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Rhizobium-Mediated Induction of Phenolics and Plant Growth Promotion in Rice (*Oryza sativa* L.)

Ravi P. N. Mishra,[1]* Ramesh K. Singh,[1] Hemant K. Jaiswal,[1] Vinod Kumar,[2] Sudarshan Maurya[2]

[1]Microbial Genetics Laboratory, Department of Genetics and Plant Breeding, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi-221 005, India
[2]Fungal Pathology Laboratory, Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi-221 005, India

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Abstract. Qualitative and quantitative estimation of phenolic compounds was done through reverse phase–high performance liquid chromatography (RP-HPLC) from different parts (leaf, stem, and root) of rice plants after inoculation with two rhizobial strains, RRE6 (*Rhizobium leguminosarum* bv. *phaseoli*) and ANU 843 (*R. leguminosarum* bv. *trifolii*) and infection by *Rhizoctonia solani*. On the basis of their retention time, the major phenolic acids detected in HPLC analysis were gallic, tannic, ferulic, and cinnamic acids. Furthermore, in all *Rhizobium*-inoculated rice plants, synthesis of phenolic compounds was more consistently enhanced than in control (uninoculated plants), where the maximum accumulation of phenolic compounds was observed in plants inoculated with RRE6 and infection with *R. solani*. Under pathogenic stress, RRE6 performed better because a relatively higher amount of phenolics was induced as compared with plants treated with ANU 843. Phenolic acids mediate induced systemic resistance and provide bioprotection to plants during pathogenic stresses. In addition, both rhizobial strains promote growth and productivity of rice plants in greenhouse conditions. This report on *Rhizobium*-mediated defense responses and growth promotion of nonlegume (such as rice) provides a novel paradigm of symbiotic plant–microbe interaction.

Rhizobia are symbiotic bacteria and belong to a versatile and physiologically robust community of N_2-fixing microorganisms. They are capable of invading and eliciting root or stem nodules on leguminous plants where they fix atmospheric nitrogen, which is of great environmental and agricultural importance. Recent reports indicate that rhizobia can and do establish endophytic association (i.e., colonize in the intercellular spaces of root) with rice in natural as well as laboratory conditions and promote its growth and productivity [3, 26, 27, 29].

Rice (*Oryza sativa* L.) is the most prominent food crop and represents the staple diet for almost half of the human population of the world. It is estimated that there will be about 8 billion people by the year 2020, of which 4.8 billion will need to be fed with 760 million tons of rice [9]. This means that the production of rice needs to be increased by 2.0% per year to meet future demands. This will require double the amount of currently applied synthetic fertilizers, which is neither economically feasible nor environmentally desirable. If rice were able to establish a more intimate and efficient symbiotic association with plant growth–promoting microorganisms, serious economic and ecological problems associated with the use of chemical fertilizers to enhance rice production could be mitigated.

Plant growth–promoting rhizobacteria (PGPR)-mediated induced systemic resistance (ISR) and bioprotection of different crops against pathogenic stress has gained considerable importance recently in controlling a wide spectrum of fungal diseases in various crops [10, 21, 25, 31]. Several species of *Pseudomonas*, *Serratia*, and *Bacillus* are known to protect plants directly by either producing antimicrobial compounds or

Correspondence to: R. P. N. Mishra; *email:* ravi_bhu05@yahoo.com

indirectly through ISR in plants [20, 30]. Krishnamurthy and Gnanamanickam [11, 12] described ISR in rice in response to inoculation with different strains of plant growth–promoting *Pseudomonas* as an important mechanism in the biologic suppression of sheath blight and blast disease of rice. The control of various fungal diseases of sunflower, okra, soybean, mung bean, and chickpea by rhizobial inoculation has also been reported [6, 16].

Rhizoctonia solani is the causal agent of sheath blight of rice, a serious devastating disease in all rice-growing countries. The disease symptoms include greenish-gray elliptical or oval-shaped spots with yellow margin mostly found on leaf sheaths, but, at times, leaf blades are also infected. Lesions may reach the uppermost leaf under favorable conditions. Severely infected rice plants produce poorly filled or empty grains, resulting in heavy yield loss.

