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Utilization of endophytic microbes for induction of systemic resistance (ISR) in soybean (*Glycine max* (L) Merril) against challenge inoculation with *R. solani*

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ABSTRACT

Microbial endophytes are known to induce systemic resistance in host plant. Present study was carried out to investigate the effects of indigenous endophytic microorganisms Pseudomonas sp., Bacillus sp., Burkholderia sp. Streptomyces sp., Actinoplanes sp., Alternaria sp., and Fusarium sp. on induction of systemic resistance (ISR) against challenge inoculation with Rhizoctonia solani in soybean (Glycine max (L) Merril). It was observed that treatment with endophytes significantly elevated the levels of marker biochemicals viz., phenols, peroxidase (PO), phenylalanine ammonia-lyase (PAL), polyphenoloxidase (PPO), □β–□1, 3-*glucanase and chitinase involved in ISR in soybean. The significant increments were recorded in protein content.Thus, present studies indicate that utilization of indigenous endophytes may exert more favorable effects on plant health which ultimately will enhance crop productivity.*

Key words: Endophytes, ISR, Soybean (*Glycine max* (L) Merril), *R. solani*.

INTRODUCTION

The classical induction of plant resistance include pathogens, plant growth promoting microbes and plant products. Biotic and abiotic inducers have been reported to enhance the resistance of plant to various pathogens (Rajendran et al., 2006). Induction of plant's defense genes by prior application of inducing agents is called induced resistance (Hammerschmidt and Kuc, 1995). Microbial endophytes promote plant growth and improve the host's capacity to withstand pathogen attack by causing organism competition, antibiosis and induction of systemic resistance. Induction of plant defence mechanisms by endophytes in the management of pests and diseases is premier area of current research. Endophytic microorganisms have attracted the attention of researchers because of their potential to serve as biocontrol agents (Strobel and Daisy, 2003; Stein, 2005; Ryan et al., 2008). Endophytes living in the healthy tissues of plants are relatively unstudied and may be the potential source of novel natural products for exploitation in agriculture, medicine and other industries (Strobel and Daisy, 2003).

Microbial endophytes are typically defined as plant associated microbes that colonize living internal tissues of plants without causing any visible symptoms or immediate over-negative effects and can be isolated from surface disinfected plant tissue (Wilson, 1995; Zinniel et al., 2002; Hung and Annapurna, 2004). Endophytic microbes include bacteria, actinomycetes, and fungi are ubiquitous in most plant species. Endophytes exist in a range of tissue types within a broad range of plants, colonizing the plant systemically, residing latently in intercellular spaces, inside the vascular tissue or within cells (Khan and Doty, 2009). Relatively steady internal environment inside the plant tissues makes endophytes more bioactive than the rhizospheric or others plant associated microorganisms (He et al., 2009). Endophytes might interact more closely with the host plant and therefore, could be efficient biological control agent in sustainable crop production and offer unique opportunity for crop protection and biological control (Melnick et al., 2008).

Although, the plant-endophyte interaction has not been fully understood, it has been reported that many isolates provide beneficial effects to their hosts like preventing disease development by synthesizing novel compounds and antifungal metabolites. Several endophytes have been shown to support plant growth and increase nutrient uptake by providing phytohormones, low molecular weight compounds, enzymes, antimicrobial substances like antibiotics and siderophores. Other beneficial effects of endophytes to plants include nitrogen fixation, increased drought resistance, thermal protection, survival under osmotic stress etc. (Khan and Doty, 2009).

Co-culturing with endophytic elicitor is an alternative way to enhance plant secondary metabolites and increase plant resistance. Mechanism of endophytic elicitor induced plant secondary metabolites production is similar to stimulation of plant resistance. Endophytes triggered ISR fortifies plant cell wall strength and alters host physiology and metabolic responses, leading to an enhanced synthesis of plant defense chemicals upon challenge by pathogens and/or abiotic stress factors (Nowak and Shulaev, 2003). The defense gene products include peroxidase (PO), polyphenol oxidase (PPO) that catalyze the formation of lignin and phenylalanine ammonia-lyase (PAL) that is involved in phytoalexins and phenolics synthesis. Other defense enzymes include pathogenesis-related proteins (PRs) such as β-1,3-glucanases (PR-2 family) and chitinases (PR-3 family) which degrade the fungal cell wall and cause lysis of fungal cell. Chitin and glucan oligomers released during degradation of fungal cell wall act as elicitors that elicit various defense mechanisms in the plants. Induction of defense proteins makes the plant resistant to pathogen invasion. Induction of defense proteins has been correlated with defense against pathogen invasion in cucumber, greengram, tobacco and tomato (Hammerschmidt and Kuc, 1995). Hence, with the view of plant health and productivity the proposed studies with special reference to indigenous endophytic microbes for induction of systemic resistance in soybean crop cultivar JS-335, against challenge inoculation with *R. solani* have been carried out.

