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Polyphenol oxidase and lysozyme mediate induction of systemic resistance in tomato, when a bioelicitor is used

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Abstract: Tomato (*Solanum lycopersicum* L.) is attacked by *Pseudomonas syringae* pv. *tomato* causing heavy damage to the crops. The present study focused on the application of aqueous fruit extracts of neem (*Azadirachta indica* L.) on a single node of aseptically raised tomato plants. Observations were done, and the changes in the activity and isoenzyme profile of polyphenol oxidase (PPO) and lysozyme, both at the site of treatment as well as away from it, were noted. The results demonstrate that neem extract could significantly induce the activities of both the enzymes as well as upregulate the *de novo* expression of additional PPO isoenzymes. Induction of systemic acquired resistance (SAR) by natural plant extracts is a potent eco-friendly crop protection method.

Key words: lysozyme, polyphenol oxidase, *Pseudomonas syringae* pv. *tomato*, *Solanum lycopersicum*, systemic acquired resistance

Introduction

Tomato (*Solanum lycopersicum* L.), one of the most popular and widely grown vegetables ranks second in the world in terms of production. It is gravely attacked by *Pseudomonas syringae* pv. *tomato* which causes epidemic breakout of bacterial speck in all the aerial parts of the plant leading to heavy economical losses all around the globe (Quattrucci *et al*. 2013). Induction of systemic acquired resistance (SAR) by the application of plant extracts has emerged as a potential alternative to the prevalent chemical pesticides used for crop disease management (Fu and Dong 2013). Foliar application of methanolic leaf extracts of metel (*Datura metel* L.) effectively reduced the incidence of sheath blight and bacterial blight diseases of rice grown in greenhouses (Kagale *et al*. 2004). Application of an aqueous extract of zimmu (*Allium sativum* L. × *Allium cepa* L.) leaves on first and second leaves of cotton plants induced systemic resistance in the distal third and fourth leaves against *Xanthomonas campestris* pv. *malvacearum* (Satya *et al*. 2007). The post-harvest deterioration of plum (*Prunus salicina* Lindl.) or Yali pear (*Pyrus bretschneideri* Rehd.) could be prevented remarkably, by treating harvested fruits with neem extract (Wang *et al*. 2010).

Systemic acquired resistance primes the host defense mechanism to upregulate the *de novo* expression of defense-related genes leading to enhanced expression and *de novo* synthesis and accumulation of pathogenesis-related 'PR' proteins in uninfected tissues, thereby protecting them against any future pathogen attack (Ramos Solano *et al*. 2008). Polyphenol oxidase (PPO) and lysozyme are two such PR proteins which are involved in the signaling of the defense responses in plants and are widely known for their vital contribution to plant defenses against pathogenic microbes. Polyphenol oxidases (PPOs) catalyze the O_2-dependent oxidation of mono and *o*-diphenols to *o*-diquinones, responsible for plant senescence, wounding, and responses to pathogens (Thipyapong *et al*. 2004). It was suggested by Chen *et al*. (2000) that plant defense enzymes like PPO could be stimulated in cucumber roots which have been colonized by non-pathogenic rhizobacteria, or in a compatible interaction between cucumber and *Pythium aphanidermatum*. It was demonstrated by Bhuvaneshwari and Paul (2012) that application of neem extract could lead to the systemic induction of defense enzymes including PPO, responsible for reduced bacterial speck symptoms in tomato.

Lysozyme catalyses the hydrolysis of the β-1,4--glycosidic linkage between *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine (NAG) alternating sugar residues in the peptidoglycan layer present in the bacterial cell wall (Wang *et al*. 2005). A plant lysozyme from bitter melon (*Momordica charantia* L.) has been reported to possess antifungal activity against *Rhizoctonia solani* and *Mucorracem osus* apart from an antibacterial action against *Escherichia coli* and *Staphylococcus aureus* (Wang *et al*. 2011). Lysozyme conferred considerable levels of resistance to potato plants against *Phytophthora infestans*, *R. solani*, and *Fusarium solani* (Rivero *et al*. 2012).

The aim of this study was to evaluate the alterations in the activities and isoenzyme profiles of PPO and lysozyme after application of a bioelicitor (aqueous fruit extracts of neem), thereby leading to enhanced resistance of the host plant against *P. syringae* pv. *tomato*.

