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Stimulation of Defense Enzymes in Tomato (*Solanum lycopersicum* L.) and Chilli (*Capsicum annuum* L.) in Response to Exogenous Application of Different Chemical Elicitors

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Abstract The objective of this study was to evaluate the response to exogenous application of different chemical elicitors on the stimulation of defense enzymes such as chitinase, peroxidase, phenylalanine ammonialyase (PAL) and polyphenoloxidase (PPO) in tomato and chilli plants leaves tissues. Chemical elicitors are compounds, which activate chemical defense systems in plants. Various biosynthetic pathways are activated by chemical elicitors in plants depending on the elicitors used. Two times exogenous application of 200 ppm of salicylic acid (SA), ascorbic acid (AA), jasmonic acid (JA) and H_2O_2 and ethanol (1 mL of 95% ethanol in 1 L distilled water) induced the four defense enzymes production in chilli and tomato plants leaves tissues and significantly reduced viral disease incidence in chilli plants compared to control. SA at 200 ppm enhanced significantly chitinase, peroxidase, PAL and PPO enzymes in leaves tissues of tomato plants. Tomato plants treated with chemical elicitors and control did not show any disease symptoms.

Keywords Ascorbic Acid, Chilli, Jasmonic acid, Peroxidase, Salicylic Acid, Tomato

1. Introduction

Chilli (*Capsicum annuum* L.) is one of the important cash crops grown in Jaffna district, Sri Lanka belonging to family of Solanaceae. It has become an essential ingredient in Sri Lankan meals. The chilli is one of the most important vegetable as well as spice crops around the world, valued for its aroma, taste, flavor and pungency [6]. Besides traditional uses, it is also being used in pharmaceuticals, cosmetics and beverages [38]. Tomato (*Solanum lycopersicum* L.) is one of the most widely cultivated vegetable crops in the world. It is an important cash crop for medium scale commercial farmers in Jaffna district of Sri Lanka. However, both quantity and quality of tomato and chilli are affected by many diseases, pests, nutrient deficiencies, and due to climatic and environmental conditions. It has become a serious threat to subsistence and commercial agriculture.

Several studies proved that exogenous application of different elicitors are useful to enhanced resistance to herbivore challenge and induce the expression of defense related genes [21, 18]. Exogenous or endogenous factors could affect host physiology, lead to rapid and coordinated activation of defense-gene in plants normally expressing susceptibility to pathogen infection [23, 26]. Studies have indicated remarkable similarities between the defense mechanisms triggered by general elicitors and the innate immunity of animals, and it is tempting to speculate that the recognition of general elicitors subsequently leads to plant innate immunity [27]. Elicitors act as signal compounds at low concentrations, providing information for the plant to trigger defense, distinguishing elicitors from toxins, which may act only at higher concentrations and / or affect the plant detrimentally without active plant metabolism [7]. This induced resistance to pathogens can be achieved by the application of various abiotic agents (chemical inducers) such as salicylic acid, potassium salts and sorbic acid [1, 3]. The present study was conducted to evaluate the defense enzymes stimulation by the application of different chemical elicitors on tomato and chilli plants.

2. Materials and Methods

Application of Chemical Elicitors

Seeds of tomato (variety KC1) and chili (variety MI2) were planted separately in plastic pots with 20.5 cm height

and 25.3 cm diameter. A rate of 2 plants/plastic pot was allowed to grow. Pots were arranged according to a completely randomized design (CRD) with six replicates per treatment. First foliar application of six treatments (Table1) were made using hand sprayer to plants at the 4-leaf stage and second application was made after 40 days of first application. A separate set of pots planted with tomato and chili separately but not treated with chemicals and water was maintained as control. Plants were maintained in the plant house at the Department of Botany, Faculty of Science, University of Jaffna, Sri Lanka.

Table1. Treatments

Treatments	Chemical elicitor	Quantity
Treatment 1 (T1)	Salicylic acid	200 ppm
Treatment 2 (T2)	Ascorbic acid	200 ppm
Treatment 3 (T3)	Jasmonic acid	200 ppm
Treatment 4 (T4)	Ethanol (95%)	1 mL in 1 L Distilled water
Treatment 5 (T5)	H_2O_2	200 ppm
Treatment 6 (T6)	Tap water	-

Disease Incidence

Plants were grown in an open green house, no pathogen was inoculated artificially. Common viral infection symptoms such as, chlorotic, necrotic, curling, mottling, stunting symptom were observed. The plants were monitored weekly after treatments were applied by recording number of leaves showing disease or damage out of total number of leaves of chilli and tomato plants.

