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RESEARCH ARTICLE

Induction of systemic resistance by *Trichoderma asperellum* against bacterial wilt of tomato caused by *Ralstonia solanacearum*

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Manuscript Info	Abstract

Trichoderma asperellum was used as a biological control agent against bacterial wilt disease caused by *Ralstonia solanacearum*. The present investigation is focused on the role of defense- related enzymes in imparting resistance to tomato against bacterial wilt pathogen *Ralstonia solanacearum.* Two isolates of *Trichoderma asperellum* (T4 and T8) exhibiting high antagonistic activity against a virulent strain of *Ralstonia solanacearum* (RS) were selected during screening for studies on several biocontrol mechanisms under greenhouse conditions. Seed treatment with *T. asperellum* isolates significantly improved the quality of seed germination and seedling vigor. Efficacy of T4 and T8 isolates, evaluated under greenhouse conditions exhibited suppression by about 50% in disease incidence of bacterial wilt and promoted growth in tomato plant. The isolates T4 and T8 were further tested for their ability to induce the production of defense-related enzymes in plants. The seed application with T4 and T8 isolates induced a significant increase in the activities of peroxidase (POX), Polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL), and $\beta-1$, 3-glucanase in treated tomato plants. Increased activities of phenylalanine ammonia lyase, peroxidase, Polyphenol oxidase, β-1, 3-glucanase were observed in T4 and T8 pre-treated tomato plants challenged with *Ralstonia solanacearum*. *Trichoderma asperellum* treatment also triggered the defense related enzymes involved in synthesis of phenols. Higher accumulation of phenolics was noticed in plants pre-treated with T4 and T8 challenged with *Ralstonia solanacearum*. Native PAGE analysis of both peroxidase (POX) and Polyphenol oxidase (PPO) was carried out for the time course of enzyme activities.

The present study shows that in addition to direct antagonism and plant growth promotion; induction and increased activity of defense related enzymes involved in the phenyl propanoid pathway collectively contributed to enhanced resistance against invasion of bacterial wilt in tomato caused by *Ralstonia solanacearum*.

1. Introduction

Bacterial wilt caused by *Ralstonia solanacearum* is one of the important tomato plant diseases globally. (Hayward, 1991, Milling et al., 2011).The worldwide importance of this disease is due to its destructive nature, wide host range, and geographical distribution of the pathogen. *Ralstonia solanacearum* is a soil-borne pathogen, highly diverse, complex species and has a large host range of more than 200 species in 50 families (Aliye et al., 2008). It occurs throughout most tomato growing areas causing severe crop damage to both greenhouse and field depending upon the varieties of tomato cultivars used and the environmental conditions (Vanitha et al., 2009).It is also known to cause disease in other economically important crops such as potato, eggplant, chilly and non

Solanaceous crops such as banana and groundnut (Anuratha et al., 1990) proving to be a chief constraint in the production of many important vegetables, fruit, and cash crops. The yield losses may vary between 10.8 and 90.6 percent depending on the environmental conditions and the stage at which infection occurs (Kisun, 1987).

Bacterial Wilt poses a constant threat to tomato in Karnataka, Madhya Pradesh, Marathwada region of Maharashtra and West Bengal in India and is amongst the most difficult diseases to control (Kucharek, 1998).The pathogen infects susceptible plants in roots, usually through wounds (Pradhanang et al., 2005) and colonizes within the xylem preventing the water movement into upper portion of the plant tissue (Kelman 1998). Although crop rotation with non-host crops might suppress soil borne populations of the pathogen (Ahmed et al., 2000), the pathogen survives in the environment in association with weed hosts, which impairs the effect of crop rotation.

 Chemical control is most effective when multiple treatments are applied but presents an array of negative side effects such as environmental pollution, detrimental health effects for farmers and consumers, and the risk of emergence of resistant pathogen strains. The development of more environmentally friendly control methods, such as biological control using antagonistic microbes can help to complement current strategies for integrated management of the disease (Mbarga et al,. 2012). Bio-control mechanisms are likely to be specific for particular antagonists that interfere with plant pathogens and pests.

Plants possess a wide array of active defense responses that contribute to resistance against a variety of pathogens. They respond to bacterial pathogen attack by activating various defense responses including changes in cell metabolism, primarily in the enzyme activities that consist of phenylalanine ammonia lyase (PAL), peroxidase (POX), polyphenol oxidase (PPO), lipoxygenase, superoxide dismutase, and β-1,3-glucanase (Babitha et al., 2004, Shivakumar et al., 2002, Girish et al., 2005) and are associated with the accumulation of several factors like defense related enzymes and inhibitors that serve to prevent pathogen infection. In this context, novel eco-friendly and safe strategies have been explored for the management of plant diseases. Thus, induced systemic resistance (ISR) is one of the important methods and is gaining worldwide importance and acceptance (Pieterse et al., 1998; VanWees et al., 2008).

Systemic induction of plant resistance in the host plant is triggered by a series of morphological and biochemical changes initiated by specific strains of fungi that colonize and penetrate plant root tissues resulting in the synthesis of defense chemicals against pathogens (Siva Prasad et al., 2013). Colonization is long lasting and results in the systemic enhancement of resistance through the release of defense elicitors.

Fungal invasion in the vascular tissue or epidermal cells of plant root leads to the accumulation of signal molecules like salicylic acid (SA) and jasmonic acid (JA) which induces the production of pathogenesis-related proteins (PR protein) by plant in defense to pathogen infection (Wasternack et al., 2006). The level of defense-related enzymes determines the degree of host resistance. Increase in activity and accumulation of these enzymes also depends on the plant genotype, physiological conditions and the pathogen.

