

Foliar application of *Burkholderia* sp. strain TNAU-1 leads to activation of defense responses in chilli (*Capsicum annuum* L.)

Srinivasan Madhavan, Vaikuntavasan Paranidharan and Rethinasamy Velazhahan*

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

*Corresponding author: Ph. 091-422-6611226, Fax: 091-422-6611437, velazhahan@hotmail.com

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ABSTRACT

Induction of phenolics, peroxidase (PO), polyphenol oxidase (PPO), chitinase and thaumatin-like proteins (TLP) in leaves of chilli (*Capsicum annuum* L.) in response to foliar application with a biocontrol agent *Burkholderia* sp. strain TNAU-1 was studied. Chilli plants, when sprayed with *Burkholderia* sp. strain TNAU-1 showed increase in phenolic content one day after application and the maximum accumulation was observed seven days after treatment. Three new peroxidase isozymes (PO-1, PO-2 and PO-3) were induced in chilli leaves upon treatment with *Burkholderia* sp. strain TNAU-1. The activity of all the peroxidases was at the maximum level three days after treatment and subsequently decreased. Protein extracts obtained from *Burkholderia* sp. strain TNAU-1 treated plants exhibited a major polyphenol oxidase (PPO-1) one day after treatment. The activity of PPO-1 subsequently decreased, but continuously present throughout the experimental period of 15 days. Western blot analysis of protein extracts from *Burkholderia* sp. strain TNAU-1 treated chilli leaves revealed that two TLPs with sizes of 23 and 25 kDa and a chitinase with an apparent molecular weight of 28 kDa were induced three days after treatment.

Key words: *Capsicum annuum*, chitinase, induced resistance, peroxidase, phenolics, polyphenol oxidase, thaumatin-like protein

INTRODUCTION

Chilli (*Capsicum annuum* L.) is an important crop in many parts of the world. Its production is affected by a number of diseases. Among the most devastating is anthracnose or fruit rot caused by *Colletotrichum* spp. This disease is prevalent in most chilli-producing areas throughout the world and often results in high yield losses (Than et al., 2008a). Several species of *Colletotrichum* viz., *C. capsici* (Butler and Bisby), *C. gloeosporioides* (Penz.), *C. acutatum* (Simmonds), *C. atramentarium* (Berk and Broome), *C. dematium* (Pers.) and *C. coccodes* (Wallr.), *Glomerella cingulata* (Stoneman) along with *A. alternata* (Keissler) have been reported as causal agents of chilli fruit rot worldwide (Than et al., 2008b).

Anthracnose is mainly a problem on mature fruits, causing severe losses via both pre and post-harvest fruit decay (Hadden and Black, 1989; Bosland and Votava, 2003). Typical anthracnose symptoms on chilli fruit include sunken necrotic tissues, with concentric rings of acervuli. In some cases, the lesions are brown and then turn black, due to the formation of setae and sclerotia.

Fruit rot reduces dry weight, capsaicin and oleoresin content of affected fruits (Mistry et al., 2008), leading to reduction in the medicinal properties of chilli. Synthetic chemical fungicides are commonly used to control anthracnose. However, this is becoming less acceptable since it increases the potential for build-up of resistance in

the pathogen to fungicides and also conflicts with the public concern for fungicide residues. The exploitation of induced resistance in plants is a promising environment-friendly strategy for controlling plant diseases. It is well known that plants have several lines of defense against invading pathogens including preformed barriers and induced responses. The latter include rapid generation of reactive oxygen species, synthesis of phytoalexins, onset of hypersensitive cell death and synthesis of pathogenesis-related proteins (Roth et al., 2000; Afroz et al., 2011).