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Materials and Methods

Host plants

Tomato seeds (Roopsi variety, Century seeds) were raised under controlled aseptic conditions and watered daily with autoclaved distilled water. Hoagland's solution was watered as a nutrient supplement.

Preparation of bioelicitor

Mature (green and hard) neem fruits were surface-sterilized with 0.9% sodium hypochlorite solution. Twenty grams sterilized neem fruits were macerated in autoclaved distilled water and centrifuged at 10,000 × g. The supernatant thus obtained, was used as the bioelicitor for spraying on the leaves.

Preparation of pathogen inoculums

Pseudomonas syringae pv. *tomato* colonies, characterized by a higher level of virulence (causing six or more lesions on a healthy tomato), were isolated from naturally-infected tomato fruits collected from the fields on King's B agar medium. It was sub-cultured on King's B broth and after 24 h its concentration was adjusted to 10^8 cfu · ml^{-1}. This was subsequently used for inoculating tomato plants without any time lag.

Treatment of plants

For the study, 8 week old plants were used. The plants were divided into five groups. The leaf on the 3rd node from the base of each plant was treated as follows:
Group 1 – sprayed with autoclaved sterile distilled water (the control);
Group 2 – inoculation with pathogen followed by bioelicitor application;
Group 3 – bioelicitor application followed by pathogen inoculation;
Group 4 – inoculation with pathogen only;
Group 5 – sprayed with bioelicitor only.

Samples were collected from treated as well as distal untreated nodes (above the 3rd node) at 0, 24, 48, 72, 96 h, and 2 weeks post treatment.

Fifty tomato plants were chosen for each treatment separately.

Disease severity

Disease severity was evaluated by inoculating the newly emerged leaves with the pathogen and visually observing bacterial speck lesions on them after 2 days of inoculation. Disease severity was scored using a disease index with a range of 0 to 3 (0 signifies a healthy-looking plant; 1 signifies 2 to 5 specks together or spread over each leaf; 2 signifies 6 to 10 specks; and 3 signifies more than 10 specks). Five plants were utilized for the evaluation of disease severity and their average was calculated.

Enzyme extraction

For the enzyme extraction, 300 mg of leaf samples were homogenized in 1.2 ml of ice-cold sodium phosphate buffer (0.1 M, pH 9.0) containing 0.001% Triton X-100, 10 mM β-mercaptoethanol, 10% (w/w) polyvinylpyrrolidone (PVP), 1 mM phenylmethylsulphonyl fluoride (PMSF), and 1 mM EDTA at 4°C. The homogenate was centrifuged at 10,000 rpm at 4°C for 20 min. The supernatant thus obtained, was used as an enzyme extract for peroxidase (POX) and lysozyme estimation. Five biological replicates (from five different plants) were taken for each sample.

Polyphenol oxidase (PPO) activity assay

The method earlier described by Bhuvaneshwari and Paul (2012) was used to carry out the PPO activity assay. The reaction mixture consisted of 0.5 ml of sodium phosphate buffer (1 M, pH 9.0), 1.25 ml of catechol (0.2 M), 0.05 ml of enzyme extract, and 0.2 ml of Type I water. The reaction mixture was incubated at 25±1°C for 5 min and terminated by the addition of 0.5 ml 10% v/v sulphuric acid. Absorbance was recorded at 420 nm using a UV--VIS spectrophotometer (Shimadzu UV-1650). Reaction mixture without the enzyme extract, served as blank. Enzyme activity was expressed as units · g^{-1} · min^{-1} fresh weight. One unit of enzyme activity was defined as the amount of enzyme required for a change in absorbance of 0.001 per minute.

Lysozyme activity assay

Substrate preparation

Five mg of lyophilized cell walls of *Micrococcus lysodeikticus* was dissolved in 1 ml of sodium acetate buffer (50 mM, pH 5.0). From this stock, 60 µl of the suspension was used to arrive at 300 µg · 3 ml^{-1} reaction mixture.

Lysozyme activity was estimated as the rate of lysis of *M. lysodeikticus* cell walls, according to the method of Sakthivel *et al*. (2010). To 250 µg of protein, 60 µl of the substrate stock as prepared above was added, and the volume was made up to 2.5 ml with sodium-acetate buffer (50 mM, pH 5.2). The reaction mixture was incubated at 37±1°C for 5 min and the reaction terminated by the addition of 500 µl of 0.1 M sodium hydroxide. The enzyme activity was monitored by recording the absorbance at 570 nm (Shimadzu UV-1650). The enzyme activity was calculated as the amount of protein required to reduce the absorbance value by 0.01 units and expressed as units · ml^{-1}.