Rhizobial symbiosis with legumes is well documented, but the finer details of rhizobial interaction with nonlegumes such as rice and its ecological significance are poorly understood and emphasized an urgency and scope for study of the same. Therefore, the aim of the present study was to investigate (i) the status and ecological significance of phenolic compounds in rice plants after inoculation with different *Rhizobium* strains and *R. solani*, and (ii) assessment of rice plant growth-promoting potential of *Rhizobium* strains. The results are presented here.

Materials and Methods

Rhizobial strains and growth conditions. RRE6 was previously isolated from rice root growing as natural endophyte [27]. ANU 843 was obtained from Prof. B.G. Rolfe, Australian National University, Australia. Both rhizobial cultures were multiplied in Yeast–Extract-Mannitol (YEM) medium [28]. The inoculum of *R. solani* was isolated from sheath blight–affected rice plants growing in the fields.

Inocula preparation and inoculation. Rhizobial strains were grown in YEM broth for 3 days at 30°C. Ten milliliters of exponentially growing cells (10^9 colony-forming units (CFUs) mL^{-1}) were collected by centrifugation for 10 min at 5000*g* at 4°C and washed with phosphate buffer (PB; pH 7.0). Cell pellets were suspended in buffer and recentrifuged at 5000*g*, for 1 min at 4°C. Rice seeds (cv Pant-12) of uniform size were coated by dipping them into the bacterial suspension. Subsequently, seeds were dried at room temperature and bacterial counts were made using serial dilution, plating onto YEM agar. The mean inoculation level was 3×10^5 cells $seed^{-1}$. Four to five seeds were sown at equal depth in plastic pot containing unsterilized clay loam soil. Each pot was reinoculated at the base of the plant with 1 mL of culture containing 10^9 CFUs mL^{-1} at 7 days of seedling emergence. For control, rice seeds were treated with PB instead of bacterial suspension.

Inoculation with *R. solani*. *R. solani* was isolated from sheath blight lesions of field-grown rice and maintained on potato dextrose agar (PDA) (potato, 250*g*; dextrose, 20*g*; agar, 15*g*; and distilled water, 1000 mL; pH 7.0) medium. To test their virulence, fungal isolates were grown on rice hull–rice grain medium. The medium contained 300 g of rice hull, 100 g of rice grain, and 200 mL of water. The mixture was placed in a 500-mL Erlenmeyer conical flask and autoclaved at 121°C for 20 min. The following day, a 4-mm agar disk from the periphery of a 5-day-old culture of *R. solani* grown on PDA in a Petri dish was transferred aseptically to the mixture and incubated for 2 weeks at room temperature (25 ± 2°C). Rice cultivar Pant-12 was grown under greenhouse conditions in pots containing clay loam soil. The temperature inside the greenhouse ranged from 20°C to 28°C and the humidity ranged from 60% to 90% relative humidity during the experimental period. When the plants were 45 days old (either treated with *Rhizobium* strains or untreated), the rice hull–rice grain inoculum (1 g) of *R. solani* was placed in between the stem and the basal leaf sheath of each tiller of the hill at about 3 to 4 cm above the water line. A similar set of rice plants were left uninoculated for their comparison with *R. solani*-treated plants.

Extraction of phenolic compounds from rice plants. Eight randomly selected plants from four pots of a single treatment were harvested from different pots and pooled to make one sample each of leaves, stem, and roots to extract the phenolic compounds. One gram of freshly harvested leaf, stem, and root samples was macerated in a clean pestle mortar, and finally, crushed samples were suspended in 5 mL of ethanol–water (80:20; vol/vol). Samples were collected in screw-capped tubes, and the suspension was subjected to ultrasonication (Microson, Misonix, USA) for 15 min at 4°C followed by centrifugation at 7500*g* for 15 min. The clear supernatant was subjected to charcoal treatment to remove pigments from each sample and was then transferred to glass tubes after filtering through Whatman filter paper no. 1. The residue was re-extracted twice, and the supernatant was pooled before evaporation under vacuum (Buchi Rotavapor Re Type BUCHI Analytical Inc., USA). Dried samples were resuspended in 1 mL high-performance liquid chromatography High performance liquid chromatography (HPLC) grade methanol by vortexing and were filtered through a Millipore filter membrane (pore size 0.45 μm) (Axiva Sichem, Delhi, India) and were stored at 4°C until HPLC analysis.

