columns sharing the same letters are not significantly different (p= 0.05; LSD test).

Effect of Biocontrol Agents on Rice Blast Disease under Glasshouse Conditions and Field Experiment: The bacterial isolates *B. pumilus* and *P. pseudoalcaligenes* were evaluated as biocontrol agents for the management of rice blast under greenhouse condition in pots having soil with following physio-chemical properties; pH: 7.79, electrical conductivity 1063 µS/cm, CEC:3 cmol, organic carbon: 5500 mg per kg, available nitrogen 200 mg per square decimeter, available Ca: 12.1cmol, available P 205: 9.5 mg per square decimeter, available K 20: 265 mg per kg, Fe: 3.1 mg per kg, Zn: 285 mg per kg, Mn: 3.7 mg per kg, Cu: 2.2 mg per kg. Both the individual isolates and their mixtures significantly reduced the disease incidence in comparison to pathogen treated control plants. The magnitude of disease reduction varied between the individual isolates and mixture of PGPR (*B. pumilus* and *P. pseudoalcaligenes*). Among all treatments the most effective was *P. pseudoalcaligenes* which showed maximum reduction of the disease incidence by reducing the disease index to 38.2% as well as enhancing other growth parameters such as plant height (77.4 cm), root length (17.1cm), number of tillers (8.2) and biomass (3.85) (Table 1). In the field study the bacterial isolates *B. pumilus* and *P. pseudoalcaligenes* were also evaluated for their effectiveness as biocontrol agents in managing of rice blast disease under field conditions. Both the individual isolates and isolate mixture significantly reduced the disease incidence compared with pathogen treated control plants. The magnitude of disease reduction varied between the individual isolates and isolate mixture. In field experiment, all PGPR non-inoculated control plants in presence of pathogen showed disease symptoms on leaves 18 days after transplantation. In contrast, symptoms were noted only in some plants inoculated with the bacterial isolates either alone or in combination and disease symptoms appeared only 23 days after transplantation. The disease incidence in all plants inoculated with PGPR was significantly lower than non-inoculated control plants. Plant inoculated with either of the PGPR alone suppressed the disease by 64-77% while the mixture of both PGPRs suppressed the disease by 58%. Plant dry weight was also recorded at 45th, 70th and 90th days after transplantation. It increased by 4.2-38% in plants inoculated with *P. pseudoalcaligenes*, 0.6-15% with *B. pumilus* and 2.7-24% in plants inoculated with both the isolates. A similar increase in yield of grain and straw was also observed (Table 2).

Induction of Defence Enzymes: There was a significantly enhanced (p=0.05) enzyme activity in both the presence and absence of the pathogen *Magnaporthe grisea*. The activity of the enzyme PAL and β-1,3-glucanase was found to be higher than with the control plants. There was significant increase (p=0.05) in enzyme activity both in the presence and absence of the pathogen *Magnaporthe grisea*. The PAL and β-1, 3-glucanase activity was found to be higher in the plants inoculated with PGPRs than the non-inoculated control plants. Induction of PAL in plants inoculated with *P. pseudoalcaligenes*, *B. pumilus* and infected with *Magnaporthe grisea* showed significant variation. Its activity was highest in plants inoculated with *P. pseudoalcaligenes* at 45 days and after 12 hours of infection. It increased by 1.3 times in the plants inoculated with *P. pseudoalcaligenes* in absence of