Native-basic PAGE and isoenzyme staining

The isozyme profiles of cytoplasmic PPO and lysozyme were analyzed by native basic polyacrylamide gel electrophoresis (PAGE) (Laemmli 1970). Since the basic PAGE was run, only the acidic isoforms' bands could be observed in the gel. For isoform analysis of each sample, 75 µg of proteins were loaded onto the native basic polyacrylamide gel. The native gel consisted of 10% resolv-

ing gel and 4% stacking gel. Electrophoresis was carried out at 70 mA/gel for 3 h at 4°C. After electrophoresis, the gels were stained for iso-PPO by equilibrating the gel in 0.1% p-phenylenediamine followed by the addition of 50 mM catechol in 0.1 M sodium-phosphate buffer (pH 7.0) (Anand *et al*. 2007). The lysozyme activity on native basic PAGE was analyzed by modifications in the method described by Sakthivel *et al*. (2010). The separating gel was incorporated with the lyophilized cell walls of *M. lysodeikticus* (0.2% w/v). After the completion of the electrophorectic run, the gel was incubated in sodium-phosphate buffer (50 mM, pH 5.0) with 1% (v/v) Triton X-100 for 2 h at 37°C, under gentle shaking. The lytic activity of lysozyme was visualized as a clear transparent zone against the dark background.

Lysozyme band elution and concentration estimation

The concentration of the in-gel-activity stained lysozyme isoenzyme was measured by excising the band and macerating the gel in 500 µl of elution buffer containing 50 mM Tris-HCl, 150 mM NaCl, and 0.1 mM EDTA (pH 7.5) in a pre-sterilized and chilled mortar and pestle. The macerated gel piece was incubated on a rotary shaker at 37°C overnight in a centrifuge tube. After incubation, the tube was centrifuged at 10,000 rpm for 10 min at 4°C and the supernatant was carefully pipetted into a new microcentrifuge tube. This was subsequently analyzed for lysozyme concentration, using Bradford's method.

Statistical analysis of the data

The data were statistically analyzed for analysis of variance (ANOVA) using the general linear model procedure and the least squares means test of the statistical software SAS (version 9.2 developed by SAS Institute Inc., Cary, NC, USA). Multiple pairwise comparison tests using least-square means were performed for post-hoc comparisons after two way ANOVA with treatment and time as the two factors along with the sample replicates. The corrections used for multiple comparisons were the Tukey's honest significantly differences (HSD) test procedure. Data for disease incidence and severity were statistically analyzed by SPSS software for windows version 16 (SPSS Inc., Chicago, Illinois, USA) using univariate general linear model procedures and one-way ANOVA, respectively, followed by post-hoc comparisons using Tukey's HSD.

Results

The disease severity analysis demonstrated that application of neem fruit extract could significantly ($p = 0.043$) reduce disease severity in tomato against *P. syringae* pv. *tomato*. Plants treated with the bioelicitor alone had ~25% lesser disease severity than the control plants. The disease severity in pathogen only inoculated plants was significantly ($p = 0.044$) higher than in the control samples. The leaves which were treated with the bioelicitor before pathogen inoculation, also had significantly ($p = 0.039$) reduced disease severity. However, in the plants treated with neem extract after pathogen inoculation, the reduction in disease severity was not significant ($p = 0.035$) (Fig. 1). The symptoms of bacterial speck on the challenge-inoculated treated plants are demonstrated in figure 2.

Results demonstrated that the bioelicitor treated plants had higher PPO activity than the control plants. A significant ($p = 0.048$) increase in PPO activity was observed at 24 h in both the 3rd node and distal leaves of plants treated with bioelicitor alone which continued up to 96 h. A significant ($p = 0.029$) increase in PPO activity was noted after 48 h in the distal leaves of plants treated with the bioelicitor followed by pathogen inoculation. The new leaves emerging after 2 weeks of neem treatments had significantly ($p = 0.035$) higher PPO activity in all the samples except for the control and the pathogen-only inoculated plants (Fig. 3).