Sample Collection for Enzyme Assay

Leaf samples were collected from tomato and chilli plants of all treatments and stored at -20 ^{o}C immediate after detachment from the plants. The frozen plant leaf samples were ground in liquid nitrogen using separate mortars and pestles. The finely ground samples were stored in eppendorf tubes at -20 ^{o}C until the assay of defense enzymes.

Assay of Defense Enzymes

Assay of Chitinase

Ground leaf sample (0.5g) was mixed with 1 mL of 0.1 M sodium acetate buffer (pH 5.0) and the mixture was vortexed for 3 min. Then sample was centrifuged at 13,000 rpm for 15 min. Volume of 0.4 mL of the supernatant was mixed with 0.2 mL of 0.1 M sodium acetate buffer (pH 5.0) and 0.2 mL of chitin azure. Then the samples were incubated at 40 ^{0}C for 10 min. An aliquot of 0.2 mL of 2 N hydrochloric acid was mixed with the sample and kept on ice for 10 min. Once again sample was centrifuged at 13,000 rpm for 15 min and the supernatant was used to read the absorbance at 575 nm [2].

Assay of Peroxidase

Ground leaf sample (0.5g) was mixed with 1.5 mL of 0.1 M sodium phosphate buffer pH 7.0 at 4 °C. Then the mixture was centrifuged at 10,000 rpm for 20 min and the supernatant was used as the enzyme source. The reaction mixture consisted of 1.5 mL of 0.05 M pyrogallol, 0.5 mL of enzyme dilute and 0.5 mL of 1 % H_2O_2. The changes in absorbance at 420 nm were recorded at 30 s intervals for 3 min. The enzyme activity is expressed as changes in the absorbance $min^{-1}g^{-1}$ leaf tissue [17].

Assay of Phenylalanine Ammonia Lyase (PAL)

An aliquot of 1mL of 0.1 M phosphate buffer was added to ground plant sample (0.5g). The samples were centrifuged at 10,000 rpm for 20 min. The supernatant was used as the enzyme source. Volume of 0.5 mL of 0.1 M phosphate buffer, 0.1 mL enzyme source and 0.4 mL de-ionized water were placed in a test tube. The reaction was initiated by the addition of 1 mL of 0.1 M L-Phenylalanine solution and incubated for 30 min at 37 °C. The reaction was stopped by adding 0.5 mL of 1 M Trichloro acetic acid. A blank reading was taken by adding 0.5 mL phosphate buffer and 0.5 mL de-ionized water. The rest of the procedure was followed for the blank test was similar to the test with sample extract. The absorbance was measured at 290 nm. A standard graph was prepared for *trans*-cinnamic acid [9].

Assay of Polyphenol Oxidase (PPO)

PPO activity was determined as per the procedure given by Mayer *et al.* [24]. Ground plant sample (0.5g) was mixed with 1.5 mL of 0.1M sodium phosphate buffer (pH7.0) and centrifuged at 10,000 rpm for 20 min. The supernatant was used as the enzyme source. The reaction mixture consisted of 200 µL of the enzyme extract and 1.5 mL of 0.1 M sodium phosphate buffer (pH7.0). To start the reaction, 200 µL of 0.01 M catechol was added. The changes in absorbance at 495 nm were recorded at 10 s intervals for 1 min. The activity was expressed as changes in absorbance $min^{-1}g^{-1}$ leaf tissue.

Data Analysis

Data were analyzed by variance (ANOVA) using a SAS statistical package (version9.1.3) and mean separation was done by Least Significance Difference (LSD).