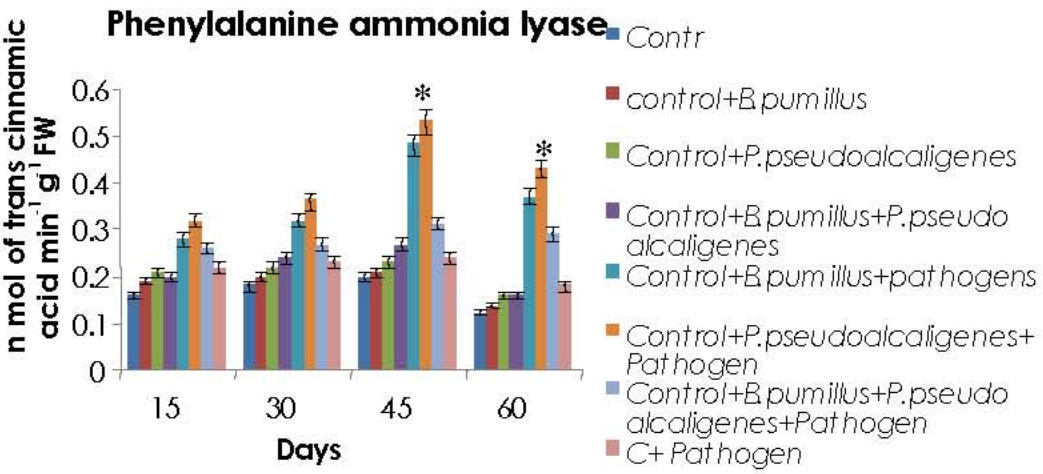

Fig. 4: PAL activity at an interval of 15 days upto 60 days of cultivation after infection with *Magnaporthe grisea*. Mean ± SE, n=5, (P= 0.05).

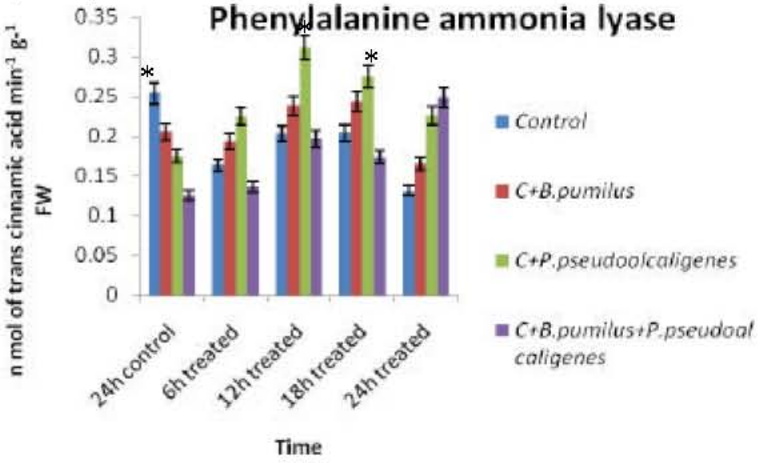

Fig. 5: PAL activity at an interval of 6 hrs upto 24hrs of infection with *Magnaporthe grisea*. Mean ± SE, n=5, (P= 0.05).