MATERIALS AND METHOD

Endophytic microorganisms and *R. solani*

In present investigation indigenous endophytic bacteria, actinomycetes and fungi isolated from soybean were utilized. The isolated endophytes were initially screened for *in vitro* antagonistic activity against *R. solani* (Zivkovic et al., 2010; Yuan and Crawford, 1995). The antagonist thus obtained were further screened for the ability to exhibit plant growth promoting ability viz., secretion of plant growth regulators (auxins (indole-3-acetic acid (IAA) and indole-3-pyruvic acid (IPyA), gibberellins (GA3) and cytokinins [isopentenyl adenine (iPa), isopentenyl adenosine (iPA) and zeatin (Z)), HCN and siderophore conditions adopting standard biochemical methodology (Strzelczyk and Pokojska, 1984; Shirling and Gottlieb,1966; El-Tarabily et al., 2009; Tien et al., 1979; Thimmaiah, 2004; Lorck, 2004; Castric and Castric, 1983; Samuel and Muthukkaruppan, 2011; Neilands, 1981; Coleman 1995; Wijesundera et al., 1995; Logeshwaran et al., 2009). The

screened endophytic isolates with dual ability of antagonistic and plant growth promotion (Table 1) were further utilized to study the effect on on induction of systemic resistance in Soybean cultivar JS-335 against challenge inoculation with *R. solani.*

Table 1. Screened endophytic isolates with dual ability of antagonism against *R. solani* and plant growth promotion

Endophytic isolates		PGP trait							
		Plant growth regulators						HCN production	Siderophore production
		Auxins		Gibberellins	Cytokines				
		IAA	IPyA	GA3	iPa	iPA	Z		
JDB3	*Pseudomonas*	+	-	+	+	-	+	+	-
JDB9	*Bacillus sp.*	+	-	+	+	-	-	+	-
JDB23	*Burkholderia*	+	-	-	-	-	-	-	+
JDA5	*Streptomyces*	+	-	-	-	-	+	+	+
JDA6	*Streptomyces*	+	-	-	-	-	-	-	+
JDA9	*Streptomyces*	+	-	-	-	-	-	+	-
JDA15	*Actinoplanes*	+	-	-	-	-	-	-	-
JDF3	*Alternaria sp.*	-	-	-	-	-	-	-	+
JDF12	*Fusarium sp*	+	-	-	-	-	+	+	+

Field experiments were conducted to study the effect of interaction between the isolated pathogens and endophytes with dual attributes on growth performance and disease incidences in soybean cultivar JS-335.

Experimental site and soil

The experiment was conducted at Agriculture Research Farm, Microbiology Research Laboratory, Tondgaon Dist. Washim, (MS) India. It is approximately 22 Km away from Washim city. The soil resembled to be the vertisol type (Figure. 1).