The profiling of PPO isoenzymes in treated tomato plants demonstrated that two PPO isoenzymes (Rf = 0.38 and 0.40) were constitutively expressed in all the samples including the control. *De novo* expression of two additional PPO isoenzymes (Rf = 0.36 and 0.42) were expressed in the distal untreated leaves of plants treated with the bioelicitor either alone or before/after pathogen inoculation. A similar expression of the additional PPO isoenzymes (Rf = 0.36 and 0.42) was also observed in the leaves treated with bioelicitor or the pathogen alone. The distal untreated leaves of plants treated with bioelicitor alone had an additionally expressed isoenzyme of PPO (Rf = 0.26). The

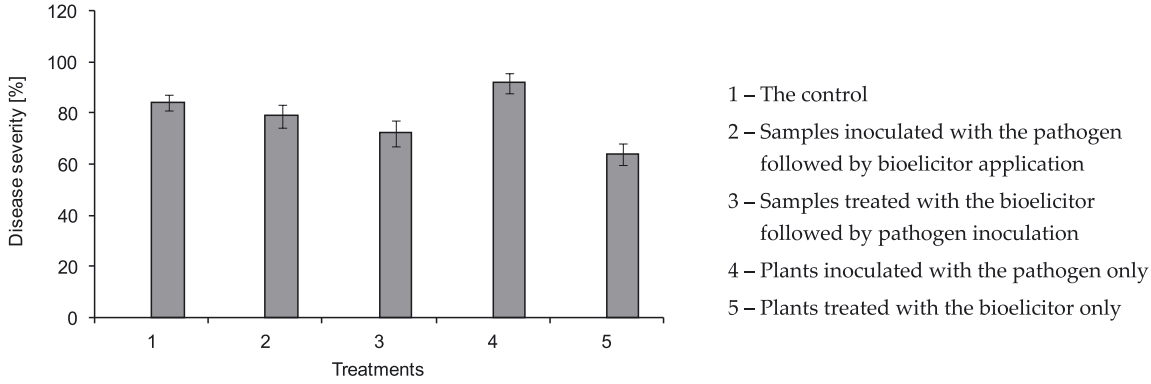

