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Pseudomonas fluorescens (Pf1) mediated chitinolytic activity in tomato plants against *Fusarium oxysporum* f. sp. *lycopersici*

R. Manikandan* and T. Raguchander

Department of Plant Pathology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore-641 003, India.

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Liquid formulation of *Pseudomonas fluorescens* strain Pf1 was found to protect tomato plants from wilt disease caused by *Fusarium oxysporum* f. sp. *lycopersici* (*FOL*). The lytic enzyme and chitinase activity were assayed in the tomato plants treated with liquid as well as talc based formulations of Pf1 and challenged with the *FOL*. The chitinase activity was significantly increased in tomato plants treated with sequential application of seedling dip + soil drench + foliar spray of liquid formulation followed by the treatment with talc product application. The increased activity was observed up to 5 days after bioformulation treatment and thereafter declined. The enzyme activity of bioformulation treatment was significantly higher than the control treatments. Isoform analysis showed that sequential application of the treatment expressed three chitinase isoforms, whereas in the control plants no isoform was observed. Western blot analysis revealed that two isoforms of chitinase with a molecular weight of 28 and 23 kDa were newly induced by liquid formulation of Pf1 treatment challenged with the pathogen. These results suggest that induction of chitinase enzymes and accumulation of these PR-proteins might have contributed to restriction of *FOL* invasion in tomato roots.

Key words: Tomato *Fusarium* wilt, PR-protein, Chitinase, Western blot analysis, Isoform.

INTRODUCTION

Plant growth-promoting rhizobacteria (PGPR) are characterized by a number of activities, which include improvement of plant establishment, increased availability of plant nutrients, enhancement of nutrient uptake, improvement of soil structure and protection against diseases (Glick, 1995 and Lynch, 1990). The soil-borne plant pathogens, *Fusarium* wilt caused by *Fusarium oxysporum* f.sp. *lycopersici* Sacc. causes considerable loss in yield. The yield losses due to *Fusarium* wilt disease which was estimated to be 40 to 50%. The disease management in tomato is widely practiced using chemicals. However, indiscriminate use of chemicals led to development of fungicidal resistance by the pathogen, environmental pollution and health hazards. In this, *fluorescent pseudomonads* have received particular attention throughout the global science because of their catabolic versatility, excellent root colonizing abilities and their capacities to produce a wide range of antifungal metabolites. In addition, it is not dose-dependent (Olivain et al., 2004). The use of *fluorescent* for controlling soil

*Corresponding authors. E-mail: manizy007@yahoo.co.in. Tel: 91-422-6611446. Fax: 91-422-6611437.

borne plant diseases has been well documented. The biocontrol agents induce systemic resistance (ISR) through fortifying the physical and mechanical strength of cell wall as well as changing physiological and biochemical reactions of host leading to synthesis of defense chemicals against challenge inoculation of pathogens. Chitinases (EC 3.2.1.14) are PR-proteins which hydrolyze chitin, a major cell wall component (3-10%) of higher fungi. Chitinase cleave a bond between C1 and C4 of two consecutive N-acetyl glucosamine (GlcNAc) either by endolytic or exolytic mechanisms. A large number of plant chitinases have been purified and characterized which are endochitinases with molecular weights ranging from 25 to 36 kDa. Many PR-proteins induced in plants treated with inducing agents have been shown to be chitinases and β-1, 3 glucanases. The production of chitinases in plants has been suggested to be a part of their defense mechanism against fungal pathogens (Schlumbaum et al., 1986). In recent years, several biocontrol agents have been shown to induce systemic resistance in plants. Accumulation of chitinases and peroxidases with the onset of ISR by PGPR has been observed in some plants. Enhanced accumulation of chitinase in tobacco and bean leaves was observed in response to application of *Pseudomonas* spp. to roots (Zdor and Anderson, 1992). Increased chitinase activity in tobacco and maximum activity in cucumber have been observed as a result of systemic resistance by *fluorescent pseudomonads* against *Pseudomonas syringae* pv. *tabaci* (Schneider and Ullrich, 1994). Nandakumar et al. (2001) found early and higher induction of chitinase in *P. fluorescens* Pf1 treated rice plants. Such enhanced induction of chitinase offered protection against *Rhizoctinia solani* in rice. Xue et al. (1998) reported that non-pathogenic treatment of binucleate *Rhizoctonia* elicited a significant and systemic increase in all cellular fractions of chitinase compared to the diseased and control bean plants. Induction of four new chitinase isoforms with molecular weights of 12, 34.5, 53.5 and 63 kDa in *Pseudomonas* treated canes challenge inoculated with *Colletotrichum falcatum* in sugarcane was also observed (Viswanathan and Samiyappan, 2001). Ramamoorthy et al. (2002b) reported the induction of 46 kDa chitinase due to *P. fluorescens* Pf1 treatment when challenge inoculated with *F. oxysporum* f. sp. *lycopersici* in tomato.