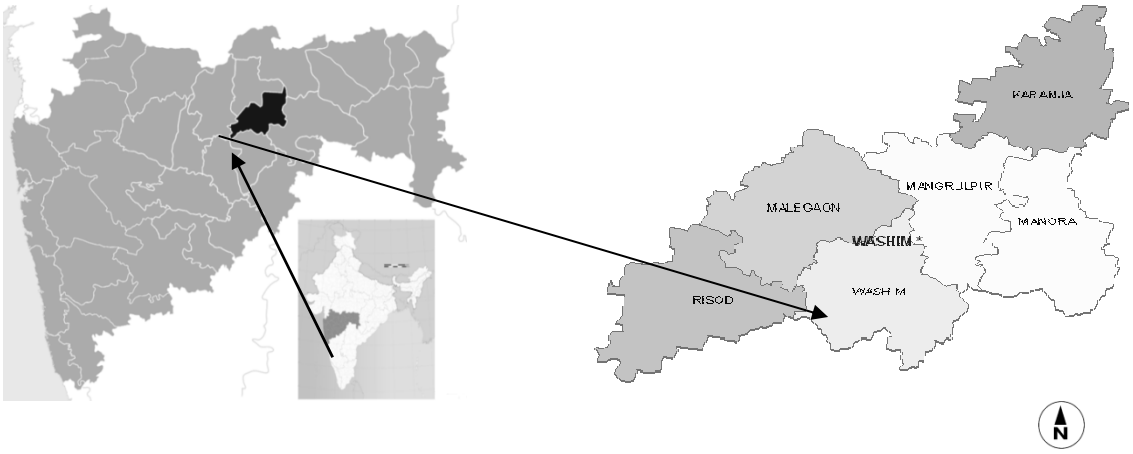

Figure 1. Location of study area

Climatic conditions

The climate of the district is characterized by hot summer and general dryness throughout the year except during the south-west monsoon season, i.e., June to September. The mean minimum temperature is 12°C and mean maximum temperature is 42°C.

Experimental details

The experimentation was carried out during Kharif season of 2012. Micro plots of size 1 m^2 were prepared and used further for experimentation adopting randomized block design with three replications The layout of the plan is presented in Figure. 2 A, B and C and details of the experiments are presented in Table 2. All the experimentation was carried out in plots amended with fungal pathogen *R. solani* sick soil with soybean cultivar JS-335 as the test crop.

(A) (B) (C)

Figure 2. Plan of layout of the experimental sites

Preparation of *R. solani* sick soil

The fungal pathogen sick soil was prepared as described by Totawar, (2001) with slight modifications. *R. solani* was enriched separately in 250 mL of potato dextrose broth and the inoculum was build upto 500 mL each. The inoculum treatment was separately given to cultivated seedlings at 15 DAS. Further the seedlings were examined for disease development at 30 DAS. The screened diseased plants were again processed for isolation of fungal pathogen. Thereafter, the process from the inoculum build up was repeated for six months so as to get the virulent soil. The virulent soil was further fortified manually @ 10 % per kg on the surface of experimental plots. The virulent soil fortified experimental plots were further considered as sick soil microplots. Whereas, microplots without fortification of fungal pathogens were maintained as control.

Treatment details

Soybean seeds were treated with endophytes alone and in combination. Test crops without endophyte treatment were maintained as control. The charcoal based endophytic bio-inoculants were produced (Chandrashekhara et al., 2007; Gopalakrishnan et al., 2012; Sudisha et al., 2006) and used for seed treatments.

Seed treatment with endophytic bio-inoculants

The seeds were surface sterilized with 2 % sodium hypochlorite for 2 min and washed with sterile distilled water and further blotted dry with sterile blotting paper. Seeds were treated with 10 % (w/v) jiggery solution and allowed to dry for 5 min. Seed treatment was done using charcoal based inoculants @ 25 gm/kg of seeds. The charcoal based inoculants were then added to seeds and mixed

uniformly so as to achieve a homogenous coat over seed. Treated seeds were stored in cool and dry place at room temperature away from sunlight. The treated seeds were sown in respective microplots. Seeds without endophytic treatments were maintained as control. The treatments were designated as RT_{1-11}, representing treatments in the *R. solani* sick soil. Necessary agricultural operations viz., thinning, hoeing, and weeding were carried out as and when required with the help of local labors.

Table 2. Treatment details *R. solani* sick soil

Treatment	Details
RT1	Seed treatment with bacterial isolateJDB3
RT2	Seed treatment with bacterial isolateJDB9
RT3	Seed treatment with bacterial isolate JDB23
RT4	Seed treatment with actinomycete isolate JDA5
RT5	Seed treatment with actinomycete isolate JDA6
RT6	Seed treatment with actinomycete isolate JDA9
RT7	Seed treatment with actinomycete isolate JDA15
RT8	Seed treatment with fungal isolate JDF3
RT9	Seed treatment with fungal isolate JDF12
RT10	RT1+RT2+RT3+RT4+RT5+RT6+RT7+RT8+RT9
RT11	Seed treatment with sterile dist. water (Control)

Studies on Induction of Systemic Resistance (ISR)

Sample preparation

Leaf samples were collected at 30^{th}, 60^{th} and 90^{th} days after sowing from the respective plot. Collected leaf samples were washed in running tap water, air dried and further homogenized with pre chilled pestle and mortar. The homogenized leaf tissues were stored at -70 °C until used for biochemical analysis.