Certain strains of Plant growth-promoting rhizobacteria (PGPR) are capable of stimulating different defense mechanisms that leads to systemic disease protection in plants, which has been termed "induced systemic resistance" (ISR) (Van Loon et al., 1998; Van Loon, 2007) and hence it is possible that even susceptible plants can be protected from diseases by enhancing their own defense mechanisms by application of PGPR. Anand et al. (2007) reported induction of systemic resistance in chilli plants against fruit rot disease by foliar application of *Pseudomonas fluorescens* isolate Pf1. Recently we observed that foliar application of powder formulation of *Burkholderia* sp. strain TNAU-1 at 0.2% (w/v) concentration at the time of flowering followed by four sprayings at 15 days interval significantly reduced the incidence of chilli anthracnose under field conditions (Madhavan et al., 2011. Unpublished). The results suggest that activation of the host biochemical defense mechanisms may be involved in the suppression of chilli anthracnose disease. In the present study we investigated the induction of defense-related enzymes viz., peroxidase, polyphenol oxidase and accumulation of phenolics and PR-proteins in leaves of chilli in response to treatment with *Burkholderia* sp. strain TNAU-1. The involvement of these enzymes in the defense response of chilli plants against *C. capsici* is discussed.

MATERIALS AND METHODS

Fungal and bacterial cultures: The fungus, *Colletotrichum capsici* was isolated from chilli fruits showing symptoms of anthracnose disease and maintained on potato dextrose agar (PDA) medium under laboratory conditions (Madhavan et al., 2010). *Burkholderia* sp. strain TNAU-1 was

obtained from the culture collection of the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, India and used in this study.

Induction of defense mechanisms: Talc-based powder formulation of *Burkholderia* sp. strain TNAU-1 was developed as described by Vijaysamundeeswari et al. (2010). To study the induction of phenolics, peroxidase, polyphenol oxidase, chitinase and TLP in *Burkholderia* sp. strain TNAU-1- treated plants, 25-day-old chilli plants (*Capsicum annum* L. cv. CO-1) were sprayed with powder formulation of *Burkholderia* sp. strain TNAU-1 (0.2%) until run-off. Control plants were sprayed with sterile distilled water. At various time intervals the leaf samples were collected and immediately frozen in liquid nitrogen and stored at -70°C until analysis.

Estimation of phenolic content: Chilli leaves (1 g) were ground to a fine powder in liquid nitrogen and extracted in 10 ml of 80% methanol for 15 min at 70°C (Swain and Hillis, 1959). For estimation of phenolic content, one ml of the methanolic extract was mixed with 5 ml of distilled water and 250 μl of Folin-Ciocalteu reagent (1 M) and the solution was incubated at 25°C . After 3 min 1 ml of saturated solution of Na_2CO_3 and 1 ml of distilled water were added and the reaction mixture was incubated at 25°C for 1 h. The absorption of the developed blue colour was measured at 725 nm using a Beckman DU64 spectrophotometer (*Beckman Instruments Inc.*, California, USA). The content of the total soluble phenols was calculated based on a standard curve obtained from a Folin-Ciocalteu reaction with phenol and expressed as phenol equivalents in $\mu\text{g g}^{-1}$ fresh mass.

Peroxidase isozyme analysis: Peroxidase isozymes were analyzed as per the procedure described by Eshel et al. (2002). The enzyme extract was prepared by homogenizing one gram of leaf sample in 5 ml of 0.1 M phosphate buffer, pH 7.0 in a chilled pestle and mortar. The homogenate was centrifuged in a refrigerated centrifuge at $12,000 \times g$ at 4°C for 20 min and the supernatant so obtained was used for PO isozyme analysis. The protein concentration in the samples was determined by the method of Bradford with bovine serum albumin as the standard (Bradford, 1976). Samples (50 μg) were mixed with sample buffer (150 mM Tris-HCl (pH 6.8), 3% bromophenol blue and 30% glycerol) and separated by non-denaturing polyacrylamide gel electrophoresis (PAGE) using a

12 % acrylamide gel. Electrophoresis of protein samples was carried out in a Mighty Small II gel electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA, USA) at a constant current of 20 mA for 2h.

After electrophoresis the gel was equilibrated for 30 min in 50 ml of 0.1 M potassium phosphate buffer, pH 7.0. Peroxidase isoforms were then visualized by incubating the gel in the dark with freshly prepared diaminobenzidine solution (10 mg in 20 ml 0.05 M phosphate buffer, pH 7.0) for 5-10 min, washing twice with water and then incubating with 10 μ l 30 % H₂O₂ in 20 ml of 0.05 M phosphate buffer, pH 7.0.