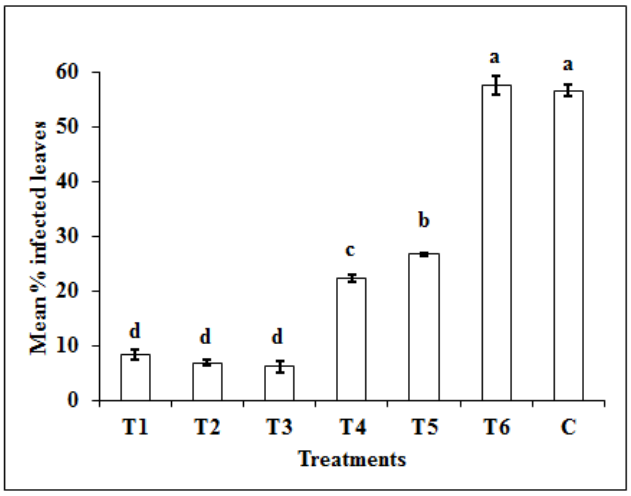
3. Results and Discussion

Chilli plants treated with chemical elicitors and distilled water and control showed viral diseases such as little leaf and leaf puckering (Figure1) but percentage infected leaves varied among treated plants and control (Figure 2). There was a significant reduction in viral diseases development in chilli plants when they were treated with chemical elicitors such as salicylic cacid (SA), ascorbic acid (AA), jasmonic acid (JA), ethanol and H_2O_2 when compared to plants treated

with distilled water and control. There was no viral disease incidents observed in tomato plants (Treated and Control). Chilli plants are infested by more than 21 insects and non-insect pests [35, 4], and some herbivores insect such as aphid, thrips and mites also acts as a vector for the transmission of plant disease causing viruses [37].

The higher percentages of disease incidence were reported by Treatment 6 and control were not significantly different in terms of percentage of infected leaves. The lower percentage of disease incidence shown by treatments Treatment 1, Treatment 2 and Treatment 3 hence chemical elicitors SA, AA and JA reduce the disease incidents in chilli plants by induce the expression of defense related genes at 200 ppm. Next to the treatments Treatment 1, Treatment 2 and Treatment 3 there were viral disease reduction observed in treatments Treatment 4 and Treatment 5 (Figure 2).

Figure 1. Disease symptoms showing chilli plants



Mean followed by same letters are not significantly different by LSD at 5% level

Figure 2. Effect of SA (T1), AA (T2), JA (T3), Ethanol 95% (T4) ,H_2O_2 (T5), Distilled water (T6) and control (C) in viral disease incidence in terms of % leaves infected on chilli plants.

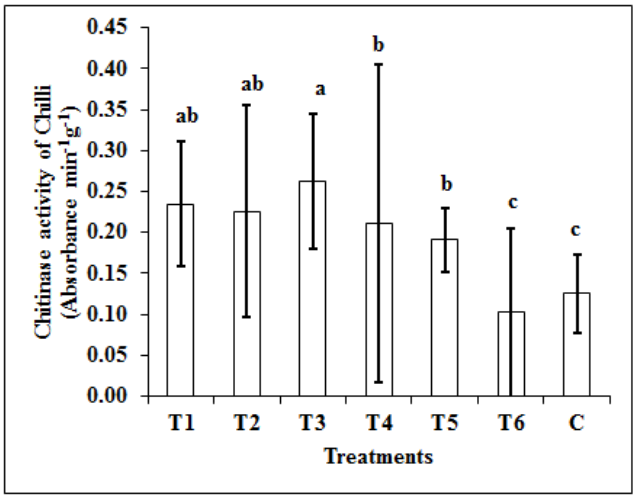


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Figure 3. Response of catalase enzymatic activity in chilli leaf tissues treated with chemical elicitors, distilled water and control.

The results from the statistical analysis showed significant differences among treatments and control. It was observed that different elicitors exerted a different effect on the chitinase on chilli and tomato plants (Figure 3 and 4). Chitinase degrades chitin, a major component of pathogen cell walls [16]. Chitinase may affect insects by damaging chitin-based structures such as the peritrophic membrane that provides a physical barrier to ingested pathogens and other substances that pose a hazard to the insect. Chitinases can also act as α-amylase inhibitors and interfere with digestion of plant parts [5]. Induction of chitinase activity may interfere with insect development, feeding and growth facilitate microbial infection, and finally cause death [34]. Wang *et al*. [39] reported that 100% larval mortality of the grain beetle, *Oryzaephilus mercator*, six days after feeding on 2% chitinase obtained from transgenic tobacco.