Estimation of marker biochemicals

Estimation marker biochemicals viz; phenols, peroxidase (PO), phenylalanine ammonia-lyase (PAL), polyphenoloxidase (PPO), β 1, 3-glucanase and chitinase in endophytes treated and control test plants was carried out as per the methods suggested by Thimmaiah, (2004) and Karande, (2008). The protein content was estimated by Bradford method (Thimmaiah, 2004). The amount of phenolics was expressed as μg catechol g^{-1} fresh tissues, enzyme peroxidase activity was expressed as changes in the absorbance of the reaction mixture min^{-1} g^{-1} fresh weight, PAL activity was expressed as synthesis of trans-cinnamic acid in (n mol quantities) min^{-1} g^{-1} fresh weight, PPO activity was expressed as changes in absorbance of reaction mixture at 495 nm min^{-1} g^{-1} fresh weight, β-1, 3-glucanase activity was expressed as μg glucose released min^{-1} g^{-1} fresh weight and chitinase enzyme activity was expressed as ηmol GlcNAc min^{-1} g^{-1} fresh weight.

Statistical Analysis

All the observations on the interaction studies were processed by standard statistical methods (Panse and Sukhatme, 1985).

RESULTS AND DISCUSSION

Phenol activity

The phenol activity was significantly induced by the endophytic treatments against challenge inoculation with *R. solani* (Table 3). Total phenol content was found to increase initially, it varied from 156.39 – 375.44 µg catechol g^{-1} fresh tissue at 30^{th} DAS, 308.42 – 565.83 µg catechol g^{-1} fresh tissue at 60^{th} DAS and declined at 90^{th} DAS and varied from 417.15 – 520.35 µg catechol g^{-1} fresh tissue. Phenol content was observed to be significantly higher in consortial treatment RT10 (375.44, 565.83 and 520.33 µg catechol g^{-1} fresh tissue) at 30^{th}, 60^{th} and 90^{th} DAS respectively as compared to uninoculated control treatment where it was observed to be 151.44, 310.21 and 245.50 µg catechol g^{-1} fresh tissue respectively. Similarly a significant increase in phenol content was also observed in bacterial treatment RT2 (344.42, 532.45 and 481.36 µg catechol/g fresh tissue) and treatment RT1 (312.25, 503.45 and 475.63 µg catechol g^{-1} fresh tissue) at all three interval.

Table 3. Induction of phenol activity by endophytic treatments against challenge inoculation with *R. solani* in soybean

Treatment details	Mean phenol activity (µg catechol g^{-1} fresh tissue)		
	30^{th} DAS	60^{th} DAS	90^{th} DAS
RT1	312.25	503.45	475.63
RT2	344.42	532.28	481.36
RT3	248.29	454.67	417.58
RT4	253.56	490.37	427.40
RT5	263.39	498.62	442.65
RT6	278.36	490.27	452.39
RT7	156.39	308.42	248.51
RT8	292.41	501.53	463.72
RT9	305.38	495.36	458.40
RT10	375.44	565.83	520.33
RT11	151.44	310.21	245.5
F-Test	Sig	Sig	Sig
SE(m)	0.49	0.53	0.55
CD (5%)	1.43	1.55	1.6

Peroxidase activity

A significant induction of peroxidase activity was observed with all endophytic treatments against challenge inoculation with *R. solani* (Table 4). The extent of induction was varied with treatment and was observed to increase upto 60^{th} DAS however, it declined at 90^{th} DAS. The PO activity ranged between 261.43-398.43 ΔA_{420} min^{-1} g^{-1}at 30^{th} DAS; from 422.31 – 665.32 ΔA_{420} min^{-1} g^{-1}at 60^{th} DAS and from 322.46 – 527.14 ΔA_{420} min^{-1} g^{-1}at 90^{th} DAS. Consortial treatment RT10 (398.43, 665.32 and 527.14 ΔA_{420} min^{-1} g^{-1}at) was observed to be significantly high in inducing PO activity over control RT11 (256.30, 402.36 and 311.51 ΔA_{420} min^{-1} g^{-1}) at all three interval. Similarly a significant increase in PO activity was also observed in bacterial treatment RT1 (367.31, 602.25 and 471.34 ΔA_{420} min^{-1} g^{-1}) at 30^{th}, 60^{th} and 90^{th} DAS.