Polyphenol oxidase isozyme analysis: The enzyme extract prepared for peroxidase isozyme analysis was used in polyphenol oxidase isozyme analysis. Separation of proteins (50 μ g) was achieved by non-denaturing polyacrylamide gel electrophoresis (PAGE) of samples using a 12 % acrylamide gel as described above. Following electrophoresis, the gel was equilibrated for 30 min in 50 ml of 0.1 M potassium phosphate buffer, pH 7.0. Polyphenol oxidase isoforms were then visualized by incubating the gel in the dark with freshly prepared *p*-phenylene diamine solution (10 mg in 20 ml 0.1 M phosphate buffer, pH 7.0) for 5-10 min, washing twice with water and then incubating with 0.2 M catechol in the same buffer. The addition of catechol was followed by a gentle shaking which resulted in appearance of dark brown discrete protein bands (Jayaraman et al., 1987).

Immunoblot analysis: Leaf samples (1g) were homogenized in liquid nitrogen and extracted in 5 ml of 0.1M phosphate buffer, pH 6.5, containing 0.5 mM phenylmethylsulfonyl fluoride (Velazhahan et al., 1998). The homogenate was centrifuged at 4°C for 15 min at 12,000 \times *g* and the supernatant was collected. The protein concentration in the samples was determined by the method of Bradford with bovine serum albumin as the standard (Bradford, 1976). For immunoblot analysis, 100 μ g of proteins were separated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in a Mighty Small II gel electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA, USA) with 12% acrylamide resolving gel and 4% acrylamide stacking gel according to the method of Laemmli (1970). A constant current of 20 mA per gel (0.75 mm x 8 cm x 7 cm) was applied. After

electrophoresis, the proteins were electroblotted onto a polyvinylene difluoride (PVDF) membranes (Immobilon-P membrane; Millipore, Bedford, MA) for 30 min at 140 mA in a Bio-Rad semi-dry transblot apparatus (Bio-Rad, Hercules, CA, USA) using 48 mM Tris buffer, pH 9.2, containing 39 mM glycine, 1.3 mM SDS and 20% (v/v) methanol in accordance with the manufacturer's instructions. The membrane was blocked for 3 h at room temperature (28 \pm 2°C) in 3.0% (w/v) bovine serum albumin in Tris-buffered saline (10 mM Tris-HCl, pH 7.9, 140 mM NaCl) (TBS) containing 0.05% (v/v) Tween-20 (TBST). After blocking, immunoblots were washed three times for 5 min each with TBST and incubated for overnight in TBST containing specific primary antibody for chitinase and TLP at 1:1000 dilution. Antiserum raised against a barley chitinase/ grapes TLP (a gift of Dr. S.Muthukrishnan, Kansas State University, Manhattan, Kansas, USA) was used as primary antibody for chitinase and TLP respectively. The membranes were washed five times with TBST for 5 min. It was then incubated in TBST containing horseradish peroxidase (HRP) -conjugated goat anti-rabbit IgG (Bio-Rad, Hercules, CA, USA) at a 1:1,000 dilution for 3 h, washed thrice for 5 min each with TBST and twice with TBS. Binding of the secondary antibody was detected by reaction of the antibody-HRP-conjugate with freshly prepared substrate solution consisting of 15 μ l of 30% H₂O₂, 5 ml of 0.3% (w/v) 4-chloro-1-naphthol (Bio-Rad, Hercules, CA, USA) in methanol, and 25 ml of TBS for 3-5 min. Presence of TLP and chitinase were detected by observing the development of bands in the membrane. Apparent molecular masses of proteins were determined by comparison with molecular weight standards (Rainbow marker, RPN 756, Amersham Pharmacia, NJ, USA).

RESULTS

Phenolic content: Chilli leaves when sprayed with the powder formulation of *Burkholderia* sp. strain TNAU-1 showed dramatic increase in phenolic content one day after treatment and the maximum accumulation of phenols was observed seven days after treatment (Figure 1). The phenolic content subsequently declined but remained significantly at higher levels when compared with control at all sampling stages.