Trichoderma species are found in soil and on decaying wood, vegetable matter and as well as in plant rhizospheres (Kubicek et al., 2008; Jaklitsch, 2009) and have the ability to use a broad range of compounds and secrete a wide variety of enzymes which in turn are capable of breaking down recalcitrant plant polymers into simple sugars for energy and growth. They are used as biocontrol agents as they aid in reduction of soil borne diseases of various crops (Lumsden et al., 1989) and include more benefits on plants such as promoting plant growth, increased nutrient uptake from the soil, and decreasing the activity of the soil borne pathogens that have a deep impact on plant growth (Harman et al., 2004).

A single Trichoderma strain may induce systemic effects in numerous plant species against a number of diseases (Harman et al., 2004). *T. asperellum* have multiple mechanisms of action, including coparasitism via production of β-1-3 glucanases and β-1-4 glucanases, antibiotics, competition, solubilization of inorganic plant nutrients, induced resistance and inactivation of the pathogen's enzymes involved in the infection process.

Phenylalanine ammonia lyase (E.C. 4.1.3.5), the first enzyme in the phenyl propanoid pathway, catalyses the conversion of L-phenylalanine to trans- cinnamic acid which in turn enters different biosynthetic pathways leading to lignin synthesis. This defense mechanism is used for protection against pathogen invasion. Thus, changes in PAL activity are the key events in controlling the synthesis of phenyl propanoids and hence PAL is one of the most extensively studied enzymes in plants. Induction of PAL as a response to pathogen infection is well documented in various host-pathogen interactions (Geetha et al., 2005, Kavitha et al., 2012).

Peroxidases (E.C.I.ll.I.7) play the most important role in the plant's biochemical defense against microbial pathogens. It is involved in substrate oxidation, cell wall lignification, photosynthesis, respiration and growth regulation. (Srivastava, 1987) and play key roles in plant-pathogen interactions.

Polyphenol oxidase (E.C.1.14.18.1), a copper containing antioxidant enzyme, oxidizes phenolics to highly toxic quinines which are apparently toxic to pathogens and thereby contribute to disease resistance. Quinones also play a

significant role in the lignin biosynthesis (Umesha et al., 2006). PPO has been implicated as functioning in the defense mechanism against insects and plant pathogens (Das et al., 2004).

Pathogenesis-related proteins are host coded proteins that are induced by pathogens and abiotic stresses (Van Loon et al., 1998). One of such PR protein is β -1,3-glucanase (PR-2) (Kauffmann et al., 1987) which has the potential to hydrolyze β -1,3-glucan, which is the major component of pathogen cell wall, leading to direct inhibition of growth of several plant pathogens(Leah et al., 1991). Glucanases have an indirect role in stimulating plant defense by releasing oligosaccharides from the pathogen cell walls by their enzymatic action which act as "elicitors" or inducers of several defense genes (Ryan, 1987).

The objectives of the present study include the characterization and evaluation of the *Trichoderma asperellum* isolated from rhizosphere soil samples of healthy tomato plants against bacterial wilt caused by *Ralstonia solanacearum* under greenhouse conditions and the induction of defense enzymes such as phenylalanine ammonia lyase, Peroxidase, Polyphenol oxidase, β-1,3glucanase by *Trichoderma asperellum* in seedlings challenge inoculated with *R. solanacearum*.

2. Materials and methods:

2.1 . Isolation and identification of *Ralstonia solanacearum* and *Trichoderma asperellum*

Ralstonia solanacearum was isolated from infected soil and plant materials collected from diseased tomato fields (Narasimha Murthy et al., 2012). *T. asperellum* was isolated from rhizosphere soil samples of healthy tomato plants and screened against *R. solanacearum* (Narasimha Murthy et al., 2013).The identification of the selected strains were confirmed by molecular methods based on 16s rRNA and ITS sequencing for *R. solanacearum* and *T. asperellum* respectively. NCBI - BLAST search was performed and the top hit sequences were multiple aligned and phylogenetic tree was constructed using CLUSTAL X2 2.1 (Windows version) software by Neighbor-Joining (NJ) analysis with 1,000 bootstrap replications based on the algorithm of Waterman, (1986). The sequences were deposited to NCBI database.

2.2. Effect of *T. asperellum* on tomato seed germination and seedling vigor index and their evaluation under greenhouse conditions

The effect of *T. asperellum* strain on seed germination and vigor index of seedlings was evaluated under laboratory conditions. Suppression of bacterial wilt of tomato and enhancement of plant growth under greenhouse conditions was evaluated (Narasimha Murthy et al., 2013).

2.3. Induction of defense mechanisms and experimental design

a). Collection of plant samples: Seedlings raised from untreated and *T. asperellum* treated seeds were carefully uprooted at different time intervals viz., 0, 1, 3, 5, 7, 9, 11, 13 and 15 days after challenge inoculation with the pathogen. Challenge inoculation was carried out on 20 day old seedlings. Four plant samples were collected from each replication of the treatment separately and used for analysis.

The following treatments were included in the experiment (1) Seedlings raised from untreated seeds(C); (2) seedlings raised from untreated seeds and challenge inoculated with *R. solanacearum* (T_1) (3) seedlings raised from *T. asperellum* treated seeds (T_2); (4) seedlings raised from *T. asperellum* treated seeds and challenge inoculated with *R. solanacearum* (T_3).