HPLC analysis. Rice plant samples prepared for phenolic estimation were analyzed through HPLC according to Singh et al. [25] with an HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with two Shimadzu LC-10 ATVP reciprocating pumps, a variable UV-VIS detector (Shimadzu SPD-10 AVP), an integrator, and Winchrom software for data recording and processing (Winchrom, India). Reverse-phase chromatographic analysis was carried out in isocratic conditions by using a C-18 reverse-phase HPLC column [(250 x 4.6-mm id, particle size 5 μm) Luna 5 μC-18 (2), Phenomenex, US] at 25°C. Running conditions included mobile phase methanol: 0.4% acetic acid (80:20, vol/vol), flow rate 1.0 mL/min, injection volume 5 μL, and detection at 290 nm. Samples were injected three times in the sample loop, and the mean of the peak areas of individual compounds was taken for quantification. Tannic, gallic, ferulic, cinnamic chlorogenic, and salicylic acids were used as internal and external standards. Phenolic compounds and salicylic acid present in the samples were identified by comparing retention time (Rt) of standards and well as by coinjection. Concentrations were calculated by comparing peak areas of reference compounds with those in the samples run under the same elution conditions.

Plant growth promotion experiment. For the plant growth experiment, only one healthy plant was allowed to grow in each pot, and data related to various plant growth parameters such as plant height, number of panicles, plant dry weight, and grain yield were recorded at maturity.

Statistical analysis. The experiment was conducted in completely randomized design, and data were analyzed by analysis of variance with the Excel (Microsoft, version 5.0) software package. Treatment means were compared at 95% and 99% probability level ($P = 0.05$ and 0.01), and the same set of data was further analyzed to calculate the least significant difference at $P = 0.05$ and 0.01, respectively.

Results

HPLC analysis of the different rice plant parts after inoculation with two *Rhizobium* sp. (*R. leguminosarum* bv. *phaseoli* RRE6 and *R. leguminosarum* bv. *trifolii* ANU 843) as well as *R. solani* revealed six to nine peaks. Of these, four peaks were detected by HPLC and identified as gallic acid (Rt. 2.86 min), tannic acid (Rt. 2.72 min), ferulic acid (Rt. 3.40 min), and cinnamic acid (Rt. 4.46 min) (Fig. 1). Appearance of the peaks was consistently uniform in all of the three plant parts (leaf, stem, and root) of a single treatment. Quantitative estimation of the detected peaks exhibited significant variation among different treatments. Among all the four phenolic acids detected, gallic acid was the most abundant in leaves and consistently present in all rice parts in all treatments including controls. However, its maximum accumulation was detected in all parts of rice plants that received RRE6+*R. solani* treatment at 48 and 96 h. ANU 843+*R. solani* treatment could also induce higher amount of gallic acid in rice plants, but the quantity was less in comparison with RRE6+*R.solani* treatment. Interestingly, in all of the treatments gallic acid content was several times more than the control at each time interval (i.e., 24, 48, and 96 h) (Table 1).

Maximum amount of tannic acid was detected in leaves and stem of rice plant after 48 h of treatment with RRE6+*R. solani*. However, its maximum amount in roots was recorded after 48 h in treatment ANU 843+*R. solani*. After 24 h of inoculation, the maximum amount of tannic acid was detected in roots, leaves, and stems in treatments *R. solani*, RRE6+*R. solani*, and ANU 843+*R. solani*, respectively. However, after 96 h of inoculation the highest concentration of tannic acid was detected in leaves and stems of rice in treatments RRE6+*R. solani* and ANU 843+*R. solani*, respectively (Table 1).

Furthermore, in the presence of rhizobial strains a rapid induction in ferulic acid was also detected in rice plants when exposed to pathogenic stress. Its maximum accumulation was detected in all parts of rice plants that were subjected to RRE6+*R. solani* treatment at each three time intervals (24, 48, and 96 h). ANU 843+*R. solani* treatment could also induce the accumulation of a higher amount of ferulic acid, but it was less compared to that of RRE6+*R. solani* (Table 1).