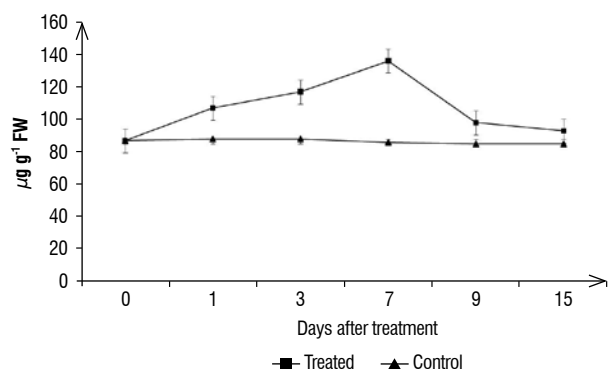


Figure 1. Time course increase in phenolic content in chilli leaves in response to treatment with *Burkholderia* sp. strain TNAU-1. Phenolic content in control and treated chilli plants was determined colorimetrically. Bars represent standard deviations of the means.

Peroxidase isozyme profile: Three new peroxidase isozymes (PO-1, PO-2 and PO-3) were induced in chilli leaves upon treatment with *Burkholderia* sp. strain TNAU-1 (Figure 2). PO-1 appeared one day after treatment and the intensity of the protein reached the maximum level three days after treatment. In addition, induction of two more PO isoforms (PO-2 and PO-3) could be detected three days after treatment with *Burkholderia* sp. strain TNAU-1. The activity of all the peroxidases was at the maximum level three days after treatment and subsequently decreased. The activity of PO was very low or at undetectable level in control leaves.

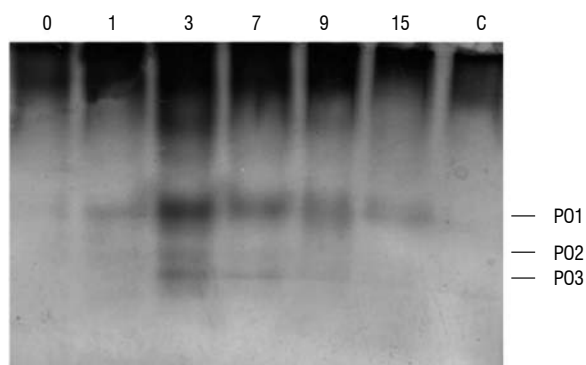


Figure 2. Peroxidase isoenzymes in protein extracts from chilli leaves treated with *Burkholderia* sp. strain TNAU-1. Fifty micrograms equivalent of protein was loaded on each lane in a 12 % non-denaturing gel. Following electrophoresis, the gel was incubated for 30 min with 0.1 M potassium phosphate buffer, pH 7.0, and peroxidase isoforms were visualized by incubating the gel in the dark with freshly prepared diaminobenzidine solution for 5-10 min, washing twice with water and then incubating with 10 µl 30 % H₂O₂ in 20 ml of 0.05 M phosphate buffer, pH 7.0. The numbers indicate days after treatment. Abbreviation used: C-control.

Polyphenol oxidase isozyme pattern: Protein extracts obtained from *Burkholderia* sp. strain TNAU-1 treated plants exhibited a major polyphenol oxidase (PPO-1) one day after treatment (Figure 3). The activity of PPO-1 subsequently decreased, but continuously present throughout the experimental period of 15 days. The activity of PPO-1 was very low or at undetectable level in control leaves.

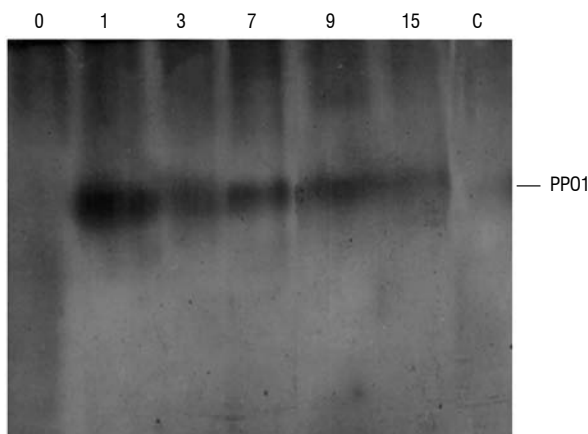


Figure 3. Polyphenol oxidase isoenzymes in protein extracts from chilli leaves treated with *Burkholderia* sp. strain TNAU-1. Fifty micrograms equivalent of protein was loaded on each lane in a 12 % non-denaturing gel. Following electrophoresis, the gel was incubated for 30 min with 0.1 M potassium phosphate buffer, pH 7.0, and polyphenol oxidase isoforms were visualized by incubating the gel in the dark with freshly prepared *p*-phenylene diamine solution for 5-10 min, washing twice with water and then incubating with 0.2 M catechol resulting in appearance of dark brown discrete protein bands. The numbers indicate days after treatment. C- control.