For the enzyme assay, one gram of tomato plant sample was homogenized with 2 ml of 50 mM sodium phosphate buffer (pH 6.0) at 4°C. The homogenate was centrifuged for 2.0 minutes at 10,000 rpm. The supernatant was used as a crude extract for enzyme activity. Crude enzyme extract in 0.1 M sodium phosphate buffer (pH 7.0) was used for the estimation of Peroxidase (POX), Polyphenol Oxidase (PPO), Phenylalanine Ammonia Lyase (PAL)and β 1, 3-glucanase.Enzyme extract was stored in deep freezer (-70°C) and utilized for further biochemical analysis.

Protein content of the crude extracts was determined using the Bradford (1976) protein assay, with bovine serum albumin (BSA) as a standard.

b). Peroxidase assay (POX, E.C.1.11.1.7):

Peroxidase (POX) activity was carried out according to the procedure described by Hammerschmidt et al., (1982). The reaction mixture consisted of 2.5 ml of a mixture containing 0.25 per cent (v/v) guaiacol in 0.01 M sodium phosphate buffer of pH 6.0 and 0.1 M hydrogen peroxide. Crude enzyme preparations were diluted to give changes in absorbance at 470 nm with differences in 0.1 to 0.2 absorbance units/min. The enzyme activity was expressed as changes in the O.D. min^{-1} g^{-1} protein.

c). Polyphenol oxidase assay (PPO, E.C. 1.14):

Polyphenol oxidase (PPO) activity was determined as per the procedure given by Mayer et al., (1965). The reaction mixture consisted of 1.5 ml of 0.1 M sodium phosphate butter (pH 6.5) and 200μl of the enzyme extract. To start the

reaction, 200µl of 0.01 M catechol was added and enzyme activity was expressed as change in absorbance at 495 nm min^{-1} g^{-1} proteins.

d). Phenylalanine ammonia lyase assay (PAL, E.C. 4.1.3.5):

PAL activity was assayed by a modified form of a procedure of Lisker et al., 1983. The reaction mixture contained 1 ml enzyme extract, 0.5 ml substrate, 50 mM L-phenylalanine and 0.4 ml 25 mM Tris-HC1 buffer (pH 8.8). After incubation for 2 h at 40°C, the activity was stopped by the addition of 0.06 ml 5 N HC1 and the absorbance was read at 290 nm against the same volume of reaction mixture without L-phenylalanine that served as blank. The enzyme activity was expressed as trans-cinnamic acid min^{-1} g^{-1} protein.

e). Estimation of β-1, 3 glucanase (E.C. 3.2.1.39):

β-1, 3glucanase activity was estimated according to the method described by Pan et al., (1989) with glucose as standard. Laminarin was dissolved in 0.05 M sodium acetate buffer (pH 5.2) to get concentration of 0.1% and was used as the substrate. 50µl of Crude enzyme was incubated with substrate for 15 min at 37°C. The enzyme-substrate reaction was stopped by adding 0.5 ml of DNS reagent by boiling for 10 min and 2 ml distilled water was added later to each tube and the product released was estimated for reducing groups at 540 nm. The specific activity of β-1, 3-glucanase was expressed as µg glucose released min^{-1} g^{-1} protein. (Vinay B.R et al., 2013)

f). Estimation of total Phenolics:

One gram of plant sample was homogenized in 10 ml of 80% (v/v) methanol and agitated for 15 min at 70°C (Zieslin and Ben-Zaken, 1993). One ml of the methanolic extract was added to 5 ml of distilled water and 250 µl of Folin-Ceocalteau reagent (1 N) and the solution was kept at 25°C. The absorbance of the developed blue color was measured using a spectrophotometer at 725 nm. Catechol was used as the standard. The amount of phenolics was expressed as µg catechol mg^{-1}.

Native-PAGE analysis of POX and PPO enzymes:

The isoform profiles of POX and PPO were examined by Native polyacrylamide gel electrophoresis (Native-PAGE) following the procedure of Laemmli (Laemmli, 1970) with modifications. Tomato seedlings, both treated and control were collected for POX and PPO enzymes. The protein extracts were prepared by homogenizing 1 g of seedlings in 1 ml of 0.1 M sodium phosphate buffer (pH 7.0) and centrifuged at 18,000 rpm for 15 min at 4°C. The protein content of the samples was estimated. Samples were loaded onto 8% (w/v) polyacrylamide gels in a vertical mini gel electrophoresis unit (Bangalore Genei, Bangalore, India). The electrode buffer was Trisbase (3.0 g Tris-base, 7.2 g glycine and 1l distilled water). Electrophoresis was performed at a constant voltage of 50 V initially for 1 h and thereafter to 100 V to complete electrophoresis.

2.4. Activity staining for POX after electrophoresis

POX isoforms were visualized by soaking the gels in staining solution containing 100 mg benzidine dissolved in 1 ml of absolute alcohol and made up to 40 ml using distilled water. A clear solution was obtained when 500 µl of glacial acetic acid was added to the above mixture. Undissolved particles of benzidine were removed by filtering the solution through cotton. At the end, 250 µ1 of H$_2$O$_2$ was added to the filtered solution and gels were incubated in the above solution until blue bands appeared (Schrauwen 1966).

2.5. Activity staining for PPO after electrophoresis

The isoforms of PPO activity staining was performed by incubating the gels in 50 mM Tris buffer (pH 6.8) containing 500 mg catechol and 300 mg of L-3, 4- dihydroxyphenylalanine (L-DOPA) on a rotary shaker. After 10 min of incubation, dark bands indicative of PPO isozymes appeared in the gel (Kavitha et al 2008).

3. Statistical analysis

All data from laboratory and greenhouse experiments were analyzed separately for each experiment and were subjected to analysis of variance (ANOVA) (SPSS, version 16). Significant effects of treatments were determined by the F values (P≤0.05). Treatment means were analyzed using Scheffe post hoc test.