Fig. 1. Disease severity in bioelicitor treated tomato plants

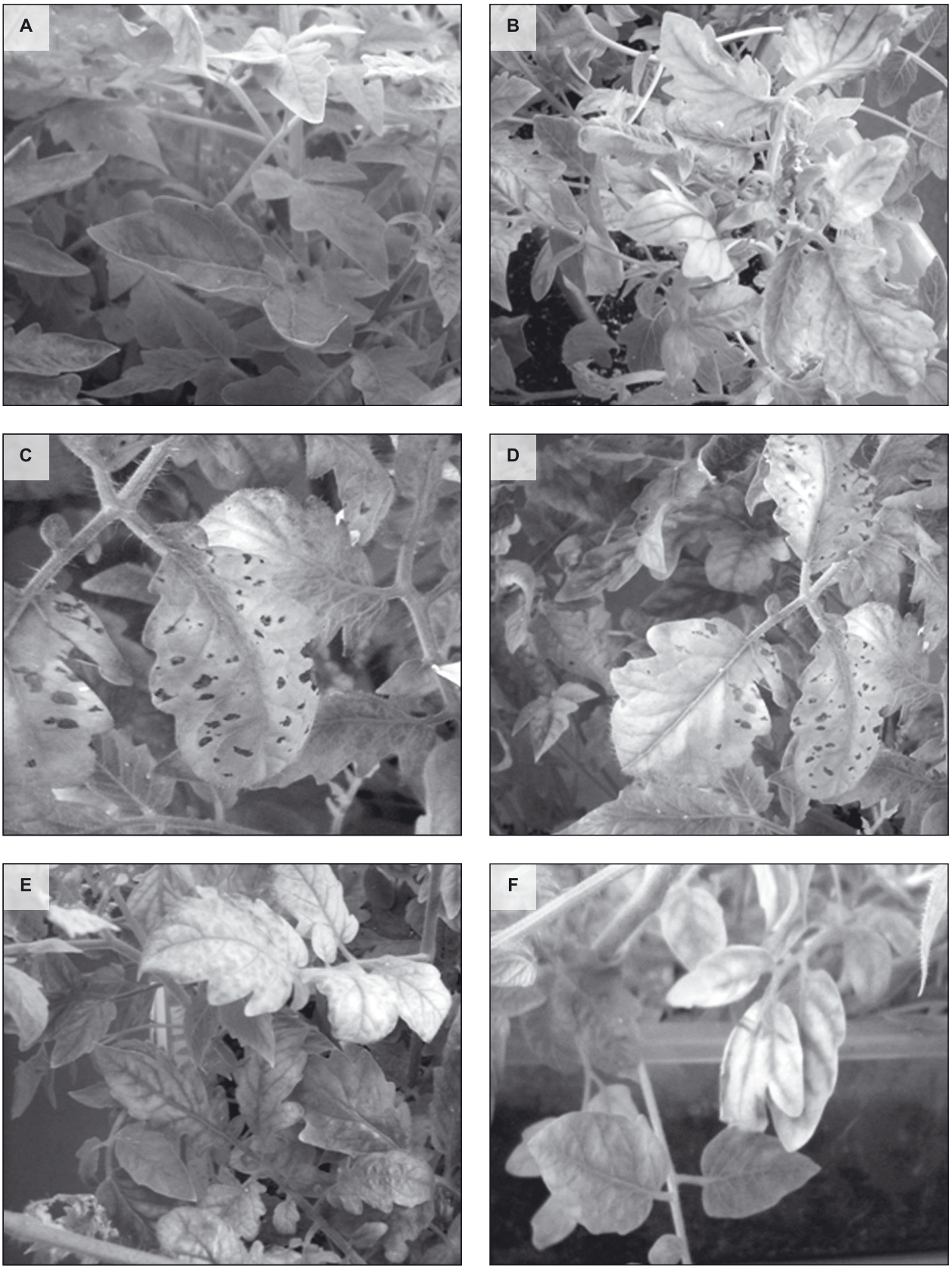

Fig. 2. Bacterial speck symptoms on newly emerged leaves of challenge-inoculated plants already treated with the bioelicitor/pathogen:
A – The water sprayed control;
B – Group 1 plants, challenge-inoculated with the pathogen;
C – Group 2 plants, challenge-inoculated with the pathogen;
D – Group 3 plants, challenge-inoculated with the pathogen;
E – Group 4 plants, challenge-inoculated with the pathogen;
F – Group 5 plants, challenge-inoculated with the pathogen.

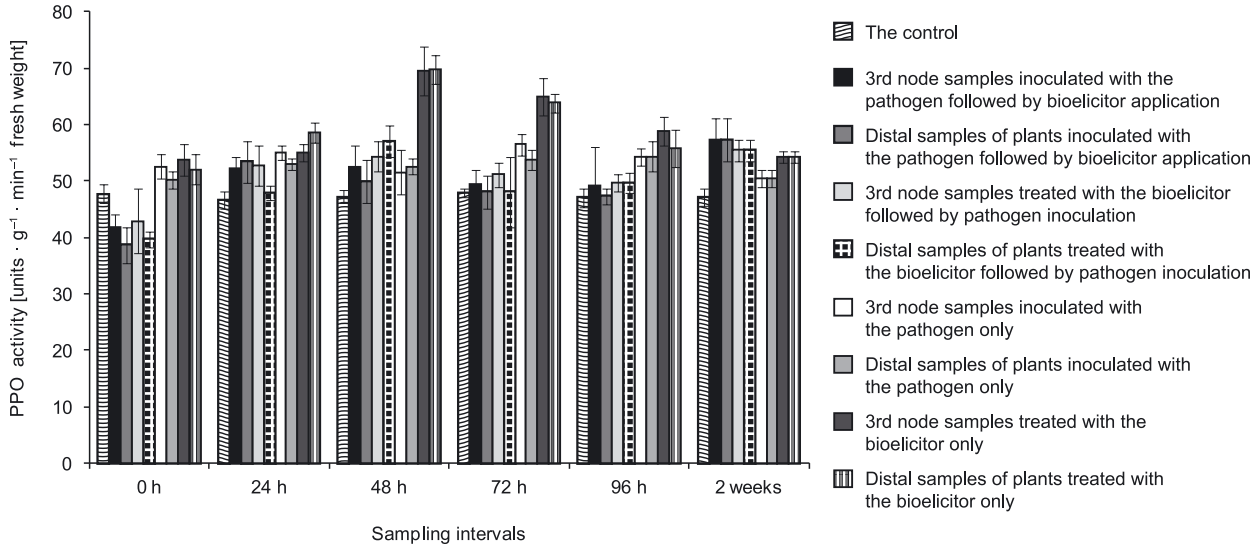

Fig. 3. Polyphenol oxidase (PPO) activity after bioelicitor application in tomato plants