Induction of TLP: Western blot analysis of protein extracts from control and *Burkholderia* sp. strain TNAU-1 treated chilli leaves revealed that two proteins with sizes of 23 and 25 kDa cross reacting with grapes TLP antibody were induced three days after treatment (Figure 4). The levels of both the proteins were at the maximum at three days after treatment, slightly decreased seven days after treatment and came to basal level thereafter. No PR-5 proteins could be detected in the control plants.

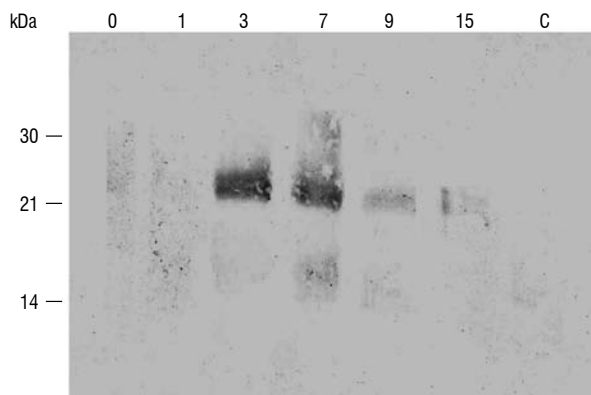


Figure 4. Induction of PR-5 proteins in chilli leaves in response to treatment with *Burkholderia* sp. strain TNAU-1. Aliquots of total proteins (100 μ g) extracted from control (lane C) and *Burkholderia* sp. strain TNAU-1 treated chilli leaves were analyzed by Western blotting after SDS-PAGE using grapes TLP antiserum. The numbers indicate days after treatment. Molecular weight markers are shown on the left.

Induction of chitinase: Immunoblot analysis of protein extracts from control plants revealed that the barley chitinase antiserum was able to recognize two proteins with apparent molecular weights of 30 kDa and 35 kDa in chilli leaves (Figure 5). Upon treatment with *Burkholderia* sp. strain TNAU-1 a chitinase with an apparent molecular weight of 28 kDa was induced three days after treatment. The level of the 28 kDa chitinase decreased thereafter.

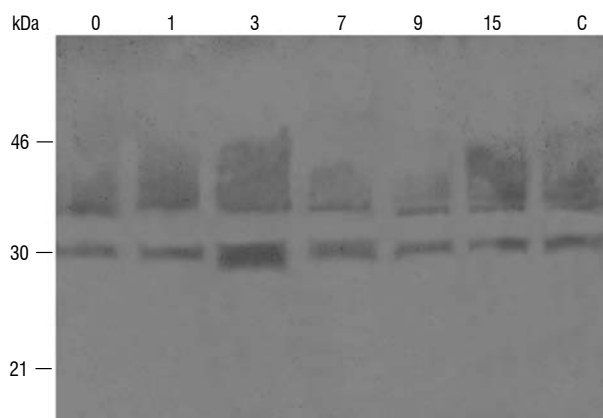


Figure 5. Induction of chitinase in chilli leaves in response to treatment with *Burkholderia* sp. strain TNAU-1. Aliquots of total proteins (100 μ g) extracted from control (lane C) and *Burkholderia* sp. strain TNAU-1-treated chilli leaves were analyzed by Western blotting after SDS-PAGE using barley chitinase antiserum. The numbers indicate days after treatment. Molecular weight markers are shown on the left.

