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Evaluation of *Pseudomonas fluorescens* and *Trichoderma harzianum* isolates in inducing systemic resistance (ISR) in maize against *Rhizoctonia solani* f. Sp. *Sasakii*

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Abstract
The induction of defence related enzymes leading to systemic resistance when treated with bioagents like *P. fluorescens* and *T. harzianum* alone and in combination both as seed treatment and foliar application and challenge inoculated with *R. solani* f. sp. *sasakii* the causal agent of banded leaf and sheath blight of maize was studied. On inoculation with *R. solani* the defence related enzymes like peroxidase and phenylalamine ammonia lyase (PAL) significantly increased in all the treatments at different time intervals when compared to control. Induced systemic resistance through as inferred biochemical analysis revealed the increased activities of the enzymes, *viz.* peroxidase (PO) and phenylalamine ammonia lyase (PAL) in the treatments of *P. fluorescens* and *T. harzianum* application as seed treatment and foliar spray alone and in combinations.

Keywords: *Pseudomonas fluorescens* and *Trichoderma harzianum*, maize, peroxidase, phenylalamine ammonia lyase (PAL)

Introduction
Maize (*Zea mays* L.) is one of the most versatile emerging crops having wider adaptability under varied agro-climatic conditions. Globally, maize is known as queen of cereals due to its high genetic yield potential. Among the potential factors that limit maize production, fungal diseases are reported to cause extensive crop yield reduction in many countries and are considered as a priority in disease management practice (Agrios, 2005) [1]. Of different fungal diseases affecting maize cultivation, banded leaf and sheath blight (BLSB) incited by *Rhizoctonia solani* f.sp. *sasakii* Exner (*Thanatephorus sasakii* (Shirai) Tu & Kimbrough) (Tu and Kimbrrough, 1978) [22] is an economically significant disease causing huge losses in all crop growing areas of the world. The disease causes direct losses, resulting in premature death, stalk breakage and ear rot, indirect losses by not only reducing the grain yield but also in grain quality in terms of human consumption. Increased incidence of BLSB has been observed in rice fallow maize crop (zero tillage) in different districts of Andhra Pradesh. Effective management of BLSB in maize is possible only when the pathogen is eliminated completely or the propagules are brought down below economic threshold limits at field level. Control measures used were partly effective because *R. soiani* is able to produce sclerotia that can persist in the soil for at least two years (Ou, 1985) [18]. The pace of development and durability of resistant varieties had been slow and unreliable, chemical control also has its own limitations such as escalated costs, low benefit-cost ratio, selectivity, health hazards, and environmental pollution etc. Considering these limitations, alternatively, use of biocontrol agents is a viable option towards management of plant diseases in a sustainable manner. However, micro-organisms have a relatively narrow spectrum of activity and often exhibit inconsistent performance resulting in limited commercial use of biocontrol approaches for suppression of plant pathogens (Harman 2000 and Chaube *et al*., 2002) [12, 7]. Among several fungal and bacterial biocontrol agents tested, *Trichoderma* and fluorescent *Pseudomonas* are the most researched groups and used against aerial, root and soil pathogens (Van Loon *et al*., 1998) [23].

The use of *Trichoderma* and *Pseudomonas* as seed, root and rhizospheric applications of late has received much attention, as they provide protection through mycoparasitism, competition for nutrition and space, antibiotic production and induced resistance (Van Loon *et al*., 1998) [23] and also Stimulate plant growth. Hence the present experiment was conducted to evaluate the induced systemic resistance ability of *P. fluorescens* and *T. harzianum* at Regional Agricultural Research Station, Lam, Guntur, Andhra Pradesh.

Materials and Methods

Greenhouse studies were conducted to evaluate the efficacy of *P. fluorescens* as well as *T. harzianum* to assess their abilities to induce systemic resistance (ISR) in maize. Maize hybrid, Pioneer 30 V 92 seeds were surface sterilized for 10 minutes in 0.05% sodium hypochlorite solution and rinsed five times with sterilized distilled water. These seeds were treated with liquid formulation of biocontrol agents (@10ml/Kg seed and CFU 10^8/ml) and planted in plastic pots (30 cm diameter) consisting of sterilized soil. Seeds were sown in pots @3/pot and arranged on GH benches in a Randomized Complete Block Design (RCBD) with seven treatments and replicated three times and the details of the treatments given below. Suitable agronomic measures were practiced throughout the study. Foliar application of *Pseudomonas fluorescens* @ 5ml/L and *T. harzianum* @ 5ml/L for plants (as per the treatment details) was imposed on 5 th leaf of plant until run-off at 30 days after sowing (DAS).

Table 1: Details of the treatments used in the experiment given below.