Many of the studies are reported for the use of powder or talc based carrier of PGPR, having the shelf life of 3 months. However, only a few reports are available on the development of liquid formulation. Comparing with powder or talc based carrier, liquid based formulation of PGPR has the advantages of higher cell count, zero contamination, longer shelf life, greater protection against environmental stresses, increased field efficacy and convenience of handling. It is possible to add certain chemicals to promote the formation of dormant cyst cells in the liquid formulation. When the organisms are kept in a dormant cyst form and if it is mixed with the soil at the time of use, the dormant form gives rise to active cells, which helps to increase the shelf life to more than one year and tolerance to adverse conditions (Vendan and Thangaraju, 2006). The liquid based formulation having extended shelf life using *Trichoderma asperellum* against *Fusarium* head blight was reported (Kolombet et al., 2008). Manikandan et al. (2010) reported that liquid formulation of strain Pf1 has six month shelf life and enhanced disease resistance in tomato plants against *Fusarium* wilt disease. The present study has been carried out to evaluate effect of *P. fluorescens* Pf1 for the induction of chitinolytic activity involved in the phenylpropanoid pathway for the bioprotection of tomato plants against subsequent infection by *F. oxysporum* f. sp. *lycopersici*.

MATERIALS AND METHODS

Pf1-Liquid formulation and *Fusarium oxysporum* f. sp. *lycopersici* fungus

Pseudomonas fluorescens strain Pf1 was obtained from the Culture Collection Centre, Department of Plant Pathology, Tamil Nadu Agricultural University, India and used for this study. Liquid based Pf1 formulation was prepared in nutrient broth amended with glycerol as per the procedure described by Manikandan et al. (2010). *F. oxysporum* f. sp. *lycopersici* causing agent of wilt disease, was maintained on sterilized sandy loam soil mixed with maize powder at 19:1 (w/w) plates.

Glasshouse study

A glasshouse study was carried out to test the effect of liquid and talc based bioformulations of strain Pf1 on defense activity against *F. oxysporum* f. sp. *lycopersici*. In this study, individual and combined applications of seedling dip, soil drenching and foliar spray were evaluated for liquid based bioformulation and compared with talc based formulation. Tomato seeds were sown in plastic pots (45 × 60 cm) containing pot mixtures (red soil: sand: cow dung manure = 1:1:1 w/w/w sterilized at 121°C, 15 psi for 2 h for two consecutive days). The seedlings were removed 25 days after sowing and dipped in liquid formulation (500 ml ha^{-1} dissolved in 10 L of water) for 30 min. The seedlings were transplanted onto 30 cm diameter pots filled with pot mixture inoculated with 50 ml of conidial suspension of pathogen per pot (Ramamoorthy et al., 2002b) under glass house conditions. At 30 days after planting, 2 ml of the liquid formulation (2×10^9 CFU ml^{-1}) per pot was applied as soil drench. Similarly, seedling dip (200 g dissolved in 10 L of water) and soil application (5 g per pot) was given with talc based bioformulation strain Pf1 as described by Saravanakumar et al. (2009). At the same time foliar application of 0.2% Pf1 liquid as well as talc formulation was applied. Mancozeb (0.2%) was used as chemical control, while plants inoculated with the pathogen alone served as the inoculated control. Totally ten treatments and thirty pots with three seedlings per pot and three replications were maintained for each treatment.