Table 4. Induction of peroxidase activity by different treatments against challenge inoculation with *R. solani*

Treatment details	Mean peroxidase activity (ΔA_{420} min^{-1} g^{-1})		
	30^{th} DAS	60^{th} DAS	90^{th} DAS
RT1	367.31	602.25	471.34
RT2	342.30	595.74	481.51
RT3	261.43	422.31	363.25
RT4	283.73	493.46	322.46
RT5	262.41	573.66	428.25
RT6	273.30	589.22	439.44
RT7	264.42	428.60	379.16
RT8	271.99	588.57	373.33
RT9	378.37	590.43	448.62
RT10	398.43	665.32	527.14
RT11	256.30	402.36	311.51
F-test	Sig	Sig	Sig
SE(m)	0.96	0.63	0.86
CD (5%)	2.81	1.87	2.53

Phenylalanine amino lyase activity

All the endophytic treatments significantly induced PAL activity against challenge inoculation with *R. solani* (Table 5). The PAL activity was observed to increase upto 60^{th} DAS but declined at 90^{th} DAS. It ranged between 161.30 – 297.88 n mol trans-cinnamic acid min^{-1} g^{-1} at 30^{th} DAS; from 242.29 – 445.18 n mol trans-cinnamic acid min^{-1} g^{-1} at 60^{th} DAS and from 191.29-381.57 n mol trans-cinnamic acid min^{-1} g^{-1} at 90^{th} DAS.

A significantly higher induction of PAL activity was observed in consortial treatment RT10 (297.88, 442.65 and 427.33 n mol trans-cinnamic acid min^{-1} g^{-1}) over uninoculated control RT11 (134.34, 248.42 and 189.18 n mol trans-cinnamic acid min^{-1} g^{-1}) at 30^{th}, 60^{th} and 90^{th} DAS respectively. The bacterial treatments RT1 (267.60, 442.31 and 372.41 n mol trans-cinnamic acid min^{-1} g^{-1}), RT2 (242.14, 445.18 and 381.57 n mol trans-cinnamic acid min^{-1} g^{-1}) and fungal treatment RT9 (223.65, 410.21 and 342.37 n mol trans-cinnamic acid min^{-1} g^{-1}) also significantly induced PAL activity at all three interval. However, the PAL activity in actinomycete treatment RT7 (242.29 n mol trans-cinnamic acid min^{-1} g^{-1}) was observed to be lower as compared to control RT11at 60^{th} DAS.

Table 5. Induction of PAL activity by different treatments against challenge inoculation with *R. solani*

Treatment details	Mean PAL activity (n mol trans-cinnamic acid min^{-1} g^{-1})		
	30^{th} DAS	60^{th} DAS	90^{th} DAS
RT1	267.60	442.31	372.41
RT2	242.14	445.18	381.57
RT3	161.30	323.85	263.60
RT4	183.49	293.55	222.38
RT5	162.26	302.49	228.01
RT6	173.53	289.45	239.60

RT7	136.54	242.29	191.29
RT8	171.17	328.61	273.46
RT9	223.65	410.21	342.37
RT10	297.88	442.65	427.33
RT11	134.34	248.42	189.18
F-test	Sig	Sig	Sig
SE(m)	0.67	0.64	0.58
CD (5%)	1.97	1.88	1.7

Protein content

The protein content was significantly enhanced in the endophytic treatment against challenge inoculation with *R. solani* (Table 6). Protein accumulation varied and it ranged from 1.67 – 2.59 mg/100g at 30th DAS and it increased at 60th DAS. It was 2.33 - 4.24 mg/100g at 60th DAS and declined 1.49 – 3.11 mg/100g at 90th DAS. In consortial treatment RT10 a significantly higher protein (2.53, 4.24 and 3.11 mg/100g) was accumulated followed by bacterial treatment RT1 (2.59, 4.13 and 2.93 mg/100g) over control RT11 (1.51, 1.71 and 1.51 mg/100g) at 30th, 60th and 90th DAS. Protein accumulation was observed to be lower in treatment RT5 at 30th DAS and RT7 at 90th DAS as compared to respective uninoculated control.