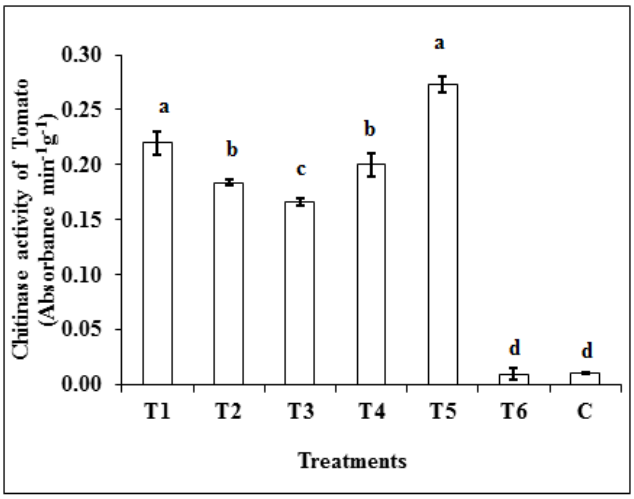

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Figure 4. Response of chitinase enzymatic activity in tomato leaf tissues treated with chemical elicitors, distilled water and control.

Peroxidases participate in a variety of defense mechanisms [20], to ameliorate oxidative burst a common event in defense response [19]. Peroxydase activity of tomato tissue was significantly different among treatments and control (Figure 5). Though there was no significant variation among treatments and control in chilli leaf tissues in the activity of peroxidase enzyme. Peroxoydase is one of the enzymes contributing to plant resistance to pathogens [33]. Plant peroxidase activity seems to be under the strict control depending on the development stage and the environmental stimulus [15]. Maksimov *et al*. [22] reported that the activation of peroxidase is correlated to the defense responses of fruit in presence of pathogens. Peroxidases are induced in tomato plants following pathogen and insect damage.

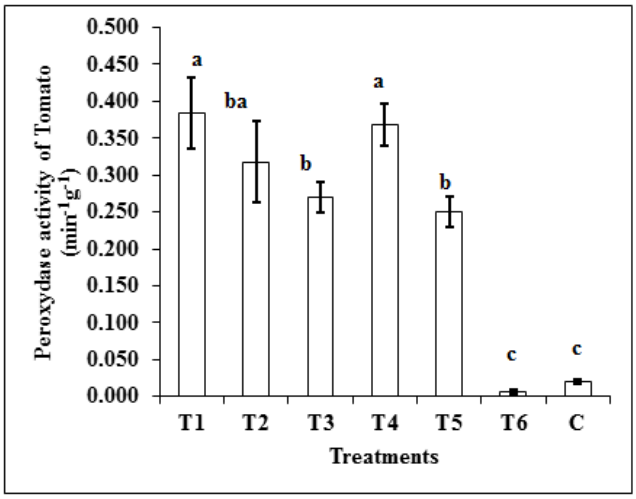

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Figure 5. Response of peroxydase enzymatic activity in tomato leaf tissues treated with chemical elicitors, distilled water and control.

Peroxidases are involved in production and polymerization of phenolics, lignification, and hypersensitive responses, limiting the possibility of disease spread [8].

PAL activity in tomato tissues differed significantly among different treatments and control (Figure 6). In chilli leaf tissues, no significant difference was observed in PAL activity among treatments and control.

A significant difference was observed in PPO activity among treatments and control in tomato leaf tissues (Figure 7) but no significant difference was observed in PPO activity among treatment and control in chilli plants leaves tissues.

The levels of defense related enzymes (PAL, POX, PPO *etc*.) play a crucial role with respect to the degree of host resistance [30]. These are highly important in the defense mechanism against pathogens, by increasing antimicrobial activity and may be directly involved in controlling pathogen development [25]. Increase in these defense related enzymes also cause reduction in disease severity [14, 10, 36].