Sample collection

Roots samples were collected from individual treatments to study the induction of defense enzymes such as chitinase in response to

soil borne pathogens in tomato plants under glasshouse conditions. Three roots from treated plants for each treatment were collected starting from 0^{th} day, 1^{st} day upto 13^{th} day at 48 h intervals.

Enzyme extraction

The root tissues were collected from treated and control tomato plants and immediately extracted with 2 ml of 0.1 M sodium citrate buffer (pH 5.0) at 4°C. The homogenate was centrifuged for 20 min at 10,000 rpm. Protein extracts prepared from tomato tissues were used for estimation of chitinase enzyme.

Assay of chitinase

The colorimetric assay of chitinase (EC 3.2.1.14) was carried out according to the procedure developed by Boller and Mauch (1988). One gram of tomato tissue was extracted with 5 ml of 0.1 M sodium citrate buffer (pH 5.0). The homogenate was centrifuged for 10 min at 20,000 g at 4°C and the supernatant was used as enzyme source.

Preparation of reagents

Preparation of colloidal chitin

Colloidal chitin was prepared by treating 1 g of crabshell chitin powder with acetone to form a paste, then slowly adding 20 ml of concentrated hydrochloric acid (HCl) while grinding in a mortar with the temperature maintained at 5°C. After several minutes, the syrupy liquid was filtered through glass wool and poured into vigorously stirred 50% aqueous ethanol to precipitate the chitin in a highly dispersed state. The residue was sedimented and resuspended in distilled water several times to remove excess acid and alcohol and then dialysed against tap water. Chitin content of the suspension was determined by drying a sample *in vacuo* and adjusted with distilled water to a final concentration of 10 mg ml^{-1} (dry weight/volume) and stored at 5°C for further use (Berger and Reynolds, 1958).

Preparation of snail gut enzyme

Six hundred milligrams of the commercial lyophilized snail gut enzyme (Helicase, Sepracor, France) was dissolved in 10 ml of 20 mM potassium chloride (KCl) and chromatographed on a Sephadex G-25 column (38 x 1.5 cm) using a 10 mM KCl solution, containing 1 mM EDTA and adjusted to pH 6.8 for equilibration and elution. The first 20 ml eluted after the void volume was collected (Boller and Mauch, 1988).

Preparation of p-dimethylaminobenzaldehyde (DMAB) reagent

The DMAB reagent was prepared by the procedure described by Reissig et al. (1955). Stock solution of DMAB was prepared by mixing 8 g of DMAB in 70 ml of glacial acetic acid along with 10 ml of concentrated HCl. One volume of stock solution was mixed with 9 volumes of glacial acetic acid immediately before use.

Assay

The reaction mixture consisted of 10 µl of 0.1 M sodium acetate buffer (pH 4.0), 0.4 ml enzyme solution and 0.1 ml colloidal chitin. After incubation for 2 h at 37°C, the reaction was stopped by centrifugation at 3,000 *g* for 3 min. An aliquot of the supernatant (0.3 ml) was pipetted into a glass reagent tube containing 30 µl of 1 M potassium phosphate buffer (pH 7.0) and incubated with 20 µl of 3% (w/v) snail gut enzyme for 1 h. After 1 h, the reaction mixture was brought to pH 8.9 by the addition of 70 µl of 0.1 M sodium borate buffer (pH 9.8). The mixture was incubated in a boiling water bath for 3 min and then rapidly cooled in an ice-water bath. After addition of 2 ml of DMAB, the mixture was incubated for 20 min at 37°C; immediately thereafter, the absorbance was measured at 585 nm in spectrophotometer (Agilent technologies). *N*-acetylglucosamine (GlcNAc) was used as a standard and the enzyme activity was expressed as n moles GlcNAc equivalents min^{-1} g^{-1} plant tissue.