Table 6. Induction of protein content by different treatments against challenge inoculation with *R. solani*

Treatment details	Protein content (mg/100g)		
	30th DAS	60th DAS	90th DAS
RT1	2.59	4.13	2.93
RT2	2.06	3.43	2.54
RT3	2.07	2.35	1.52
RT4	1.95	2.45	2.37
RT5	1.67	2.46	1.59
RT6	2.33	2.81	1.60
RT7	2.07	2.33	1.49
RT8	2.05	2.62	1.74
RT9	2.12	3.69	2.23
RT10	2.53	4.24	3.11
RT11	1.71	1.51	1.51
F-test	Sig	Sig	Sig
SE(m)	0.17	0.29	0.59
CD (5%)	0.51	0.84	1.22

Polyphenol oxidase activity

PPO activity was significantly induced against challenge inoculation with *R. solani* in all the endophytic treatment (Table 7). The PPO activity varied from 21.71 –42.34 ΔA_{495} min^{-1} g^{-1} at 30th DAS; from 43.37 - 69.42 ΔA_{495} min^{-1} g^{-1} at 60th DAS. However it declined at 90th DAS and ranged from 34.27 - 54.49 ΔA_{495} min^{-1} g^{-1}. The consortial treatment RT10 (42.34, 63.2 and 54.49 ΔA_{495} min^{-1} g^{-1}) significantly induced PPO activity over uninoculated control RT11 (12.39, 29.33 and 23.40 ΔA_{495} min^{-1} g^{-1}) at all three interval. Similarly, a significant increase in PPO activity was observed in bacterial treatments RT1 (33.42, 60.35 and 47.34 ΔA_{495} min^{-1} g^{-1}), RT2 (35.45, 63.56

and 46.20 ΔA_{495} min^{-1} g^{-1}) and actinomycete treatment RT7 (31.42, 57.31 and 42.23 ΔA_{495} min^{-1} g^{-1}) at 30^{th}, 60^{th} and 90^{th} DAS.

Table 7. Induction of polyphenol oxidase activity by different treatments against challenge inoculation with *R. solani*

Treatment details	Mean polyphenol oxidase activity (µg catechol g^{-1} fresh tissue)		
	30^{th} DAS	60^{th} DAS	90^{th} DAS
RT1	33.42	60.35	47.34
RT2	35.45	63.56	46.20
RT3	23.59	55.50	47.32
RT4	26.64	47.35	38.55
RT5	22.49	43.37	34.27
RT6	21.71	44.33	35.33
RT7	31.42	57.31	42.23
RT8	22.23	48.28	36.61
RT9	22.55	49.35	35.48
RT10	42.34	69.42	54.49
RT11	12.39	29.33	23.40
F-test	Sig	Sig	Sig
SE(m)	0.61	0.56	0.52
CD (5%)	1.8	1.64	1.54

β - 1, 3-glucanase activity

All the endophytic treatments tested significantly induced β - 1, 3-glucanase activity against challenge inoculation with *R. solani* (Table 8). The β - 1, 3-glucanase activity increased upto 60^{th} DAS and declined at 90^{th} DAS. It varied from 110.62 - 299.09 µg glucose min^{-1} g^{-1} at 30^{th} DAS; from 232.50 - 431.38 µg glucose min^{-1} g^{-1} 60^{th} DAS and from 192.38 -340.19 µg glucose min^{-1} g^{-1} at 90^{th} DAS. The consortial treatment RT10 (299.09, 431.38 and 340.19 µg glucose min^{-1} g^{-1}) significantly induced β - 1, 3-glucanase activity followed by RT1 (278.27, 420.16 and 314.86 µg glucose min^{-1} g^{-1}) and RT2 (240.52, 450.50 and 319.25 µg glucose min^{-1} g^{-1}) over control RT11 (132.53, 247.51 and 180.42 µg glucose min^{-1} g^{-1}) at 30^{th} 60^{th} and 90^{th} DAS respectively. However, the β - 1, 3-glucanase activity in treatments RT3, RT5, RT8 at 30^{th} DAS and in RT4 at 60^{th} DAS were lower as compared to respective uninoculated control.

Table 8. Induction of β - 1, 3-glucanase activity by different endophytic treatments against challenge inoculation with *R. solani*

Treatment details	Mean β - 1, 3-glucanase activity (µg glucose min^{-1} g^{-1})		
	30^{th} DAS	60^{th} DAS	90^{th} DAS
RT1	278.27	420.16	314.86
RT2	240.52	450.50	319.25
RT3	110.62	327.41	238.49
RT4	137.49	232.50	228.41
RT5	121.34	323.85	280.64
RT6	136.74	293.46	235.45
RT7	140.48	280.59	192.38

RT8	111.37	388.42	230.47
RT9	280.42	429.42	270.31
RT10	299.09	431.38	340.19
RT11	132.53	247.51	180.42
F-test	Sig	Sig	Sig
SE(m)	0.4	0.61	0.42
CD (5%)	1.17	1.79	1.23