When SA, AA, JA, ethanol 95% and H_2O_2 were used as elicitors, statistically significant increase of the activities of chitinase, PAL, peroxydase and PPO were observed in the leaf tissues of chilli and tomato plants. The influence of jasmonic acid on the mechanisms of plant resistance to arthropods is associated with the products of induced expression of numerous genes [32, 28]

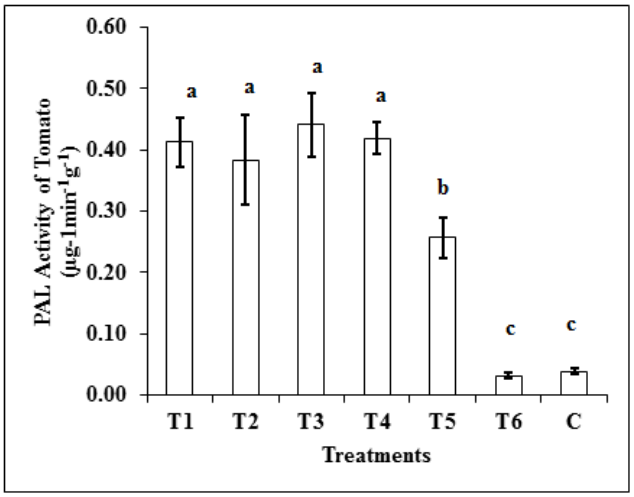

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Figure 6. Response of PAL enzymatic activity in tomato leaf tissues treated with chemical elicitors, distilled water and control.

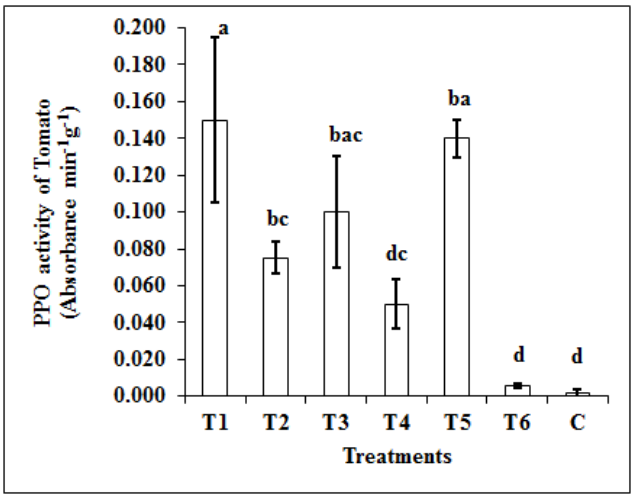

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Figure 7. Response of PPO enzymatic activity in tomato leaf tissues treated with chemical elicitors, distilled water and control.

Jasmonic acid enhanced resistance by regulating expression of defense related genes [21, 18]. Moreover, JA is also involved with different physiological activities such as seed germination, tuber formation, tendril coiling, leaf senescence, stomata opening, fruit ripening and root growth, and also plays crucial roles in plant defense responses against insect damage and microbial pathogens attack [40]. Systemic resistance mechanisms are induced in crop plants by treatment with chemical inducer salicylic acid [13, 31].

In chilli leaf tissues, no significant difference were observed in peroxydase, PAL and PPO activity among treatments and control. All the treatments (1, 2, 3, 4 and 5) prduced defence enzymes because of the activation of defense system in chilli plants by exogenous chemical elecitors. Due to the viral infection plants treated with distilled water and control also produced four defense enzymes. An important finding from our study revealed that all tested chemical inducers had positive effects on plant disease control in chilli plants. Tomato plants did not show symptoms it could be the tomato seeds are resistant to disease or the environmental conditions are not suitable for development of disease in tomato plants. Elicitors can be used to activate plant defensive systems at desired times; however, generally they should be applied prior to having a pest-problem so that the plant will have the best opportunity for resisting pests [11]. Elicitors will probably not be effective in all plants since defensive systems vary with plant variety.

Similar results also gave evidence to the role of H_2O_2 in activation of an array of host defense mechanisms including induced activity of enzymes as peroxidase and chitinase accompanied by a significant increase in the lignin and suberin content [29]. Moreover, H_2O_2 plays also an essential role in lignifications, and cross linking of cell wall proteins with phenolic acids, leading to reinforcement of cell walls at the site of pathogen attack positively influences the local and systemic accumulation of SA that is correlated with the enhancement of peroxidase activity [12].

4. Conclusions

Two time exogenous application of SA, AA, JA, ethanol 95 % and H_2O_2 showed stress symptoms indifferent defense enzymes activities (Chitinase, Peroxidase, phenylalanine ammonia lyase and polyphenol oxidase) in chilli and tomato plant but in chilli plants reduced percentage of viral disease incidence and severity.

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