Preparation of glycol chitin

Glycol chitin was obtained by acetylation of glycol chitosan (Trudel and Asselin, 1989). Five gram of glycol chitosan was dissolved in 100 ml of 10% acetic acid by grinding in a mortar. The viscous solution was allowed to stand overnight at 22ºC. Methanol (450 ml) was slowly added and the solution was vacuum filtered through a Whatman no. 4 filter paper. The filtrate was transferred into a beaker and 7.5 ml of aceticanhydride was added with magnetic stirring. The resulting gel was allowed to stand for 30 min at room temperature and then cut into small pieces. The liquid extruding from the gel pieces was discarded. Gel pieces were transferred to pistel morter and homogenized for 4 min at top speed. This suspension was centrifuged at 12000 rpm for 15 min at 4ºC. The gelatinous pellet was resuspended in about one volume of methanol, homogenized and centrifuged as in the preceding step. The pellet was resuspended in distilled water (500 ml) containing 0.02% (w/v) sodium azide and homogenized for 4 min. This was the final 1% (w/v) stock solution of glycol chitin.

Activity gel electrophoresis

Chitinase activity was detected in the polyacrylamide gel electrophoresis according to Trudel and Asselin (1989) with modification. Gels were incubated in 150 mM sodium acetate buffer at pH 5.0 for 5 min and then in 100 mM sodium acetate buffer at pH 5.0, containing 0.01% glycol chitin for 30 min at 37°C. The gels were finally transferred into a solution containing 0.01% (w/v) Calcofluor white M2R (Fluorescent brightener) in 500 mM Tris HCl (pH 8.9). After 5 min the brightener solution was removed and the gels were rinsed with distilled water for more than 1 h. Lytic zones were visualized and photographed under UV light in gel documentation system (Alpha Innotech Corporation).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

One gram of powdered leaf sample was extracted with one millilitre of 0.1M sodium phosphate buffer (pH 7.0) under 4°C. The homogenate was centrifuged for 20 min at 10000 rpm and the supernatant was used for the SDS-PAGE (Laemmli 1970). Hundred microgram of protein from different treatments was taken and mixed with 10 µl of sample buffer in a microfuge tube, boiled for 4 min and incubated at 4°C for 30 min. Then the samples containing equal amount of proteins were loaded into the wells of polyacrylamide gels (Sigma-Aldrich Techware system, Sigma, USA). The medium range molecular weight markers (Bangalore Genei, India) were used and electrophoresis was carried out at constant voltage of 75 volts for 2 h. The gels were stained with 0.2% Coomassie brilliant blue (R250) solution. Based on the R*f* value of each protein band stained, the molecular weight was calculated.

Table 1. Induction of Chitinase activity (nmoles GlcNac equivalents min^{-1} g^{-1} of fresh tissue) in tomato plants treated with Pf1 liquid formulation against *Fusarium oxysporum* f. sp. *lycopersici* under glasshouse condition.

Treatments	0 DAT	1 DAT	3 DAT	5 DAT	7 DAT	9 DAT	11 DAT	13 DAT
T_1 - Seedling dip with liquid formulation	1.64^{ab}	1.65^{ef}	1.67^{e}	1.68^{f}	1.68^{ef}	1.67^{d}	1.65^{de}	1.61^{d}
T_2 - Seedling dip with talc formulation	1.65^{a}	1.67^{e}	1.67^{e}	1.68^{f}	1.68^{ef}	1.67^{d}	1.64^{de}	1.61^{d}
T_3 - Seedling dip with Chemical (Mancozeb)	1.53^{bcd}	1.53^{fg}	1.54^{ef}	1.54^{fg}	1.55^{fg}	1.54^{ef}	1.53^{ef}	1.53^{d}
T_4 - T_1 + soil drenching with liquid formulation	1.60^{abc}	1.98^{c}	2.67^{c}	2.68^{c}	2.32^{c}	1.98^{c}	1.80^{c}	1.75^{b}
T_5 - T_2 + soil drenching with talc formulation	1.62^{abc}	1.81^{d}	2.36^{d}	2.47^{d}	2.11^{d}	1.77^{d}	1.71^{cd}	1.64^{bcd}
T_6 - T_3 + soil drenching with Chemical (Mancozeb)	1.53^{a-d}	1.55^{efg}	1.65^{e}	1.66^{f}	1.65^{ef}	1.64^{de}	1.63^{de}	1.63^{cd}
T_7 - T_4 + foliar spray with liquid formulation	1.63^{abc}	2.42^{a}	3.22^{a}	3.26^{a}	2.86^{a}	2.48^{a}	2.23^{a}	2.21^{a}
T_8 - T_5 + foliar spray with talc formulation	1.62^{abc}	2.21^{b}	2.94^{b}	2.99^{b}	2.64^{b}	2.27^{b}	2.11^{b}	2.19^{a}
T_9 - T_6 + foliar spray with Chemical (Mancozeb)	1.52^{cd}	1.58^{ef}	1.68^{e}	1.88^{e}	1.78^{e}	1.76^{d}	1.75^{cd}	1.74^{bc}
T_{10} - Inoculated Control	1.45^{d}	1.45^{g}	1.47^{f}	1.47^{g}	1.47^{g}	1.46^{f}	1.46^{f}	1.58^{d}