DISCUSSION

Plants possess various biochemical defense mechanisms to protect themselves from pathogenic microorganisms and many of the defense reactions are due to the activation of defense-related genes. The level of accumulation of defense chemicals or the rapidity of the induction of defense genes in a host plant is correlated with the degree of its disease resistance (Lawrence et al., 1996). Activation of the plant's own defense system with the aid of biotic and abiotic inducer is a novel technology in the management of plant diseases. Several plant growth-promoting rhizobacteria (PGPR) are known to induce systemic resistance in plants against fungal (Van Peer et al., 1991; Wei et al., 1991; Thangavelu et al., 2003; Yan et al., 2002), bacterial (Vidhyasekaran et al., 2001) and viral pathogens (Raupach et al., 1996; Murphy et al., 2000). Induced resistance in plants by application of PGPR is often associated with increased activity of peroxidase, lignin deposition, increase in phenolic content and induction of pathogenesis-related (PR)-proteins (Ramamoorthy et al., 2001). Verhagen et al. (2004) reported a substantial change in the expression of 97 genes out of more than 8,000 genes surveyed in the roots of *Arabidopsis* in response to treatment with ISR-inducing bacterium *P. fluorescens* WCS417r by using microarray analysis. In the present study the biochemical alterations in chilli plants in response to treatment with *Burkholderia* sp. strain TNAU-1 were investigated.

Many phenolic compounds are known to be antimicrobial, function as precursors to structural polymers such as lignin, or serve as signal molecules to activate plant defense genes (Dakora, 1996). Increase in phenolic content in plants has been correlated with increased resistance to pathogens (Velazhahan and Vidhyasekaran, 1994). Singh et al. (2003) demonstrated that foliar application of *Pseudomonas fluorescens* strain Pf4 and *P. aeruginosa* strain Pag enhanced the phenolic acids viz., gallic, chlorogenic and cinnamic acids content as well as total phenolics within 24 h after application in chickpea. In the present study chilli leaves when sprayed with powder formulation of *Burkholderia* sp. strain TNAU-1 showed dramatic increase in phenolic content one day after treatment and the maximum accumulation of phenols was observed seven days after treatment with *Burkholderia* sp. strain TNAU-1. The phenolic content subsequently declined but remained significantly at higher levels when compared with control at all sampling stages. The increase in phenolic

content in chilli in response to treatment with *Burkholderia* sp. strain TNAU-1 in the present study might have contributed to increased physical and mechanical strength of host cell wall and thereby increased resistance to *C. capsici*.

Peroxidases are involved in the lignification, suberification, polymerization of hydroxy proline rich glycoproteins, regulation of cell wall elongation, wound healing and plant defense (Yoshida et al., 2003). Furthermore, peroxidase itself was found to inhibit spore germination and mycelial growth of *Pseudocercospora abelmoschi* and *P. cruenta* (Joseph et al., 1998). Caruso et al. (2001) demonstrated that a basic hemeperoxidase (WP1) with a molecular mass of 36 kDa purified from wheat kernels inhibited germ tube elongation of *Fusarium culmorum*, *Trichoderma viride* and *Botrytis cinerea*. Zdor and Anderson (1992) observed an increase of peroxidase activity as well as an increase in the level of mRNAs encoding for PAL and chalcone synthase in the early stages of interaction between bean roots and various bacterial endophytes. The results of the present study indicate that three new peroxidase isozymes (PO-1, PO-2 and PO-3) were induced in chilli leaves upon treatment with *Burkholderia* sp. strain TNAU-1. The activity of all the peroxidases was at the maximum level three days after treatment and subsequently decreased. The activity of PO was very low or at undetectable level in control leaves. The occurrence of new PO isoforms is suggestive of their synthesis *de novo* as a result of *Burkholderia* sp. strain TNAU-1 treatment. The increased PO in chilli leaves treated with *Burkholderia* sp. strain TNAU-1 might be involved in lignin biosynthesis which in turn might have contributed to disease resistance.