A similar result was obtained in the case of cinnamic acid. Consistent maximum induction of cinnamic acid was recorded in all parts of rice plants in RRE6+*R. solani* treatment at each of the time intervals (24, 48, and 96 h). ANU 843+*R. solani* was again the second most effective treatment that could induce a higher amount of cinnamic acid (Table 1).

The most interesting result was the consistent high accumulation of all phenolic acids in almost all plant parts in the treatment combination where rhizobial inoculation was challenged by *R. solani*. The induction of phenolics in these treatments (RRE6+*R. solani* and ANU 843+*R. solani*) was multifold higher than control (uninoculated) and plants inoculated with *R. solani*, RRE6, or ANU 843 alone (Table 1).

A significant increase in plant height, dry weight, and grain yield was also recorded in the greenhouse rice plant inoculation experiment using RRE6 and ANU 843 as a source of inoculum. Maximum increase in growth and grain yield was observed in plants inoculated with RRE6 (Table 2).

Discussion

Rhizobial association with legumes is well documented, and exploitation of this beneficial nitrogen-fixing root nodule symbiosis represents a hallmark of successfully applied agricultural microbiology [2]. However, scanty information is available regarding the mechanism of association between nonlegumes and rhizobia. Because the biochemical parameters (e.g., phenolic compounds) induced by *Rhizobium* are the determinants of ISR in the host against pathogenic attack, the levels of phenolics were measured to correlate and explain the relationship between the level of phenolics and protection of rice from *R. solani.*

Phenolic acids are carbon-based compounds present in all plants investigated to date. Some of them occur constitutively, whereas others are formed in response to pathogenic attack and are associated with as part of an active defense response in the host [17]. The constitutive phenolics are known to confer resistance either directly or indirectly through activation of postinfection responses in the hosts [7]. Phenolic acids are perhaps the compounds most noted for their ability to bind to protein in vitro, forming soluble and insoluble complexes [4]. The phenolic–protein interactions are thought to be, in part, responsible for the putative function of phenolic acids as plant defense compounds [14]. Successful management of various devastating diseases of a number of crop plants through the application of PGPR in the greenhouse as well as in the field has been previously reported by our group [21, 24, 25].

In the present investigation, a rapid accumulation of phenolic acids, particularly gallic, ferulic, tannic, and