*DAT- Days after treatment. Values are mean of three replications. Means followed by a common letter are not significantly different at 5% level by DMRT.

Western blotting for chitinase detection

Preparation of protein samples and electrophoresis condition were the same as above. After SDS-PAGE electrophoresis, the proteins were electro blotted onto 0.45 µm PVDF membranes (Sigma, USA). The electrophoretic transfer of proteins was carried out from gel to membrane in a Bio Rad semidry transblot apparatus (140 mA, 30 min). The membranes were then stained with Ponceau S stain for 2 min to check the resolution and transfer quality. Ponceau S stain was destained with Tris Buffer Saline Tween 20 (TBST for 2 min and the membrane was blocked for 1.5 h at room temperature (28 ± 2°C) in TBST containing 2.5% (w/v) gelatin. The membrane was then soaked in the diluted primary antibody for overnight in TBST. After incubating with the primary antibody, the membrane was washed with TBST thrice for 10 to 15 min each time to remove the unbound antibody. The membrane was then incubated in secondary antibody for 3 h. Affinity purified goat anti-rabbit immunoglobulin (IgG) alkaline phosphatase conjugate was used as secondary antibody at a dilution of 1:7000. The membrane was then washed thrice with TBST and thrice in TBS for 10 to 15 min each time. Immunological reaction was visualized by soaking the membranes in alkaline phosphatase colour development reagents. Immediately after colour development the membranes were washed in distilled water and dried.

Statistical analyses

The data were statistically analyzed using the IRRISTAT version 92 developed by the International Rice Research Institute Biometrics unit, the Philippines (Gomez and Gomez, 1984). Prior to statistical analysis of variance (ANOVA) the percentage values of the disease index were arcsine transformed. Data were subjected to analysis of variance (ANOVA) at two significant levels ($P< 0.05$ and $P< 0.01$) and means were compared by Duncan's Multiple Range Test (DMRT).

RESULTS

Chitinase activity

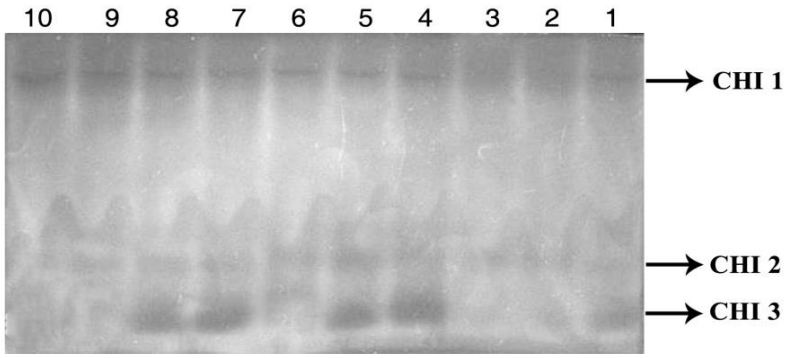
The lytic enzyme, chitinase activity was assayed in the tomato plants treated with the liquid as well as talc based formulations of strain Pf1 and challenge inoculated with the *F. o.* f. sp. *lycopersici.* The chitinase activity has significantly increased in tomato plants treated with combined application of seedling dip + soil drench + foliar spray of liquid formulation and challenge inoculated with *F. o.* f. sp. *lycopersici* followed by the treatment with talc product application of seedling dip + soil drench + foliar spray. The increased activity was observed upto 5 days after final treatment and thereafter declined. The combined application of seedling dip + soil drench with liquid formulation and the same application with the talc product showed the intermediate chitinase activity. The treatment received only seedling dip with liquid and with talc showed the enzyme activity comparatively higher than the untreated and inoculated control (Table 1).