Treatment	Details
T1	Seed treatment with *P. fluorescens* @10ml/kg seed
T2	Seed treatment with *T. harzianum* @10ml/kg seed
T3	Seed treatment with *P. fluorescens* @10ml/kg seed+ foliar application of *Pseudomonas fluorescens* @ 5ml/L
T4	Seed treatment with *T. harzianum* @10ml/kg seed+ foliar application of *T. harzianum* @ 5ml/L
T5	Seed treatment with *P. fluorescens* @10ml/kg seed + Seed treatment with *T. harzianum* @10ml/kg seed
T6	Seed treatment with *P. fluorescens* @5ml/kg seed + foliar application of *Pseudomonas fluorescens* @ 2.5ml/ Seed treatment with *T. harzianum* @5ml/kg seed+ foliar application of *T. harzianum* @ 2.5ml/L
T7	Control

After 24 hrs of foliar application of biogents, the BLSB pathogen, *R. solani* f. sp. *sasaki* was challenge inoculated by toothpick method on the third leaf of the maize plant. Few drops of sterile water were added to the inoculated sheath for maintenance of the moisture. Plants were incubated and maintained on a greenhouse bench at 26±2 ºC and leaf sampling for measurement of enzyme activity were performed at 0, 24, 48, 72, and 96 hours after inoculation of sheaths with *R. solani.* The induction of defense related enzymes *viz*., peroxidase (PO) and phenylalanine ammonia lyase (PAL) were assayed.

Enzyme Extract: The leaf sample, collected from treated and pathogen- inoculated maize plants was immediately homogenized with liquid nitrogen. One g of powdered sample was extracted with 2 ml of sodium phosphate buffer, 0.1M (pH 7.0) at 4ºC. The homogenate was centrifuged for 20 min at 10,000 rpm. Protein extracts prepared from maize tissues were used for estimation of defense enzymes (peroxidase (PO) and Phenylalanine ammonia-lyase (PAL). The supernatants (crude enzyme extract) were immediately used for determination of enzyme activities and total protein. An aliquot of the extract was used to determine the protein content using bovine serum albumin as standard (Bradford, 1976) [3].

Assay for peroxidase (PO): Assay of PO activity was carried out as described; the reaction mixture consisted of 2.5 ml of a mixture containing 0.25 percent (v/v) guaiacol in 0.01M, sodium phosphate buffer, (pH 6.0) and 0.1M hydrogen peroxide. Enzyme extract (0.1 ml) was added to initiate the reaction which was followed colorimetrically at 470 nm. Crude enzyme preparations were diluted to give changes in absorbance at 470 nm of 0.1 to 0.2 absorbance units/min. Boiled enzyme was used as blank. Activity was expressed as the increase in absorbance at 470 nm min^{-1} mg^{-1} of protein (Hammerschmidt *et al*., 1982) [10].

Assay for Phenylalanine ammonia-lyase

Phenylalanine ammonia-lyase (PAL) activity was measured as previously described by Ke and Saltveit (1986) with some modifications. Four g of maize tissue was homogenized with 16 ml 50 mM borate buffer (pH 8.5) containing 5 mM of 2-mercaptoethanol and 0.4 g insoluble polyvinyl polypyrrolidone. The homogenate was filtrated through 4 layers of cheesecloth and centrifuged at 20 000 rpm at 4ºC for 20 min. One-ml aliquots of the supernatant were assayed for PAL activity after addition of 110 ml of 100 mM L-phenylalanine and incubated at 4ºC for 30 min. The proteins were precipitated by the addition of trichloroacetic acid at 4% (w/v) before taking the readings. The samples with TCA were incubated for 5 min at room temperature and centrifuged at 10 000 rpm for 5 min. Absorbance of the supernatant was read at 290 nm at the beginning and after the incubation time. An aliquot of 1 ml of 1/20 dilution of the fraction was assayed similarly to the crude extract of maize tissue when assaying the fractions obtained from purified fusion proteins. PAL activity was calculated as mol of cinnamic acid per g of tissue produced under the specific conditions, or as m mol of cinnamic acid produced per mg of protein per hour for fractions of purified fusion protein.

Results and Discussion

The present experiment was carried out to study the induction of defence related enzymes leading to systemic resistance when treated with bioagents like *P. fluorescens* and *T. harzianum* alone and in combination both as seed treatment and foliar application and challenge inoculated with *R. solani* f. sp. *sasakii* the causal agent of banded leaf and sheath blight of maize.