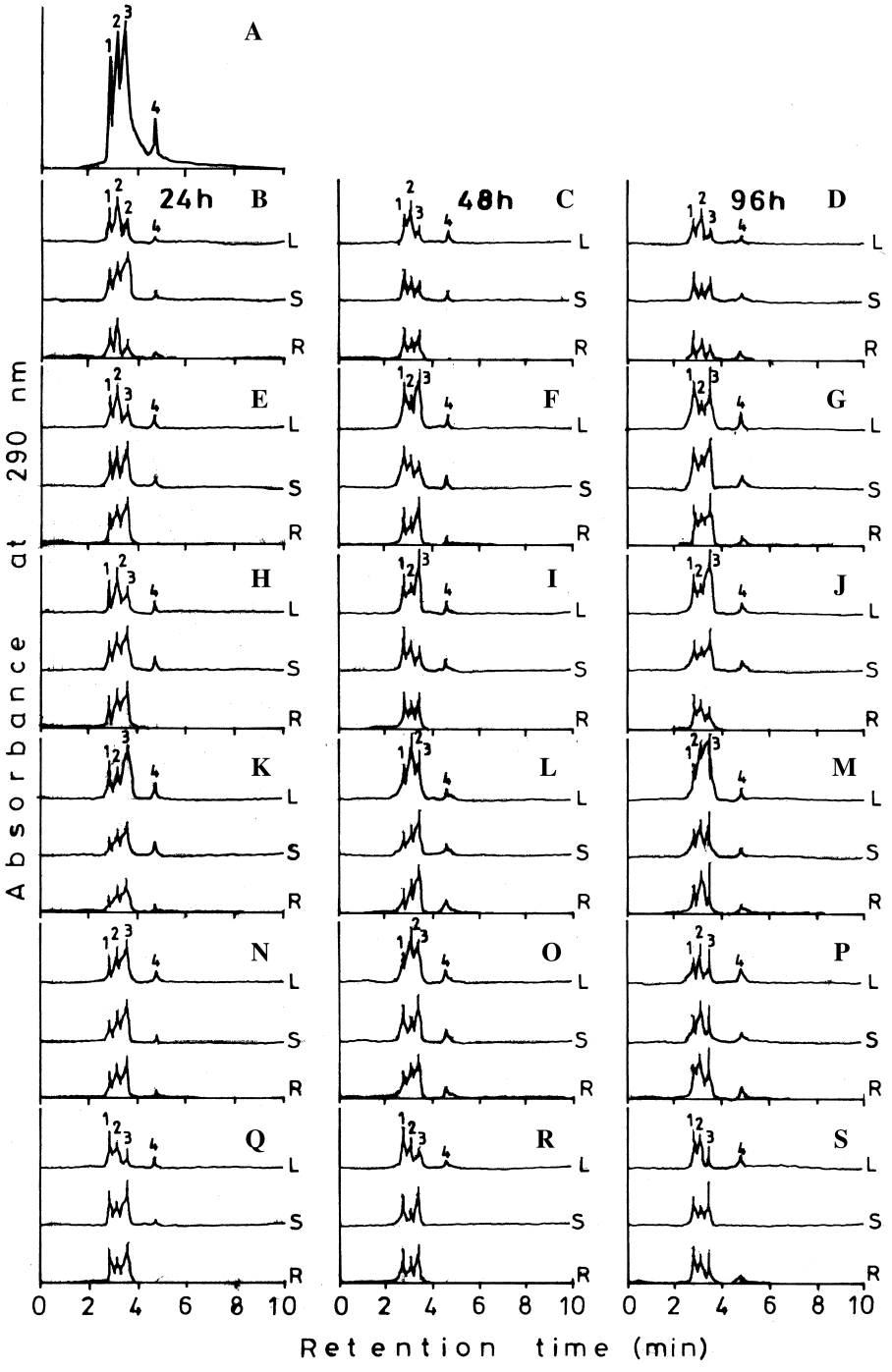

Fig. 1. High-performance liquid chromatography profile of phenolic acids induced in different parts [Leaf (L), Stem (S), and Root (R)] of rice plants after inoculation with *Rhizobium* strains and *R. solani*. Standard phenolic acids (A, 1- T.A., 2- G.A., 3- F.A., 4- C.A.), *R. solani* (B–D), RRE6 (E–G), ANU 843 (H–J), RRE6 + *R. solani* (K–M), ANU 843 + *R. solani* (N–P), and Control (Q–S).

cinnamic acids, reveals that the two *Rhizobium* strains were effective in inducing resistance in rice plants because their accumulation was enhanced in the presence of the pathogen *R. solani*. Between the two rhizobial strains, RRE6 was more efficient because more phenolics were induced in rice plants after inoculation with it. Gallic acid that shows antimicrobial activity [1] is potentially not a fungitoxin compound. Gallic acid converted into its derivative gallotannins, which are heterogeneous polymers containing numeric gallic acid molecules connected in a different manner to one another and to sugars. One of the important properties of these gallotannins is that they provide protection to the plants from bacteria and fungi [8].

Similarly, ferulic and cinnamic acids, which arise from the shikimic acid pathway, and subsequent reactions

Table 1. Amount of various phenolic acids (μg g^{-1} fresh weight) in different parts of rice plants (cv Pant-12) after inoculation with *R. solani*