4. Results

Among the ten Trichoderma isolates screened for antagonistic activity, *T. asperellum* isolates (T4 and T8) exhibiting the highest inhibition on all the test strains of *R. solanacearum* were selected for further ISR studies. Treatment with *T. asperellum* increased seed germination and seedling vigor as compared to control. The amplified PCR products were sequenced and a phylogenetic tree (Figure 1 and 2) was constructed by the blast analysis and multiple sequence alignment data. The sequences were deposited in NCBI GenBank with Accession No. (T4): KF679342 and (T8): KF679343.

Figure 1: Phylogenetic relationships of *R. solanacearum* isolates inferred by Neighbor-Joining (NJ) bootstrap tree analysis of 16S rRNA sequences. Sequences used for this comparison were obtained from GenBank.
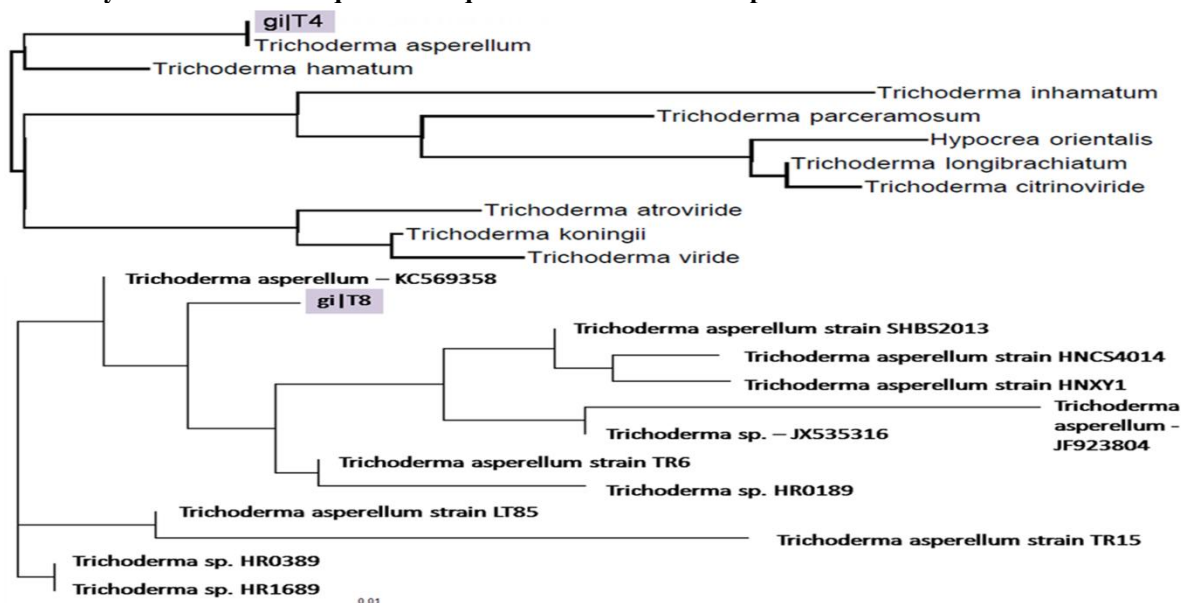

Figure 2: Phylogenetic relationships of *Trichoderma asperellum* (T4&T8) isolates inferred by Neighbor-Joining (NJ) bootstrap tree analysis of ITS sequences. Sequences used for this comparison were obtained from GenBank.

The efficacy of *T. asperellum* in control of *R. solanacearum* wilt in tomato plants under greenhouse conditions was evaluated and was found to significantly increase the plant growth promotion compared to the control. Analysis of plant height, fresh weight and dry weight of 30 days old, challenge inoculated seedlings revealed that *T. asperellum* treated seedlings showed increased growth over control plants. The fresh weight, shoot length, root length, dry weight, root growth and disease incidence were tabulated (Table.1).

Table.1: Plants growth promotion studied under greenhouse conditions were done using 30-day-old–seedlings grown from *T. asperellum* treated seeds.

Treatment	Plant height	Fresh weight	Root Length	Shoot length	Dry weight	Disease Incidence (%)
Control	15.33[d]	18.73[c]	8.30[c]	12.76[c]	4.93[c]	0.0000[a]
RS	8.23[a]	9.16[a]	4.33[a]	7.90[a]	2.43[a]	86.83[c]
T4	16.53[c]	19.76[c]	9.10[c]	13.30[c]	5.53[d]	0.00[a]
T8	15.66[c]	18.70[c]	8.83[c]	12.83[c]	5.26[c]	0.00[a]
T4 + RS	13.30[b]	15.53[b]	6.76[b]	10.30[b]	4.06[b]	35.33[b]
T8+RS	12.86[b]	15.23[b]	6.50[b]	10.00[b]	3.93[b]	38.0[b]

Distilled water treated seeds served as control. Scheffe post hoc test: Means sharing different alphabetical (a, b, c) superscripts in a column significantly different (P<0.05). RS– *R. solanacearum*.

A Pot trial was conducted to study the induction of biochemical defense mechanisms in tomato seedlings in response to treatment with *T. asperellum*.