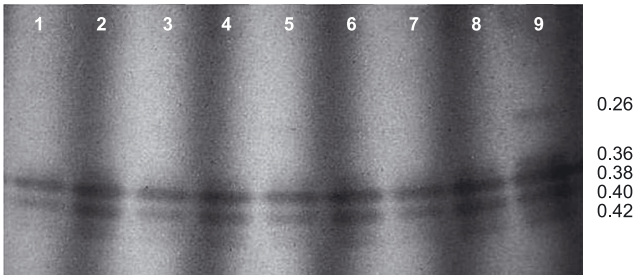

Fig. 4. Isoenzyme profile of polyphenol oxidase (PPO) in the bioelicitor treated tomato plants:
1 – 3rd node samples inoculated with the pathogen followed by bioelicitor application;
2 – istal samples of plants inoculated with the pathogen followed by bioelicitor application;
3 – 3rd node samples treated with the bioelicitor followed by pathogen inoculation;
4 – distal samples of plants treated with the bioelicitor followed by pathogen inoculation;
5 – the control;
6 – 3rd node samples inoculated with the pathogen only;
7 – distal samples of plants inoculated with the pathogen only;
8 – 3rd node samples treated with the bioelicitor only;
9 – distal samples of plants treated with the bioelicitor only.
(Numbers on the top are the lane numbers and on the right are the Rf values of corresponding isoenzyme)