Many phenols and their oxidation products such as quinones are highly toxic to the invading fungi (Sequeira, 1983). Both PO and PPO catalyze the oxidation of phenolic compounds through a PPO-PO-H₂O₂ system (Srivastava, 1987). Polyphenol oxidases (PPO) are copper containing enzyme and involved in catalyzing the oxygen-dependent oxidation of phenols to quinones (Li and Steffens, 2002; Vaughn and Duke, 2006). A number of studies have correlated the induction of PPO activity with a resistance response (Srivastava, 1987; Velazhahan and Vidhyasekaran, 1994; Thipyapong et al., 2007; Jung et al., 2011). Li and Steffens (2002) demonstrated that overexpression of PPO in transgenic tomato plants resulted in enhanced resistance to the bacterial pathogen *Pseudomonas syringae* pv. *tomato*. Thipyapong

et al. (2004) demonstrated that antisense down regulation of PPO in tomato results in enhanced susceptibility to *Pseudomonas syringae* pv. *tomato*. The results of the present study indicate that protein extracts obtained from *Burkholderia* sp. strain TNAU-1 treated plants exhibited a major polyphenol oxidase (PPO-1) one day after treatment. The activity of PPO-1 subsequently decreased, but continuously present throughout the experimental period of 15 days. The activity of PPO was very low or at undetectable level in control leaves. The induced PPO might have involved in the resistance of chilli against *C. capsici* through oxidation of phenolic compounds to fungitoxic quinones.

Accumulation of pathogenesis-related (PR) proteins is known to be associated with systemic acquired resistance (SAR) in plants (Ryals et al., 1996). Several studies have shown that PR-proteins are also induced in plants upon treatment with *P. fluorescens* (Hynes and Lazarovits, 1989; Maurhofer et al., 1994; M'Piga et al., 1997). PR-proteins like chitinase and β -1,3-glucanase have the potential to hydrolyze chitin and β -1,3-glucan respectively, which are major components of fungal cell walls. Moreover the chitinase and glucanase release elicitors from the walls of fungi which, in turn, stimulate various defense responses in plants (Ham et al., 1991; Ren and West, 1992). Maurhofer et al. (1994) reported that PR-proteins viz., PR-1a, 1b, 1c, endochitinase and β -1,3-glucanases were induced in the intercellular fluid of tobacco leaves of plants grown in the presence of *P. fluorescens* strain CHA0. Using cytochemical labelling of chitin, M'Piga et al. (1997) demonstrated disorganization of *Fusarium oxysporum* f. sp. *radicis-lycopersici* hyphae colonizing bacterized tomato roots due to chitin degradation suggesting accumulation of chitinolytic enzymes in bacterized plants. The PR-5 family comprises proteins displaying a certain degree of homology over their amino acid sequences to thaumatin-like protein, a sweet tasting protein isolated from the fruits of the West African shrub *Thaumatococcus danielli* (for review, see Velazhahan et al., 1999). TLPs have been shown to have antifungal activity against a broad spectrum of fungal pathogens (Velazhahan et al., 1999).

Furthermore, it has been demonstrated that overexpression of chitinase and TLP in transgenic crop plants was associated with enhanced resistance to different fungal pathogens (Datta and Muthukrishnan, 1999). Immunoblot analysis of protein extracts from *Burkholderia* sp. strain

TNAU-1 treated plants in the present study revealed that a chitinase with an apparent molecular weight of 28 kDa was induced three days after treatment. Western blot analysis for TLP revealed that two proteins with sizes of 23 and 25 kDa cross reacting with grapes TLP antibody were induced three days after treatment. These results indicated that chitinase and TLPs are present at low or undetectable levels in control plants and are inducible after foliar application of *Burkholderia* sp. strain TNAU-1. Hence, it is also possible that increased resistance in chilli due to foliar application of *Burkholderia* sp. strain TNAU-1 might be related to the accumulation of PR-proteins in addition to accumulation of phenolics.

The coordinate expression of many different defense mechanisms in induced plants suggests that these defense responses may be involved in restricting the development of *C. capsici*. In recent years, many antifungal protein genes have been utilized to transform crop plants in an attempt to increase resistance to diseases (Collinge et al., 2010). However, it is suggested that the constitutive expression of defense genes may likely impose a considerable drain on energy and metabolic resources whereas inducible defenses would impose a significantly lower metabolic cost (Herms and Mattson, 1992; Heil and Bostock, 2002). Hence induction of systemic resistance in chilli when required by application of *Burkholderia* sp. strain TNAU-1 offers new prospects for ecofriendly management of chilli anthracnose disease.

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