A significant increase in PAL activity was noticed in all the treated seedlings. Treatment with *T. asperellum* (T4 &T8) significantly increased PAL enzyme activity challenge inoculated with *R. solanacearum*. The PAL activity reached the highest on 7th day after challenge inoculation and then slowly declined. However, treated tomato seedlings exhibited significantly higher PAL activity than the control plants at all-time intervals. *T. asperellum* (T4 &T8) treatment led to an increase in PAL activity of up to 81% and 78%, respectively, relative to control plants (Figure 3).
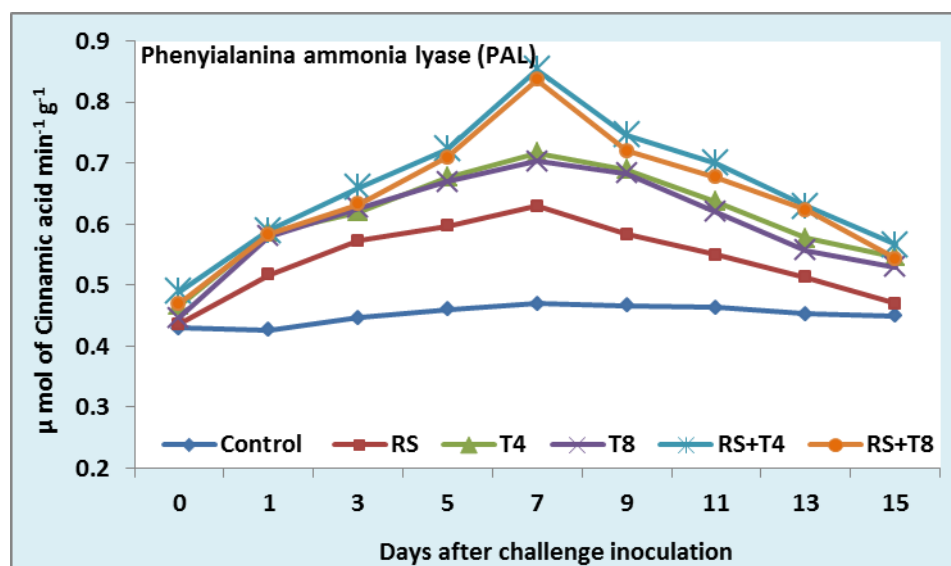

Figure 3. The effect of treatment of *T. asperellum* isolates on the activity of phenylalanine ammonia lyase (PAL) in tomato seedlings. Values are the mean of three replications and bars represent SE. C – Control RS– *R. solanacearum*.

T. asperellum (T4 &T8) treatment in tomato seedlings showed significant increase in POX activity. The activity of POX reached the highest level in all the treatments on 9th day after challenge inoculation and then slowly decreased as compared to control. The protein samples of treated seedlings of tomato were analyzed for expression of POX. A total of four samples (C – Control, RS–*R. solanacearum*, T1 – *T. asperellum* + RS, T2 –*T. asperellum*) of POX were expressed and its intensity of bands varied between control and inoculated seedlings (Figure 4).
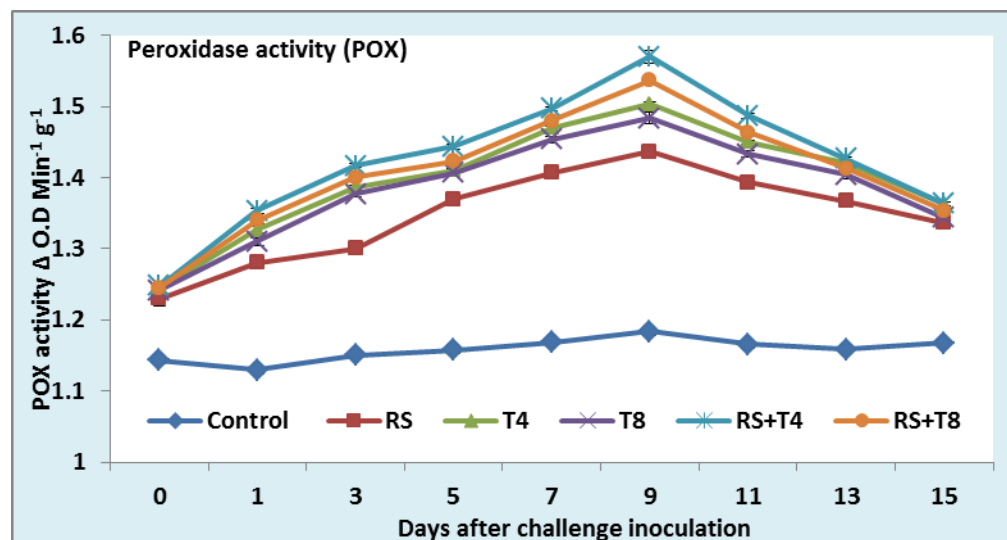
Figure 4. **The effect of soil application of *T. asperellum* (T4 &T8) on the activity of peroxidase (PO) in tomato seedling samples. Values are the mean of three replications and bars represent SE. C – Control RS–*R. solanacearum*.**