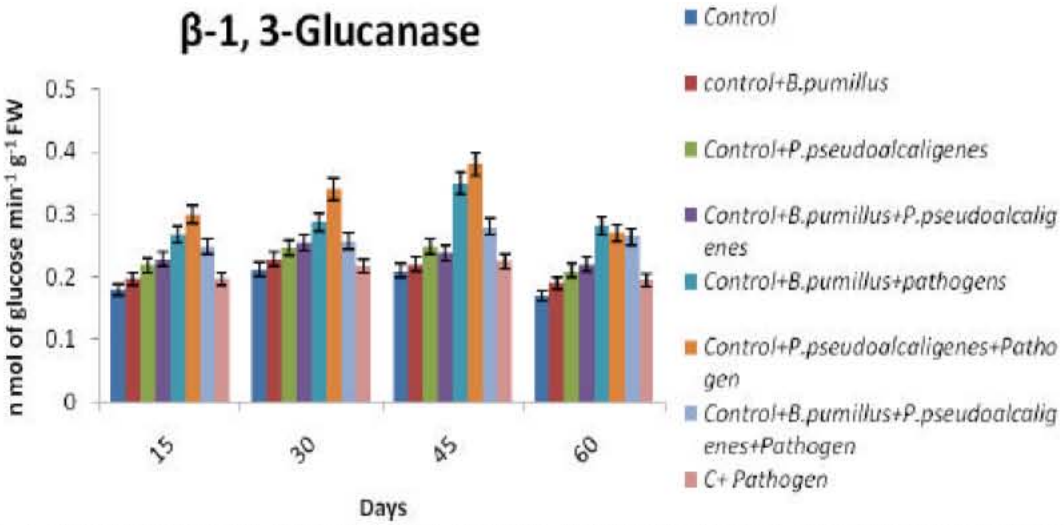

Fig. 6: β-1, 3-glucanase activity at an interval of 15 days upto 60 days of cultivation after infection with *Magnaporthe grisea*. Mean ± SE, n=5, (P= 0.05).

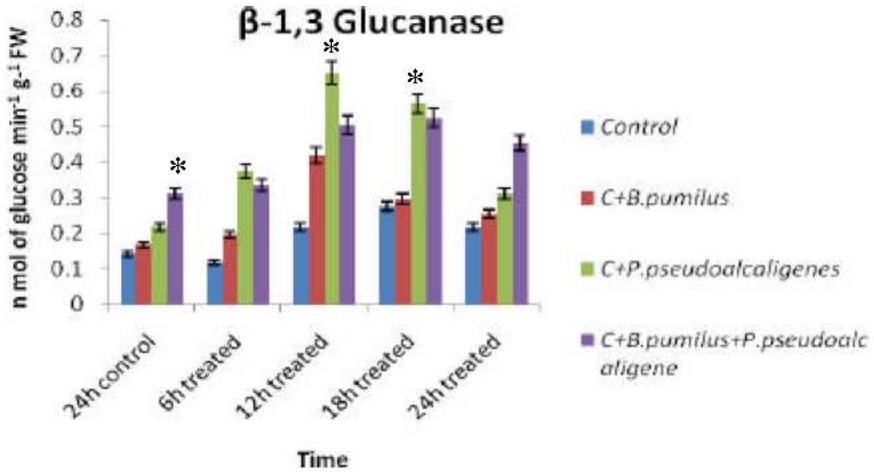

Fig. 7: β 1, 3-glucanase activity at an interval of 6 hrs upto 24hrs of infection with *Magnaporthe grisea*. Mean ± SE, n=5, (P= 0.05).

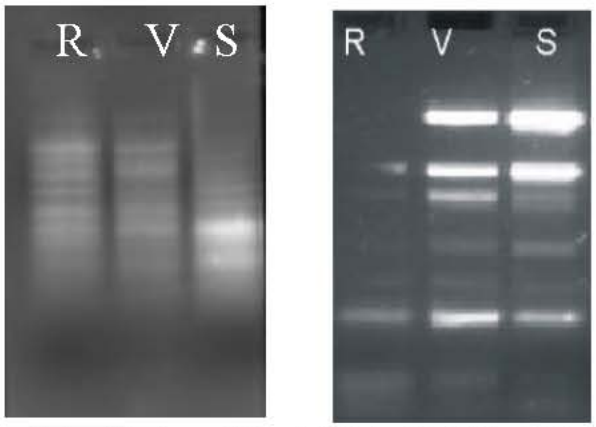

Fig. 8: Agarose gel of RAPD analysis with primer AH1 and AH2 for GJ-17(V) with the known susceptible (GAUR-100-S) and resistant (GR-8-R) variety for rice blast.