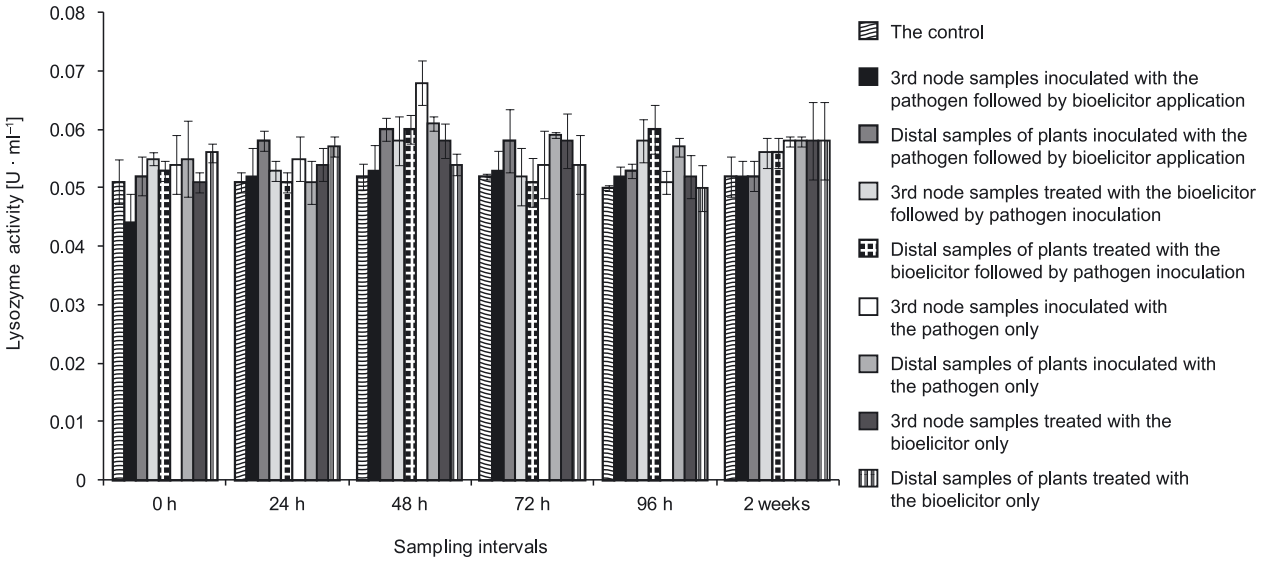

Fig. 5. Lysozyme activity after bioelicitor application in tomato plants

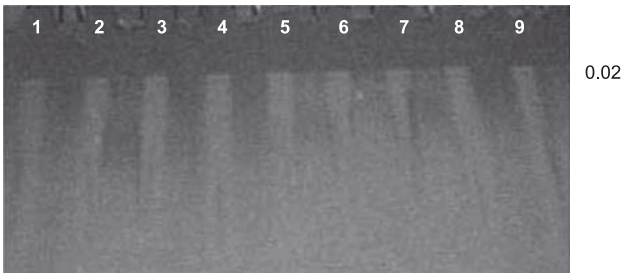

Fig. 6. Isoenzyme profile of lysozyme in the bioelicitor treated tomato plants:
1 – 3rd node samples inoculated with the pathogen followed by bioelicitor application;
2 – distal samples of plants inoculated with the pathogen followed by bioelicitor application;
3 – 3rd node samples treated with the bioelicitor followed by pathogen inoculation;
4 – distal samples of plants treated with the bioelicitor followed by pathogen inoculation;
5 – the control;
6 – 3rd node samples inoculated with the pathogen only;
7 – distal samples of plants inoculated with the pathogen only;
8 – 3rd node samples treated with the bioelicitor only;
9 – distal samples of plants treated with the bioelicitor only.
(Numbers on the top are the lane numbers and on the right is the Rf value of corresponding isoenzyme)

results clearly demonstrated that the bioelicitor could effectively lead to *de novo* expression of PPO isoenzymes in the leaves which were away from the site of the treatment (Fig. 4).

A significant ($p = 0.046$) increase in lysozyme activity was observed at 48 h in the 3rd node leaves of plants inoculated with the pathogen only. The 3rd and distal node leaves of plants inoculated with bioelicitor alone, showed significant ($p = 0.042$) increase in lysozyme activity at 48 h (Fig. 5).

However, the bioelicitor application in combination with the pathogen could not induce lysozyme activity in the host plants. The isoenzyme profiling of the samples demonstrated the presence of a single lysozyme isoenzyme ($Rf = 0.02$) (Fig. 6). The protein concentration in the band had a significant ($p = 0.028$) increase in lysozyme expression in both the 3rd and distal leaves of plants inoculated only with pathogen, and the distal leaves of plants treated with the bioelicitor.

Discussion

The use of plant extracts for disease management is gaining worldwide importance and acceptance. Crop plants acquire an enhanced defensive capacity that results in a faster and/or stronger defense reaction upon treatment with a resistance-inducing agent, known as priming. Inducing the plants own defense mechanisms through SAR is thought to be a novel plant protection strategy. In the present study, plants treated with the bioelicitor (*A. indica* fruit extracts) were significantly protected against *P. syringae* pv. *tomato*. A successful induction of resistance was mediated by increased activities of PPO and lysozyme. Pretreatment with the bioelicitor primed the *de novo* synthesis of PPO isoenzymes in the leaves which were located away from the site of application.

The results clearly demonstrated a significant reduction in the severity of bacterial speck in bioelicitor-treated plants. Greenhouse-grown tomato and pepper plants sprayed with aqueous suspensions of neem oil and then inoculated with *X. campestris* pv. *vesicatoria*, showed fewer disease symptoms than the water-treated controls (Abbasi *et al.* 2003). Reddy *et al*. (2012) recommended the use of the above treatment to control bacterial spot in field-grown tomato plants. Extracts from neem leaf, neem seed and mahogany bark when used individually could significantly reduce the severity of tuber soft root caused by *Erwinia carotovora* ssp. *carotovora* in potato plants (Bdliya and Dahiru 2006; Bdliya and Abraham 2010). It was reported by Goel *et al.* (2013, 2014) that aqueous extracts of *A. indica* were efficient in inducing defense enzymes in tomato against *P. syringae* pv. *tomato*, thus significantly reducing the severity of bacterial speck in the host plant. Neem seed and fruit extracts separately showed *in vitro* antibacterial activity against *Pseudomonas aeruginosa* and *Corynebacterium diphtheria* and could induce PPO and other defense enzymes in tomato for protection against *P. syringae* pv. *tomato* (Bhuvaneshwari *et al*. 2015).