An increase in PPO activity was also observed in tomato seedlings upon treatment with *T. asperellum*. The PPO activity increased significantly by one day after challenge inoculation and reached the highest level on 9th day. The enhanced induction of PPO was evident by activity gel electrophoresis (Figure 5).
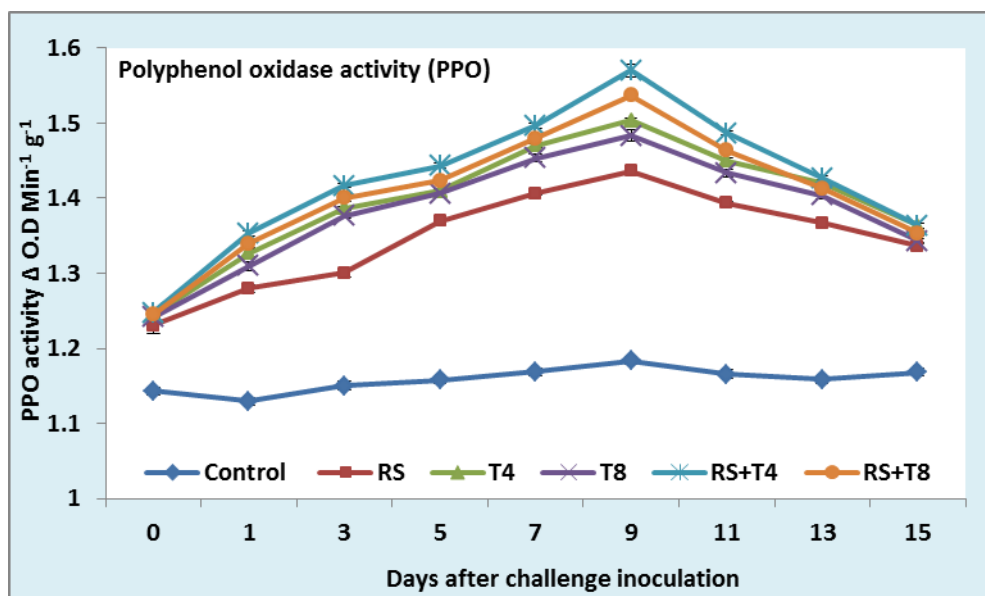

Figure 5. **The effect of soil application of *T. asperellum* (T4 &T8), on the activity of Polyphenol oxidase (PPO) in tomato seedlings. Values are the mean of three replications and bars represent SE. C – Control RS–*R. solanacearum*.**

Treatment of tomato seedlings with *T. asperellum* (T4 &T8) resulted in a high phenol accumulation in plant extracts. Seed treatment of *T. asperellum,* resulted in the maximum accumulation of phenol (620 µg g^{-1} catechol) when compared to the control (265 µg g^{-1} catechol). The accumulation of phenol increased from 1st day after challenge inoculation with *Ralstonia solanacearum* and reached the maximum level on 7th day (Figure 6).
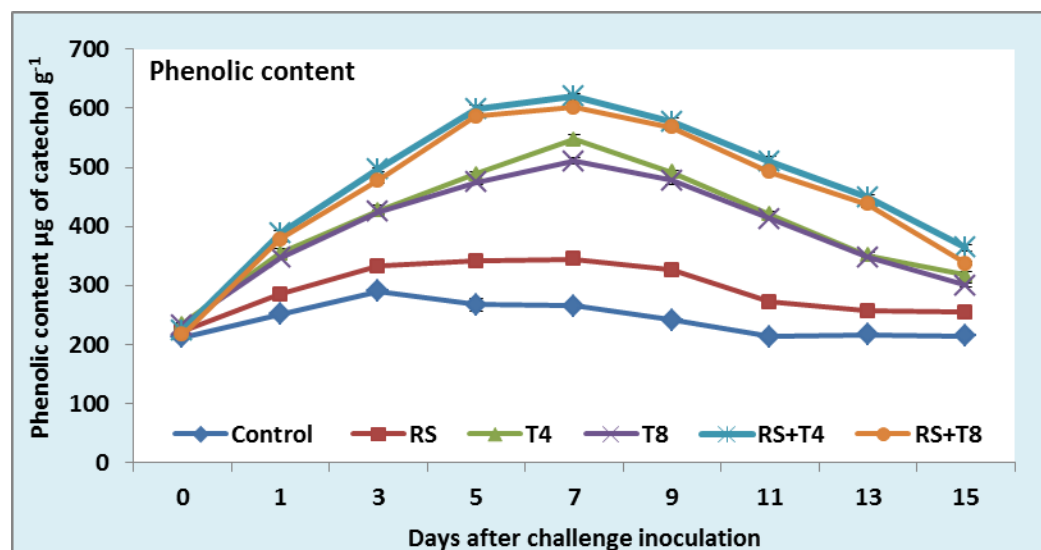
187

Figure 6.The effect of seed treatment of *T. asperellum* (T4 &T8) on the catechol concentration in tomato plant extract. Values are the mean of three replications and bars represent SE. C – Control RS–*R. solanacearum*.

An increase in β-1, 3-glucanase activity was also observed in tomato seedlings treated with *T. asperellum* and *R. solanacearum*. The β-1, 3-glucanase activity increased within 1 day after challenge inoculation, reached the highest level at 9th day and declined thereafter. Application of *T. asperellum* resulted in increase of β-1, 3-glucanase activity compared to the control (Figure 7).
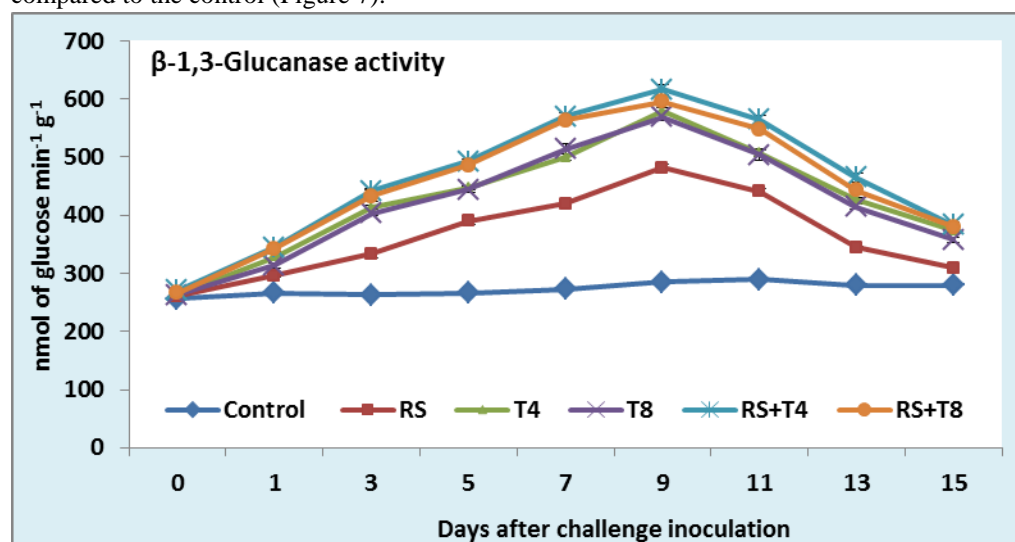

Figure 7.The effect of soil application of *T. asperellum* (T4 &T8) on the activity of β-1, 3-glucanase in tomato plant extract. Values are the mean of three replications and bars represent SE. C – Control RS–*R. solanacearum*.