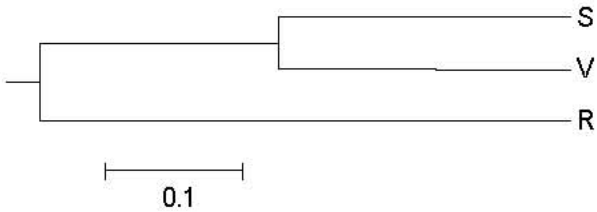

Fig. 9: The phylogenetic tree constructed using Tree explorer 2.12 software indicated that the Paddy variety GJ-17 (V) is closely related with susceptible variety GAUR-100 (S) for rice blast.

pathogen and 2 times in the plants inoculated with *P. pseudoalcaligenes* in presence of pathogen compared with non-inoculated control plants at 15th day. While it increased by 1.1 times in the plants inoculated with *P. pseudoalcaligenes* in absence of pathogen and 2.6 times in the plants inoculated with *P. pseudoalcaligenes* in presence of pathogen compared with non-inoculated control plants at 45th day. PAL activity was found to be moderated in plants inoculated with *B. pumilus* (about 2.4 times) and inoculated with both the PGPRs (about 1.2 times) in presence of pathogen as compared to non-inoculated control plants (Fig. 4 and 5).

In the case of β-1, 3-glucanase, the activity was again highest on 45th day and after 18 hours of infection in the plants inoculated with *P. pseudoalcaligenes*. Enzyme activity was 1.2 times high in plants inoculated with *P. pseudoalcaligenes*, 1 times higher in plants inoculated *B. pumilus* and 1.1 times higher in combination of both the

isolates in absence of pathogen compared to non-inoculated control plants. While it was 1.8 times high in plants inoculated with *P. pseudoalcaligenes*, 1.6 times higher in plants inoculated *B. pumilus* and 1.3 times higher in combination of both the isolates in presence of pathogen compared to non-inoculated control plants (Fig. 6 and 7).

RAPD analysis of GJ-17: RAPD analysis of paddy variety GJ-17 (V) was done with the known susceptible (GAUR-100-S) and resistant (GR-8-R) variety for rice blast. The PCR product will be analyzed on agarose gel (Fig. 8). The tree were constructed by using Tree explorer 2.12 software clearly indicated that the Paddy variety GJ-17 (V) is closely related with susceptible variety GR-8 (S) (Fig. 9).

DISCUSSION

Microbes play an important role in seed germination and seedling establishment. A large number of evidence suggests that PGPR enhance the growth, seed emergence and crop yield and contribute to the protection of plants against certain pathogens and pests [25-28]. The relationship between bacteria and plants has been well-documented [29]. When microorganisms invade plants, major physiological changes occur and plant-defence enzymes, including PR proteins, are reported to accumulate, which leads to the onset of induced systemic resistance. A number of metabolites of the bacteria under consideration trigger induced systemic resistance under biotic and abiotic stresses. The present study strongly supports the development of biocontrol strategies using an endophytic bacteria strain to reduce the damage caused by plant pathogens in economically important crops like paddy [30]. In the present study, the association of bacteria with the paddy root was confirmed by TTC staining. 2,3,5-triphenyl-2H-tetrazolium chloride (TTC), is a stable water-soluble hydrocyclic organic salt that can be easily reduced to form a highly coloured (red) insoluble product called formazon. It stains living cells (respiring) with red colour whereas dead cells remain colourless. Therefore, the red colour of the microorganism colony could be attributed to the formazon complex formed in it. The *in vitro* growth inhibition of *Magnaporthe grisea* is correlated with *in vivo* pathogenicity suppression. Between the two isolates of bacteria, *P. psuedoalcaligenes* showed a larger zone of inhibition in its vicinity than *B. pumilus*. Marjan *et al.* demonstrated that was a direct correlation between *in vitro* antagonism and *in vivo* disease suppression by *Pseudomonas* in radish [31]. The present study supports the fact that *in vitro* growth inhibition has a relationship with *in vivo* disease suppression, as observed during the biocontrol assay also. Both the individual strains and strain mixtures significantly reduced the disease incidence but effect of endophytic bacteria *viz. P. pseudoalcaligenes* showed maximum reduction of the disease incidence. Growth parameters such as plant height, root length, number of tillers and biomass increased in its presence. Bacteria indirectly enhanced plant growth *via* suppression of phytopathogens by a variety of mechanisms, which include the ability to produce siderophores that chelate iron, the ability to synthesize anti-fungal metabolites, enzymes of fungal cell wall lyses or hydrogen cyanide, which suppresses the growth of fungal pathogens. It also has the ability to successfully compete with pathogens for nutrients or specific niches on the root and the ability to induce systemic resistance is also reported [32,3].