Isoform pattern of chitinase

Treatment of the tomato plants with combination of seedling dip + soil drench and seedling dip + soil drench + foliar spray of Pf1 liquid formulation expressed three chitinase isoforms (Chi1, Chi2 and Chi3), whereas in the control plants no isoform was observed (Figure 1).

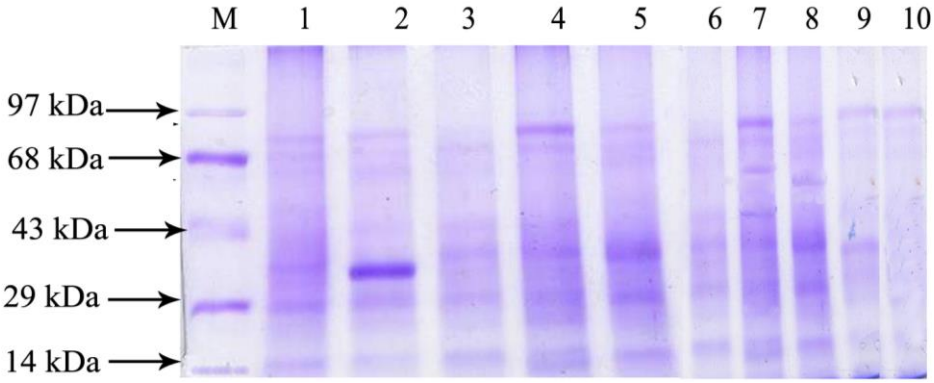
SDS-PAGE analysis of crude protein of Pf1 treated tomato plants challenged with *F. o.* f. sp.*lycopersici*

The protein banding pattern was studied from the tomato plants treated with liquid and talc based formulation of strain of Pf1after challenge inoculation of *F. oxysporum* f. sp*. lycopersici.* The banding pattern of protein was predominant in the treatment with seedling dip + soil drench + foliar spray of Pf1liquid formulation followed by same method of treatment with talc formulation. The proteins of 14, 29, 35 and 68 kDa were expressed in irrespective of the treatments. However, 72 kDa protein appeared in the combined application and also 35 kDa protein had appeared with high intensity (Figure 2).



1. T_1 - Seedling dip with liquid formulation
2. T_2 - Seedling dip with talc formulation
3. T_3 - Seedling dip with Chemical (Mancozeb)
4. T_4 - T_1 + soil drenching with liquid formulation
5. T_5 - T_2 + soil drenching with talc formulation
6. T_6 - T_3 + soil drenching with Chemical (Mancozeb)
7. T_7 - T_4 + foliar spray with liquid formulation
8. T_8 - T_5 + foliar spray with talc formulation
9. T_9 - T_6 + foliar spray with Chemical (Mancozeb)
10. T_{10} - Inoculated Control

Figure 1. Expression of chitinase isoforms in Pf1 liquid formulation treated tomato plants challenge inoculated with *Fusarium oxysporum* f. sp. *lycopersici*

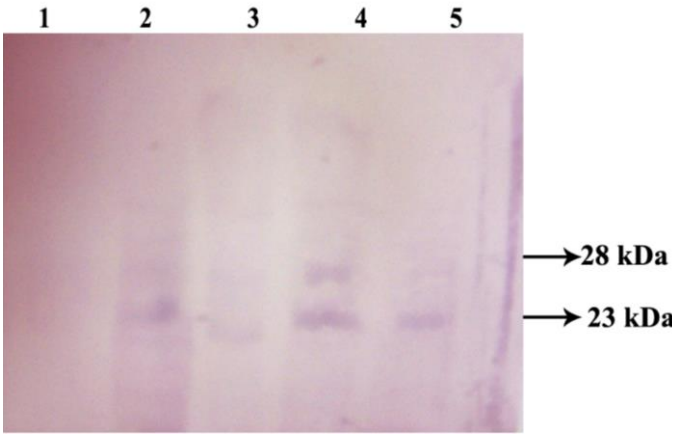


1. T_1 - Seedling dip with liquid formulation
2. T_2 - Seedling dip with talc formulation
3. T_3 - Seedling dip with Chemical (Mancozeb)
4. T_4 - T_1 + soil drenching with liquid formulation
5. T_5 - T_2 + soil drenching with talc formulation
6. T_6 - T_3 + soil drenching with Chemical (Mancozeb)
7. T_7 - T_4 + foliar spray with liquid formulation
8. T_8 - T_5 + foliar spray with talc formulation
9. T_9 - T_6 + foliar spray with Chemical (Mancozeb)
10. T_{10} - Inoculated Control