The protein samples of seedlings of tomato were analyzed for expression of PPO. A total of four samples (C – Control, RS– *R. solanacearum*, T1 – *T. asperellum* + RS, T2 – *T .asperellum*) of different treatments were expressed and the band intensities varied between control and inoculated seedlings. Native PAGE analysis revealed that five POX isoforms designated as POX1, POX2, POX3, POX4 and POX5 were observed in *T. asperellum* tomato plant extracts. The extracts challenged with pathogen *R. solanacearum* and the expression of POX4 and POX5 were more prominent in T3 treatments. Protein extracts from control exhibited only 2 isozymes with lesser intensity when compared to treatments (Figure 8). Four PPO isoforms- PPO1, PPO2, PPO3 and PPO4 were observed in tomato plants treated with *T. asperellum* challenged with *R. solanacearum*. PPO2 and PPO3 exhibited higher activity when compared to control. In control PPO1, PPO2, PPO3 and PPO4 isoforms were expressed with lower intensity than
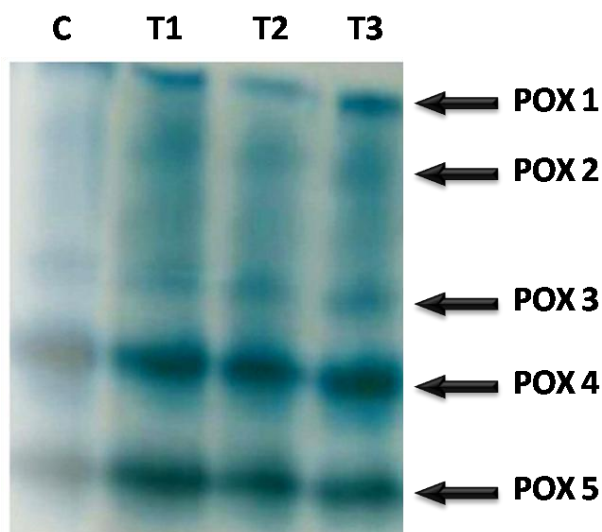
treatments (Figure 9).

Figure 8 Native PAGE profile of POX activity in tomato plant extracts. C – Control, T1 –treatment with RS, T2 – treatment with *T. asperellum*, T3 – treatment with RS + *T. asperellum* (C – Control, RS– *R. solanacearum*)
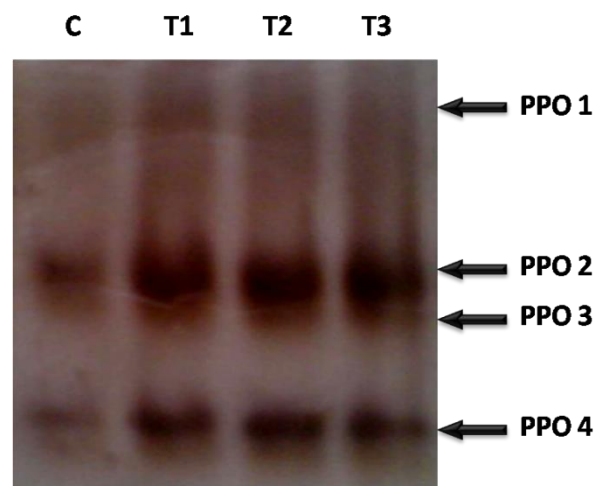

Figure 9 Native PAGE profile of PPO activity in tomato plant extracts. C – Control, T1- treatment with *T. asperellum*, T2 — treatment with RS, T3 – treatment with RS + *T. asperellum* (C – Control RS–*R. solanacearum*)

5. Discussion:

Trichoderma spp. are effective biocontrol agents for a number of soil-borne plant pathogens and plays a major role in controlling plant diseases. Some isolates are also known for their ability to induce systemic resistance in plants against different foliar pathogens (Yedidia, et al., 1999).

Plants are able to defend themselves upon numerous phytopathogen attacks by producing a wide range of defense enzymes that enhance both cellular protection and disease resistance. The aim of the current study was to check the effect of *T. asperellum* on tomato seed germination and evaluate its efficacy under greenhouse conditions and to be utilized as a potential antagonist against the *R. solanacearum* associated with bacterial wilt of tomato. *T. asperellum* has shown to possess antagonistic activity against bacterial wilt pathogen *R. solanacearum* both in laboratory by excreting lytic enzymes such as β1, 3- glucanases which can lyse the cell wall of the pathogen and also in green house conditions (Narasimha Murthy et al., 2013).