Chitinase activity

Chitinase activity was induced significantly in all the endophytic treatments against challenge inoculation with *R. solani* (Table 9). However the degree of induction varied between different treatments. Chitinase activity varied from 4.93 – 7.64 n mol GlcNAc min^{-1} g^{-1} at 30^{th} DAS; from 5.81- 8.43 n mol GlcNAc min^{-1} g^{-1} at 60^{th} DAS however, a reduction in chitinase activity was observed at 90^{th} DAS and it ranged from 4.45 -7.34 n mol GlcNAc min^{-1} g^{-1}. The individual bacterial treatment RT3 (7.64, 8.43 and 7.34 n mol GlcNAc min^{-1} g^{-1}) significantly induced chitinase activity followed by consortial RT10 (6.73, 7.43 and 6.23 n mol GlcNAc min^{-1} g^{-1}) over uninoculated control RT11 (5.22, 6.32 and 5.17 n mol GlcNAc min^{-1} g^{-1}). However chitinase activity at 30^{th} DAS in treatment RT2, RT6, RT7 and RT9 and at 60^{th} DAS in treatment RT4 and RT6 and at 90^{th} DAS in treatment RT2, RT5 and RT6 were observed to be lower as compared to respective control.

Table 9. Induction of chitinase activity by different treatments against challenge inoculation with *R. solani*

Treatment details	Mean chitinase activity (n mol GlcNAc min^{-1} g^{-1})		
	30^{th} DAS	60^{th} DAS	90^{th} DAS
RT1	5.98	7.00	5.26
RT2	5.20	6.53	5.05
RT3	7.64	8.43	7.34
RT4	5.31	6.11	5.24
RT5	5.55	6.34	4.45
RT6	4.99	5.81	4.89
RT7	5.13	7.28	6.22
RT8	6.42	7.23	5.22
RT9	4.93	6.98	5.64
RT10	6.73	7.43	6.23
RT11	5.22	6.32	5.17
F-test	Sig	Sig	Sig
SE(m)	0.07	0.09	0.07
CD (5%)	0.21	0.26	0.19

Plants have endogenous defense mechanisms that can be induced in response to attack by insects and pathogens. It is well known that the defense genes are inducible genes and appropriate stimuli or signals are needed to activate them. Inducing the plant's own defense mechanisms by prior application of a biological inducer is thought to be a novel plant protection strategy. The synthesis of many secondary metabolites in plants is widely accepted to be part of the defense responses of plants. These metabolites serve as relief mechanisms to grasses resisting biotic and abiotic stresses, including fungal diseases (Kuldau and Bacon, 2008).

The different biochemical markers are involved in protecting the plant from pathogens and the elevated levels of marker biochemicals implies their role against the soil-borne fungal pathogens in soybean. The phenolic compounds may contribute to enhance the mechanical strength of host cell wall and may also inhibit the fungal growth, as phenolics are fungi toxic in nature. PAL is the first enzyme in phenylpropanoid metabolism. PAL activity could be induced in plant pathogen interactions and fungal elicitor treatment (Ramanathan et al., 2000). PAL is a key enzyme in the production of phenolics and phytoalexins in cucumber (Daayf et al., 1997). PO is a key enzyme in the biosynthesis of lignin (Bruce and West, 1989). Increased activity of cell wall bound peroxidases has been elicited in different plants such as cucumber (Chen et al., 2000), rice (Reimers et al., 1992), tomato (Mohan et al., 1993). PR-proteins are host-coded proteins induced by different types of pathogens and abiotic stresses (van Loon, 1997). Synthesis and accumulation of PR proteins have been reported to play an important role in plant defense (Maurhofer et al., 1994; Van Loon, 1997). Maurhofer et al., (1994) reported that induction of systemic resistance by *P. fluorescens* was correlated with the accumulation of *β*-1,3-glucanase and chitinase. These enzymes act upon the fungal cell wall resulting in degradation and loss of inner contents of cells (Benhamou et al., 1996). The enzymatic degradation of the fungal cell wall may release non-specific elicitors (Ham et al., 1991; Ren and West, 1992) which in turn elicits various defense reactions. The fungal cell wall elicitors have been reported to elicit various defense reactions in greengram (Ramanathan et al., 2000).