The present investigation proved that application of bioelicitor could significantly increase PPO activity and effectively induced expression of its acidic isoforms. Appearance of additional isoenzymes in bioelicitor-treated plants indicate that either the isoenzymes were expressed after bioelicitor application or the already expressed but inactive isoenzymes were activated by it. This could possibly be due to activation of the PPO-mediated phenylpropanoid pathway resulting in the synthesis of quinones from cytoplasmic phenols and production of microtoxic relative oxygen species (ROS). Enhanced PPO activity could have promoted accelerated cell death of the cells surrounding the infection site, thus preventing the spread of the pathogen. Also, cross linking of carbohydrates, glycoproteins, and lignin in the cell walls might have occurred, thereby reducing pathogen ingress. Successful establishment of SAR can thus be attributed to the multifaceted defensive functions of PPO in tomato. Tyagi *et al*. (2000) suggested that the increase in the number of PPO isoforms due to *Alternaria triticana* infection

in wheat, led to increased contents of oxidised quinone derivatives, thus increasing resistance of wheat towards the pathogen. Li and Steffens (2002) reported that quinones generated in PPO over-expressing tomato plants could hinder the ingress of the bacterial pathogen *P. syringae* pv. *tomato* by generating microtoxic ROS to directly inhibit the pathogen growth inside the cells. Polyphenol oxidase has been observed to be instrumental in imparting resistance to potato against soft rot infection by oxidation of chlorogenic acid. This acid inhibits the cell wall degrading-activity of *Pectobacterium* sp. (Ngadze *et al.* 2012). Upon challenge with *P. syringae* pv. *tomato*, PPO-over-expressing tomato plants showed reduced bacterial growth, whereas PPO suppressed-lines had a higher disease incidence (Goel *et al*. 2014).

Lysozyme activity was significantly increased in the plants when treated with the bioelicitor or the pathogen alone. The virulence compounds (effector proteins) of the pathogen possibly triggered the breakage of vacuoles and the discharge of lysozyme into the host cytoplasm. This could possibly have acted as an effective second line of defense when the pathogen causes tissue damage. Once the vacuole is disrupted, lysosome is released into the cell cytoplasm, thereby attacking the pathogen. This may occur in the hypersensitive reaction of the plant where a small group of plant cells around an invading pathogen die and release their contents (Van Loon *et al*. 2006). It was suggested by Busam *et al*. (1997) that expression of VCH3, a type III chitinase possessing lysozyme activity, could successfully induce SAR in *Vitis vinifera* against *Plasmopara viticola*. Silverleaf whitefly feeding on the host plants significantly induced lysozyme activity both locally and systemically in the hosts, leading to the plants having increased resistance against the pest (Mayer *et al*. 2002). The presence of lysozyme in mung bean seeds and *M. charantia* L. which exhibited broad spectrum antibacterial properties was reported by Wang *et al*. (2005; 2011). The presence suggests lysozyme's important role in constitutive host defense mechanisms against microbial pathogens. However, no new isoenzymes of lysozyme were expressed upon elicitor application. Since neem extract could not induce lysozyme activity when applied in combination with the pathogen, it can be hypothesized that the pathogen effector proteins probably interfere with the elicitation properties of the bioelicitor. This appears to be in agreement to the findings of Rico and Preston (2008), who reported that such effector proteins can potentially inactivate plant surveillance mechanisms and signaling pathways, thus allowing the survival of the pathogen on the leaf surface. Hauck *et al*. (2003) demonstrated that AvrPto, an effector molecule of *P. syringae* pv. *tomato* strain DC3000, could downregulate the expression of a set of genes in *Arabidopsis* which encoded cell wall and defense proteins, thus increasing the susceptibility of the host towards the pathogen. Coronatine, another effector molecule synthesized by *P. syringae* pv. *tomato*, acts as a molecular mimic of methyl jasmonate. Thus, coronatine inhibits the activation of jasmonic acid signaling pathway responsible for defense response during stress conditions (Nomura *et al*. 2005).

The results of the present study demonstrate that bioelicitor (neem extract) was able to reduce disease severity in tomato plants by inducing PPO and lysozyme mediated-resistance in the plants, which play a critical role in host defense. However, it would be important to identify the chemical constituents in the extract which are responsible for this induction. Also, it would be of great interest to study the interaction of these compounds with the host cell-wall receptors leading to activation of certain pathways. These pathways are ultimately responsible for enhancement of the defense enzymes' activities and the upregulated expression of the concerned genes.

Conclusions

Neem extract could significantly enhance PPO activity at the site of application as well as in the systemic tissues away from it. *De novo* expression of additional PPO isoforms was also observed in host plants. Lysozyme activity was induced by the bioelicitor when applied alone. However, application in combination with the pathogen reduced its efficiency, indicating the interference of the pathogen in the elicitation properties of the neem extract. Neem fruit extract could prove to be a potential biocide for eco-friendly plant protection strategies.

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