The *P. pseudoalcaligenes*-treated rice plant better indicates its ability to induce defence enzymes than the rhizosphere bacterium *Bacillus pumilus* in presence of pathogen *Magnaporthe grisea*. The combination of both (*P. pseudoalcaligenes* and *B. pumilus*) showed only a moderate response. Increased PAL activity was observed in the leaves of bacteria-inoculated plants treated with *Magnaporthe grisea*. An early induction of PAL is very important for biosynthesis of lignin precursors from L-phenylalanine. General phenyl propanoid metabolism is defined as the sequence of reactions involved in the conversion of L-phenylalanine to activated cinnamic acids. The first enzyme of this pathway is PAL, which catalyzes the trans-amination of ammonia from L-phenylalanine to form trans-cinnamic acid, which enters into different biosynthetic pathways for the production of phenolics and phytoalexins [15]. The β-1,3-glucanase is well known as a defence-related enzyme in plants. Fluorescent *Pseudomonas* has been reported to induce systemic resistance in several crops by activating defence-related proteins and genes [20,33]. Such systemic resistance induction by *Pseudomonas* was associated with induction of β-1,3-glucanase and chitinase. These defence proteins have the potential to hydrolyze the major components of fungal cell walls *viz.*, chitin and β-1,3-glucan, respectively [34,35]. There is an interesting observation that induction of PAL and β-1,3-glucanase was at different time interval, experiment also indicates that induction of PAL was early to β-1,3-glucanase during infection with pathogen. The higher induction of PAL and β-1,3-glucanase in the plant in the presence of *P.*

pseudoalcaligenes may be because of better association of these bacteria with plants as well as lesser competition than the rhizosphere bacteria.

The RAPD analysis also clearly indicates that paddy Variety GJ-17 was susceptible for rice blast as it was closely related with susceptible variety GR-8 (S). but it was able to survive in presence of pathogen *M. grisea* due to association of PGPR with the plant.

The field experiment also shows that *P. pseudoalcaligenes* better able to protect the plant from the pathogen than the *B. pumilus* and the mixture of both the PGPR. At the same, there was also higher dry mass and yield in the plant inoculated with *P. pseudoalcaligenes*. Pierson and Weller reported that combination of *Pseudomonas* strains increased wheat yield. Our result does not show much variation to the greenhouse bioassay [35,36] Our results indicate the potential use of PGPR, specially the endophytic PGPR helping in induction of defence enzymes was better in vegetative stage than in the juvenile stage and in booting stage [38]. In the juvenile stage, the induction of defence enzymes takes place in response to bacteria, which will persist a lifetime to defend against all the pathogens. Present study also indicates that endophytic PGPR better able to induce systemic resistance in plant compared to rhizosphere and combination of both [39]. Van Wees *et al.* suggested that a single bacterium might induce systemic resistance through more than a single mechanism [40]. Thus, the bacteria are ideal organisms to deliver crop protection, because they can be applied to seed or mixed with soil during seedling stage or transplanting. In addition to direct control of soil pathogens, the study demonstrated that bacteria represented an attractive alternative to chemical pesticides for systemic protection against pathogens. A major advantage of bacteria is that once systemic resistance is induced, the natural defence mechanism of the plant is operative for prolonged periods even if population of inducing bacteria decline across time [2]. Therefore, certainly bacteria represent a potentially valuable crop protection tool in high-value cropping systems like cereals and vegetables, where high use of chemicals leads to contamination.

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