In present investigation, the role of endophytic microbes in inducing the systemic resistance is analyzed under field conditions. It was observed that endophytes significantly induced the systemic resistance in soybean against challenge inoculation with fungal pathogens. The results on present study are in support with other workers. Benhamou et al., (2000) reported that the endophytic bacterium *Serratia plymuthica* raised levels of phenolics in cucumber roots, affording protection against *Pythium ultimum*. ISR by fluorescent pseudomonads was associated with the production of chitinase, which appeared to be a promising means to manage red rot of sugarcane (Viswanathan and Samiyappan, 1999). Phenolic compounds enhanced the mechanical strength of the host cell walls and also inhibited the invading *Xam.* Seed treatment with *P. fluorescens* 63 caused levels of phenolics to rise in tomato root tissue (M'Piga et al., 1997).

Radjacommare, (2000) reported that *P. fluorescens* strain Pf1 raised levels of PPO isozymes in rice against sheath blight and leaffolder. The chitinases and the 1,3-glucanases (which are classified under the PR- 3 and PR-2 groups of the PR proteins respectively) are reported to be associated with greater resistance in plants against pests and diseases . PAL increased in cucumber treated with the fluorescent pseudomonad to protect it against *P. aphanidermatum,* and this increase was related to enhanced resistance (Chen et al., 2000). Higher levels of PO have been correlated with enhanced ISR in several plants. The roles of chitinases and peroxidases against various pathogens in plants have been reported by Kandan et al., (2002), Chen et al., (2000) and Ramamoorthy et al., (2002) with their direct or indirect role in inducing ISR (Dalisay and Kuc, 1995).

Khan *et al,* (2013) demonstrated that co-synergism of endophyte *Penicillium resedanum* LK6 with salicylic acid helped *Capsicum annuum* in osmotic stress mitigation. They found that endophyte and SA, in combination, reduced the production of ROS by increasing the total polyphenol, reduce glutathione, catalase, peroxidase and polyphenol oxidase as compared to control plants. Osmotic stress pronounced the lipid peroxidation and superoxide anions formation in control plants as compared to endophyte and SA-treated plants.

PO participates in a variety of plant defence mechanisms, and is involved in plant resistance against certain diseases (Silva et al., , 2008; Dutsadee and Nunta, 2008). PAL is the key enzyme of phenols in plants, and PPO can oxidise various phenols into quinones. Both are involved in the resistance-related reactions of plants (Xu and Dong, 2005). PAL has been reported to be upregulated in

Catharanthus roseus cell cultures induced by *Aspergillus niger* elicitor (Juan et al., , 2002; Chen et al., , 2009).
ISR elicited by the endophytes *B. pumilus* strain SE34, *S.marcescens* strain 90-166, and *Pseudomonas fluorescens* strain 89B-61 has been shown to reduce the severity of blue mold of tobacco, caused by *Peronospora tabacina* (Zhang et al., , 2002a, 2002b, 2004). Strains SE34, 90-166, and 89B-61 also significantly reduced disease severity in the detached leaf (injection of a bacterial suspension into petioles) and microtiter plate bioassays (application of bacterial suspensions to roots). Sporulation of the pathogen was significantly reduced by both strains in the detached leaf bioassay. Application of the endophytes as a seed treatment alone elicited significantly enhanced tobacco plant growth but not disease protection study (Zhang et al., , 2004). When the strains were applied as seed treatments followed by a soil drench, both plant growth promotion and ISR were elicited.

CONCLUSION

Combined use of plant growth promoting microorganisms is based on the principles of natural ecosystems, which are sustained by their constituents, that is, by the quality and quantity of their inhabitant and specific ecological parameters *i.e.*, the greater the diversity and number of inhabitants, the higher the order of their interaction and more stable the ecosystem. Present study shows that endophyte induction of systemic resistance (ISR) against challenge inoculation with *Rhizoctonia solani* in soybean (*Glycine max* (L) Merril). Seed treatment with endophytes significantly improced the levels of marker biochemicals viz., phenols, peroxidase (PO), phenylalanine ammonia-lyase (PAL), polyphenoloxidase (PPO), □β–□1, 3-glucanase and chitinase involved in ISR in soybean. Considering the inconsistency, limitations and failures of traditionally used agronomic practices, organic farming and use of PGPRs, the use of endophytes may prove to be beneficial in context of controlling plant diseases and promoting plant growth. The utilization of endophytic microbes with combined potential of plant disease control and growth promotion may result in increased plant growth production, nutrient uptake and protection through induction of systemic resistance.

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