Glucanases are involved in direct control of a pathogen, while peroxidases are used primarily for the synthesis of secondary metabolites and are known to be induced by various types of stresses including pathogen infection

(Delannoy et al., 2003; Sasaki et al., 2005). Increased peroxidase activity, high level of mRNAs encoding for phenylalanine ammonia lyase was reported during the interaction between host plants and various bacterial endophytesas indicated by Zdor and Anderson (1992). In our study, a significant increase in PAL activity till the seventh day was observed in theT4 &T8 treated seedlings challenge inoculated with *R. solanacearum* indicating the induction of resistance in host plants. The PAL activity slowly decreased following the seventh day. The control seedlings with or without pathogen infection reported the lowest PAL activity without much variation (Fig 3). However, the seedlings inoculated with the T4 &T8 isolates alone (without challenge inoculation) also exhibited high PAL activity of upto 81% and 78% in comparison with the control.

Peroxidase is an useful marker of plant development, physiology, infection and stress (Welinder, 1992).Vidhyasekaran (1997) reviewed that the peroxidase activity was more in the plants with infection by the pathogens and has great role in inhibit the pathogen development. Interestingly, peroxidase activity of our treated tomato plant extracts was higher than that of the control even in the absence of *R. solanacearum* infection. The POX activity reached the peak level in the entire treatment on day 9 after challenge inoculation and this increase trend was maintained throughout the time intervals (Figure 4).These results are in conformity with a previous study (Van Wees *et al.*, 2008) in which the authors illustrated that defense responses in primed plants are not activated directly but are accelerated upon attack by pathogens or insects, resulting in faster and stronger resistance to the invader encountered.

This study also noticed that priming tomato seedlings withT4 and T8 isolates upon subsequent stimulation by *Ralstonia solanacearum* attack, induced PPO gene expression and their levels increased significantly in 24 hours, and that these expression levels were found to be highest on day 9 post inoculation (Fig. 5). The significant increase in the PPO gene expression levels were further confirmed by activity gel electrophoresis in which PPO2 and PPO3 isoforms exhibited higher activity compared to control. Our results are in affirmation with the study of (Sriram et al., 2009, Nawrocka et al., 2011) wherein a gradual increase in polyphenol content was observed when treated with *T. harzianum* T10 or *T. harzianum*T61.

The present study also finds the high accumulation of phenols in plant extracts treated with *T. asperellum* isolates when compared to the control, the maximum level being attained on the seventh day (Figure 6).

The activity of GLU was significantly higher inT4 and T8 plus pathogen-inoculated plants with a relevant increase from the first day to the ninth day and declined thereafter. All the treated seedlings showed a constant increase inβ-1, 3-glucanase activity when compared to the control (Figure 7).

Additionally, the expression levels of PAL, POX and PPO genes were significantly higher than the control plus pathogen-inoculated plants, while no induction was observed in uninoculated control seedlings. Our findings suggest that both pathogen and PGPFs are needed to induce expression of POX and GLU, whereas priming of PGPFs alone is sufficient for PAL gene induction. This finding is well supported by the study of Shoresh et al., (2005), who reported high induction of *PAL* in cucumber plants treated with *T. asperellum*.

Similarly, systemic resistance was enhanced in response to *R. solanacearum* challenged in tomato due to high accumulation of defense enzymes (Vanitha et al., 2009).

The accumulation of PAL, POX, and GLU mediated by the *T. asperellum* was reported in tomato (Kavitha et al, 2008). Further, Vanitha and Umesha (2009) demonstrated that rapid and high induction of *PAL* and *POX* was noticed in *Pseudomonas fluorescens*-pretreated tomato seedlings, which were inoculated with *R. solanacearum*. The interaction between tomato and *Verticillium dahlia* elicited enhanced activities of POX and PAL, phenylpropanoid metabolism, and synthesis of lignins (Gayosoet al 2010).

In conclusion, the current investigation has lead to the identification of two *T. asperellum* isolates T4 and T8 which were able to trigger the susceptible tomato cultivar into developing resistance against bacterial wilt pathogen. Further, these results also provide evidence of interconnecting ISR and defense responses. The induction of SA-responsive genes (POX and GLU) was unregulated when tomato plants were in contact with the pathogen *R. solanacearum*, and also stimulated the immune response of tomato before and after challenging with *R. solanacearum* pathogen by activating PAL, a defense gene which confers ISR. From the results obtained, it can be established that *T. asperellum* is a potential biocontrol agent to control bacterial wilt of tomato caused by *R. solanacearum*. The treated plants had improved plant health by enhanced nutrient uptake from the soil under greenhouse conditions. These two isolates are useful in the development of protection against bacterial wilt to impart high-yielding tomatoes. Further studies on field trials are required to establish *T. asperellum* as an effective and successful biocontrol agent under natural conditions.

6. Conclusion:

Trichoderma species are among the most-promising biocontrol fungi against many fungal plant pathogens. In our present study, we have assessed the potential of two *T. asperellum* isolates used as biological agents to control *R.*

solanacearum. The results indicate that *T. asperellum* isolates are effective against tomato wilt pathogen *R. solanacearum*.

Different enzyme activities such as PAL, POX and PPO which are indicative of cell metabolism when there is an interaction between the host plant and pathogen were determined. Our results have exhibited that there is a significant correlation between the induction of systemic resistance and the activities of these enzymes. Our study signifies that *T. asperellum* can induce resistance in tomato and proves to be an effective biocontrol agent against *R. solanacearum* and is found to decrease wilt incidence in tomato plants under greenhouse conditions.

The results obtained so far have shown a wide variety of reactions triggered by *T. asperellum*. Such induction of all available defense mechanisms seems to be the optimal defense tactic against different pathogens (Bolton, 2009). This beneficial impact of the fungi encourages further studies of new strains in experimental systems with different plants and pathogens aimed to determine the ability of *Trichoderma* strain to induce defense response and resistance in plants in a variety of biotic and abiotic conditions.

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