On inoculation with *R. solani* the defence related enzymes like peroxidase and phenylalamine ammonia lyase (PAL) significantly increased in all the treatments at different time intervals when compared to control. Induced systemic resistance through as inferred biochemical analysis revealed the increased activities of the enzymes, *viz.* peroxidase (PO) and phenylalamine ammonia lyase (PAL) in the treatments of

P. fluorescens and *T. harzianum* application alone and in combinations and the results are presented in Table 2.

Table 2: Changes in peroxidase (PO) and phenylalanine ammonia lyase (PAL) activity in maize leaf sheaths due to seed treatment with *Pseudomonas fluorescens* and *Trichoderma harzianum* alone and in combination, challenge inoculated with *Rhizoctonia solani* f.sp. *sasakii*

Treatment	PO activity (change in absorbance (OD/min/g)					PAL activity(change in absorbance (OD/min/g)					PDI
	Days after inoculation					Days after inoculation					
	0	1	2	3	4	0	1	2	3	4	
T1- Seed treatment with *P. fluorescens* @10ml^{-1}kg	1.17	1.27	1.43	1.43	1.37	0.31	0.47	0.63	0.47	0.43	68.0
T2- Seed treatment with *T. harzianum* @10ml^{-1}kg	1.07	1.17	1.27	1.23	1.27	0.31	0.43	0.47	0.40	0.31	67.3
T3- Seed treatment+ foliar application of *P. fluorescens* @ 5ml^{-1}	1.17	1.47	1.83	1.87	2.00	0.31	1.07	1.13	1.17	1.22	44.7
T4- Seed treatment + foliar application of *T. harzianum* @ 5ml^{-1}	1.13	1.33	1.63	1.77	1.93	0.31	0.93	0.97	0.99	1.07	51.7
T5- Seed treatment *P. fluorescens*@ 5ml^{-1}kg+ *T. harzianum* @ 5ml^{-1}	1.10	1.93	2.13	2.20	2.27	0.31	1.23	1.23	1.27	1.29	62.7
T6- Seed treatment+ foliar application of *P. fluorescens* @ 5ml^{-1} + *T. harzianum* @ 5ml^{-1}	1.13	2.17	2.43	2.57	2.73	0.31	1.43	1.63	1.68	1.72	37.7
Control	1.10	1.17	1.07	1.07	1.23	0.31	0.31	0.32	0.31	0.31	89.0
SEm+	0.03	0.03	0.03	0.03	0.03	0.00	0.02	0.02	0.03	0.02	0.17
CD	NS	0.09	0.09	0.11	0.11	NS	0.06	0.08	0.09	0.05	5.34

* Changes in absorbance /min/mg of protein

Peroxidase Activity

Data pertaining to peroxidase activity as influenced by different treatments of *P. fluorescens a*nd *T. harzianum* alone and in combination on maize seed, leaves and sheaths at different intervals of time *viz.*, 0, 24, 48, 72 and 96 h are furnished in table 2. At zero hour there was no significant difference in enzyme activity in different treatments.

But there after 24 h of inoculation maximum peroxidase activity was recorded in treatment combination of all four treatments *i.e*, seed treatment and foliar application of both *P. fluorescens a*nd *T. harzianum* (T6) (2.17 OD/min/g), which was followed by the seed treatment with both *P. fluorescens a*nd *T. harzianum* (T5) (1.93 OD/min/g). The least peroxidase activity was recorded in the pathogen inoculated control (1.17OD/ OD/min/g). At 48 h and 72 h the same trend was followed but at 96 h there was decline in the peroxidise activity in T1 and T7.

Treatment which includes combination of seed treatment and foliar application of both *P. fluorescens a*nd *T. harzianum* had recorded significantly highest enzyme activity (2.57 OD/min/g). The second best treatment was seed treatment with both *P. fluorescens a*nd *T. harzianum* with 2.20 OD/min/g. The pathogen inoculated control treatment recorded significantly lower enzyme activity compared to all the treatments and started declining from 48h after inoculation (1.07 OD/min/g). At 72 h, peroxidase activity was further increased in all the treatments except pathogen inoculated control, which recorded significantly the lowest enzyme activity (1.07 OD/min/g). In general, the peroxidase activity increased from 24h up to 92 h (except the pathogen inoculated control (T7) and (T1) seed treatment with *P. fluorescens*) and declined thereafter in all the inoculated treatments. Whereas in case of pathogen inoculated control, it gradually declined from 48h itself.

Phenylalanine ammonia lyase (PALase) activity in maize plants

Results of phenylalanine ammonia lyase activity was influenced by different treatments including *P. fluorescens* and *T. harzianum* application alone and in combinations at different intervals *viz*; 0, 24, 48, 72 and 96 h are presented in Table 2. There was no significant difference in enzyme activity in different treatments at zero h after inoculation of the pathogen. At 24 h there was significant increase in phenylalanine ammonia lyase activity due to different treatments when compared to the inoculated control. Combination of seed treatment and foliar application of *P. fluorescens* and *T. harzianum* in combination recorded significantly higher PALase activity (1.72 change in cinnamic acid/min/g) followed by seed treatment with *P. fluorescens* and *T. harzianum* in combination method (1.29) change in cinnamic acid/min/g) at 96 h. And the least enzyme activity was recorded in the inoculated control (0.31 change in cinnamic acid/min/g).