		24 h				48h				96 h			
Treatments	Plant parts	G.A.	T.A.	F.A.	C.A.	G.A.	T.A.	F.A.	C.A.	G.A.	T.A.	F.A.	C.A.
R. solani	Leaf	75.1 (0.41)[a]	72.5 (0.40)	47.0 (0.40)	10.1 (0.04)	61.6 (0.45)	53.2 (0.42)	38.1 (0.41)	24.3 (0.20)	50.3 (0.40)	40.2 (0.41)	30.1 (0.24)	10.3 (0.20)
	Stem	57.4 (0.40)	55.1 (0.45)	60.1 (0.41)	15.6 (0.18)	59.1 (0.37)	62.5 (0.51)	42.2 (0.42)	13.5 (0.16)	30.6 (0.40)	49.3 (0.45)	45.7 (0.44)	10.6 (0.20)
	Root	60.3 (0.41)	50.1 (0.32)	45.2 (0.31)	9.2 (0.16)	40.6 (0.23)	57.5 (0.51)	50.21 (0.43)	ND	35.2 (0.39)	40.1 (0.41)	30.6 (0.40)	10.6 (0.08)
RRE6	Leaf	79.5 (0.45)	68.2 (0.41)	61.9 (0.42)	21.3 (0.18)	63.2 (0.41)	80.3 (0.45)	135.3 (1.01)	24.2 (0.19)	68.2 (0.42)	80.5 (0.61)	100.0 (0.93)	26.5 (0.23)
	Stem	63.5 (0.42)	62.5 (0.41)	70.2 (0.40)	13.2 (0.15)	68.0 (0.43)	78.9 (0.41)	60.2 (0.40)	23.0 (0.20)	55.3 (0.35)	71.3 (0.40)	72.2 (0.38)	13.8 (0.17)
	Root	64.2 (0.42)	54.2 (0.43)	66.1 (0.40)	ND	68.5 (0.41)	60.3 (0.41)	78.2 (0.39)	10.6 (0.13)	60.5 (0.41)	66.3 (0.40)	75.1 (0.40)	12.6 (0.10)
ANU 843	Leaf	70.1 (0.40)	60.7 (0.42)	50.1 (0.41)	17.1 (0.18)	63.0 (0.39)	75.0 (0.58)	118.2 (0.90)	19.5 (0.21)	60.1 (0.40)	78.1 (0.46)	105.5 (0.80)	19.0 (0.18)
	Stem	60.2 (0.44)	55.5 (0.40)	65.0 (0.40)	21.5 (0.12)	60.5 (0.29)	68.1 (0.46)	52.1 (0.32)	23.3 (0.16)	55.3 (0.41)	66.2 (0.40)	70.1 (0.42)	10.0 (0.16)
	Root	57.6 (0.44)	55.2 (0.40)	65.5 (0.40)	ND	60.1 (0.26)	69.0 (0.48)	68.7 (0.44)	ND	56.2 (0.39)	65.8 (0.40)	60.6 (0.43)	ND
RRE6 + *R. solani*	Leaf	102.8 (0.11)	96.3 (0.48)	150.2 (0.82)	29.2 (0.20)	262.5 (1.02)	133.2 (1.04)	221.6 (1.10)	41.5 (0.38)	186.2 (1.12)	110.2 (0.90)	200.1 (1.16)	36.2 (0.30)
	Stem	90.5 (0.40)	80.0 (0.43)	96.2 (0.63)	20.5 (0.22)	103.3 (0.70)	99.9 (0.69)	160.2 (1.02)	32.2 (0.36)	95.5 (0.70)	89.5 (0.80)	140.1 (1.12)	26.1 (0.25)
	Root	89.8 (0.38)	65.9 (0.43)	99.3 (0.60)	16.2 (0.17)	105.2 (0.76)	88.5 (0.42)	190.4 (1.11)	27.5 (0.29)	98.5 (0.73)	80.6 (0.63)	125.6 (1.09)	25.1 (0.23)
ANU 843 + *R. solani*	Leaf	95.4 (0.39)	86.0 (0.40)	110.5 (0.68)	25.1 (0.21)	192.5 (1.08)	99.0 (0.73)	168.2 (1.06)	36.1 (0.36)	125.2 (1.07)	88.2 (0.60)	120.0 (0.97)	30.2 (0.26)
	Stem	82.2 (0.41)	72.6 (0.40)	102.3 (0.76)	18.6 (0.15)	99.8 (0.70)	86.3 (0.41)	136.1 (0.99)	26.2 (0.30)	109.7 (0.96)	80.2 (0.43)	96.6 (0.72)	22.6 (0.20)
	Root	78.0 (0.41)	70.2 (0.45)	81.3 (0.65)	12.9 (0.10)	96.5 (0.70)	85.5 (0.45)	110.2 (100)	19.6 (0.20)	93.0 (0.83)	72.9 (0.40)	98.9 (0.64)	26.1 (0.20)
Control	Leaf	58.3 (0.43)	66.2 (0.40)	55.3 (0.42)	20.5 (0.19)	55.1 (0.42)	65.1 (0.43)	46.2 (0.38)	20.1 (0.21)	59.6 (0.43)	60.1 (0.39)	40.2 (0.32)	19.3 (0.16)
	Stem	40.2 (0.33)	50.1 (0.42)	79.4 (0.46)	9.5 (0.13)	35.9 (0.30)	51.9 (0.42)	61.3 (0.41)	ND	37.2 (0.40)	47.2 (0.36)	68.5 (0.40)	ND
	Root	48.1 (0.40)	54.5 (0.41)	60.7 (0.46)	ND	45.1 (0.32)	53.5 (0.43)	61.5 (0.43)	ND	49.1 (0.42)	56.6 (0.43)	52.3 (0.44)	8.6 (0.11)