Figure 2. SDS-PAGE analysis of pathogenesis related proteins induced in tomato plants treated with Pf1 liquid formulation against *Fusarium oxysporum* f. sp. *Lycopersici.*

Western blot analysis of chitinase in Pf1 treated tomato plants against *F. o*. f. sp. *lycopersici*

Western blot analysis of root extracts from tomato plants showed that chitinase antiserum was able to recognize two proteins with sizes of 23 and 28 kDa. No isoforms of chitinase was detected in control treatments in which the tomato plants inoculated with *F. o.* f. sp. *lycopersici*. Also,



1. Untreated control
2. Seedling dip with liquid formulation
3. Seedling dip with talc formulation
4. Seedling dip + soil drenching with liquid formulation
5. Seedling dip+ soil drenching with talc formulation

Figure 3. Western blot analyses of chitinase isoforms in tomato plants induced by Pf1 liquid formulation against *Fusarium oxysporum* f. sp. *Lycopersici.*

the intensity of chitinase isoforms was more in the plants when treated with combined application of seedling dip+ soil drench + foliar spray of liquid based formulation followed by the same method of application with the talc formulation (Figure 3).

DISCUSSION

Synthesis and accumulation of PR proteins have been reported to play an important role in plant defense mechanisms. Chitinases and β-1, 3-glucanases (which are classified under PR-3 and PR-2 groups of PR proteins, respectively) have been reported to associate with resistance in plants against pests and diseases. The enzymatic degradation of the fungal cell wall by hydrolytic enzymes may release non-specific elicitors which in turn elicit various defense reactions. Viswanathan and Samiyappan (1999) reported that ISR induced by *P. fluorescent* is associated with induction of chitinase which appears to be the promising technology for the management of red rot disease of sugarcane. The antifungal nature of induced chitinase in rice plants after treatment with biological control agents has been reported by Nandakumar et al. (2002). Similarly, 57 kDa chitinase showing the antifungal activity against *Pyricularia grisea* in *Pseudomonas* treated finger millet (Radjacommare et al*.,* 2004b). Chitinase isoforms of different relative mobility were induced in response to pathogens after pretreatment with PGPR strains. The induction of chitinase has also been implicated in defense against further invasion of the pathogen in leaves and sheaths of rice against *R*. *solani*. In the present study, treatment of the tomato plants with combination of seedling dip + soil drench and seedling dip + soil drench + foliar spray of Pf1 bioformulation expressed three chitinase isoforms *viz*., Chi1, Chi2 and Chi3 whereas in the control plants no isoform was observed. It indicates the reduced disease incidence might be due to the higher induction of lytic enzymes. This type of findings has been reported by various workers in different crops. The culture filterate of combination of *Pseudomonas* strains (EPB22 + Pf-1) recorded higher chitinolytic activity than the individual strain and also more number of chitinase isoforms detected by the western blot analysis (Harish, 2005).

In conclusion, the present study implies that earlier and higher accumulation of chitinase enzymes involved in phenylpropanoid metabolism and PR-proteins has been found in tomato root tissue treated with *P. fluorescens* Pf1 liquid formulation in response to invasion by *F. o.* f. sp. *lycopersici*. The plant-pathogen interactions have also triggered the activities of defense enzymes initially but later the activities drastically declined when the pathogen colonized the root tissues. Enhanced accumulation and induction of chitnase isoform, by Pf1 liquid formulatin in tomato root tissues might have collectively involved in bioprotection of tomato plants against *F*. *oxysporum* f. sp. *lycopersici*. Thus the combined application of Pf1 bioformulation in liquid *viz.,* seedling dip + soil drench + foliar spray can enhance the disease protection activity and improve the consistency of pathogen suppression

throughout cropping period.

Conflict of interests

The authors did not declare any conflict of interest.

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