At 72 h, the PAL activity increased in all the treatments except in seed treatment with *P. fluorescens* and *T. harzianum* alone treatments (0.63 to 0.47 and 0.47 to 0.40 change in cinnamicacid/min/g respectively). The PALase activity gradually increased from 24 h to 96 h in seed treatment and foliar application of *P. fluorescens* and *T. harzianum* in combination treatment.

In general, phenylalanine ammonia lyase activity increased from 24 h upto 72 h and declined thereafter in seed treatment of *P. fluorescens* and *T. harzianum* alone treatments. Whereas, in case of pathogen inoculated control, there was no change in the enzymatic activity. It is clear from the data depicted in Table 2 that the combination of seed treatment and foliar application of both *P. fluorescens* and *T. harzianum* reduced the disease by induced systemic resistance. The pathogen was inoculated at the base and the biocontrol agent was inoculated either onto seeds or foliar application on maize plants, thus spatially separated from the pathogen. Yet there was substantial disease control ranging from 28 to 49 per cent, indicating induction of systemic resistance by *P. fluorescens* and *T. harzianum.* This can be evidenced by the data on the accumulation of defense molecules triggered by the strains.

Our results corroborate studies of Bradley *et al.* (1992) [4], who reported that increased PO activity has been correlated with resistance in many species of plants and that these enzymes are involved in the polymerization of proteins and lignin or suberin precursors into plant cell walls, thus

constructing a physical barrier that can prevent pathogen penetration of cell walls or movement through vessels. Also, *T. harzianum* isolates increased PAL enzyme activity in maize seedlings. PAL induced phenyl propanoid metabolism starts with the conversion of L- phenylalanine into *trans*cinnamic acid thus supplying precursors for flavanoid pigments, lignin and phytoalexins (Massala *et al.,* 1980; Hahlbrock and Scheel, 1989) [17, 9]. An increase in PAL activity subsequently might have led to increased levels of the signaling molecule salicylic acid and the phenolic compounds in the host thereby contributing to disease resistance (Klessig and Malamy, 1994; Charitha Devi and Radha, 2012) [15, 6].

PGPRs have been known to bring about ISR by changing the physiological and biochemical reactions of the host leading to the synthesis of defense chemicals against the pathogen (Van loon, 1998) [23]. Although, all the methods of application of *Pseudomonas* sp. increased peroxidase and PALase activities in plant issue when compared to the check, the combined application method recorded the peroxidase activity (79% higher) and PALase activity (343% higher). Peroxidase is a key enzyme in the biosynthesis of lignin (Bruce and West, 1989) [5].

Biocontrol of diseases by PGPR through ISR has been very well documented in a number of other crops (Kloepper *et al*., 1995; Van Loon *et al.,* 1998; Kavitha *et al.,* 2003; Ramamoorthy and Samiyappan, 2001; Bharathi *et al.,* 2004; Gayathri and Bhaskaran, 2005) [23, 13, 2].

Maize plants raised from *P. fluorescens* treated seeds showed higher activity of peroxidase, polyphenol oxidase and PAL, when leaf sheaths were inoculated with the pathogen, *R. solani*. The bacterized seeds with *P. fluorescens* lead to accumulation of higher phenolic compounds and higher activity of PO, PPO and PAL that may play a role in defense mechanism in plants against pathogen (Sivakumar and Sharma, 2003) [21]. PGPR mediated plant growth promotion and ISR were inter-related for their PGPR strains tested. This observation could lead to the hypothesis that the main effect of such PGPR is not ISR, but rather induced physiological changes which are manifested in multiple ways primarily as growth promotion and secondarily as increase in some defense compounds. In our studies also, the highest density of the PGPR strain in the rhizosphere, when inoculated through the combined method, resulted in the maximum growth promotion and biocontrol activity, indicating a strong relationship. From the current study, it can be inferred that there is maximum disease reduction in the application of indigenous *T.* harzianum + *P. fluorescens* against maize BLSB as compared to the other treatements because of multiple beneficial traits- IAA and GA production, PHB production and ability to trigger defense molecules (ISR activity).

This may be due to various reasons, including pathogenicity of isolates, climatic adaptability, influence of the pathogen origin and even the influence of local maize hybrids used in this region (Harman, 2006; Sharon *et al.,* 2007) [11, 20].

The combined application of seed treatment and foliar spray of *P. fluorescens* and *T. harzianum* was found as the most effective method for getting successful establishment of the bioagents on the roots leading to maximum plant growth promotion and biocontrol of early incidence of BLSB disease on maize.

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