[a]Values given in parentheses represent ± standard error of mean (± SEM).
ND, not detected; G.A., gallic acid; T.A., tannic acid F.A., ferulic acid, C.A., cinnamic acid.

are reported to be antifungal and antioxidant, respectively [13]. Cinnamic acid is a key product of the phenylpropanoid pathway and serves as a precursor for the synthesis of ferulic acid. It is synthesized from phenylalanine through catalysis by phenylalanine ammonia lyase (PAL) and plays a vital role in host resistance under pathogenic stress [23]. Significantly high accumulation of cinnamic acid in *Rhizobium*-inoculated rice plants, suggests the activation of the phenylpropanoid pathway through increased PAL activity. Most importantly, this phenomenon is more prominent after inoculation with the pathogen *R. solani*. Likewise, accumulation of a high amount of tannic acid in rice plants by *Rhizobium* application as compared to control unequivocally supports the role of phenolic acid in bioprotection during pathogenic attack. However, the possibilities of involvement of some more complex molecular alterations in rice after application of *Rhizobium* resulting in the formation of defense barriers or activation of defense responses through synthesis of pathogenesis-related proteins [22] or certain other enzymes associated with host resistance cannot be ruled out.

The exact mechanism of mode of action of *Rhizobium* endophytic strains in alteration of phenolic profile and plant growth promotion is still not very clear.

Table 2. Effect of rhizobial inoculation on yield and yield components of rice (cv Pant-12) in greenhouse conditions in nonsterilized soil.

	Charactersistics				
Strains	Plant height (cm)	No. of panicles hill^{-1}	Root dry weight(g hill^{-1})	Shoot dry weight (g hill^{-1})	Grain yield (g hill^{-1})
Control	90.3	7.5	1.9	24.41	14.65
RRE6	95.3*	9.5*	2.41*	29.78**	16.82*
ANU843	95.1*	9.0*	2.32*	28.20*	16.16*
LSD					
5%	4.05	1.30	0.31	3.01	1.36
1%	6.10	2.15	0.42	4.98	2.25

*,** For each strain, values for inoculated plants are significantly different from controls at $P = 0.05$ and 0.01 levels, respectively. Values are means of six replicates.
LSD, least significant difference.

However, bacterial endophytic biocontrol agents are reported to exert their beneficial effect on crop plants in two possible ways: (i) by extensive colonization of internal plant tissues and suppression of invading pathogens by niche occupation, antibiosis, or both; and (ii) by colonization of root cortex, where they stimulate general plant defense/resistance mechanism [5]. Rhizobia can and do colonize the root interiors of rice plants and promote its growth by producing various biostimulatory agents [15, 18, 26, 29]; therefore, it is quite possible that RRE6 and ANU 843 might also be following the same ways of plant protection and growth promotion by colonizing the root tissues.

The differences in the relative efficiency of the *Rhizobium* strains have been observed in inducing synthesis of phenolic compounds, and plant growth promotion in a single variety of rice (Pant-12) and against a single isolate of *R. solani* suggests variation in their ability to react against a common host–pathogen interaction. Differential behavior of different PGPR has already been reported in other pathosystems [19]. Induction of phenolic compounds and increase in growth and productivity in rice after inoculation with *Rhizobium* strains and their probable role in protecting rice plants against *R. solani* infection is reported for the first time.

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