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Tagetes erecta leaf extract induces defense enzymes in *Solanum lycopersicum*

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ABSTRACT

Resistance of a plant against pests and pathogens is largely a function of its defense related proteins. In vivo induction of these proteins using natural extracts from different parts of plants is an environment-friendly measure of plant protection. The present study, therefore, focused on the evaluation of the ability of an aqueous extract from the leaves of Tagetes erecta (Marigold) in inducing the expression of four defense proteins Peroxidase (POX), Polyphenol Oxidase (PPO), Lipoxygenase (LOX) and Catalase (CAT) in Solanum lycopersicum (Tomato). The plants were raised under aseptic conditions. Eight weeks old plants were sprayed with the marigold extract on the third leaf from the base of each plant. Enzyme activities and isoform expression of POX, PPO, LOX and CAT were studied at both site of treatment and away from it. The results demonstrate that the extract could significantly enhance the activities of POX, PPO and CAT, and was able to induce additional isoforms of POX, PPO and LOX, which might be instrumental in enhancement of host resistance against several biotic and abiotic stresses.

Keywords: *Tagetes erecta*, Peroxidase, Polyphenol Oxidase, Lipoxygenase, Catalase

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INTRODUCTION

Tomato is a globally grown, economically-important vegetable [1]. Chemical methods to control bacterial and fungal pathogens are environmentally unacceptable [2]. Therefore, there is an explicit need to develop alternate environmentally safe methods of pest and disease control. Though many biological control methods have been developed but most of them have limitations. However, induction of Systemic Acquired Resistance (SAR) is most potent in crop plants [3]. Plant defense responses primed by SAR are followed by the enhanced expression of varied defense related proteins which provide broad-spectrum resistance against a large number of pathogens.

Extracts from different parts of *Tagetes erecta* (Marigold) are known to possess broad spectrum antimicrobial activity as well as potential to induce defense responses in plants [4]. Koul *et al*. [5] stated that extracts from *T. erecta* have strong biopesticidal potential. Root extracts of marigold (*T. erecta*) could control rootknot nematodes on mulberry [6] and *Meloidogyne incognita* on tomato plants [7]; probably by inducing defense responses in the host plants.

Peroxidases (POXs) are haem-containing glycoproteins which generally oxidize a wide variety of compounds in the presence of hydrogen peroxide (H_2O_2). Peroxidases are involved in auxin and ethylene metabolism, redox reactions in plasma membranes, cell wall modifications (lignification and suberinization) as well as in developmental and defense processes [8]. Gradual increase in POX activity in cotton after fungal infection pointed towards its possible role in acquisition of resistance [9]. The enhanced activitity of the POX may contribute to bioprotection of black gram plants against *B. tabaci* infestation [10].

Polyphenol oxidases (PPOs) are nuclear-encoded copper-containing enzymes which catalyze the O_2-dependent oxidation of mono and *o*-diphenols to *o*-diquinones, highly reactive intermediates whose secondary reactions are believed to be responsible for the oxidative browning which accompanies plant senescence, wounding, and responses to pathogens [11]. The defensive roles of PPO against disease and insect pests have been clearly established [12]. When challenged by the bacterial pathogen *Pseudomonas syringe* pv. *tomato*, PPO-over-expressing tomato plants showed reduced bacterial growth, whereas PPO suppressed lines had higher disease incidence [13].

Lipoxygenases (LOXs) are non-heme, iron-containing, monomeric proteins of about 95 to 100 kDa made of two domains. The amino-terminal domain of about 25 to 30 kDa is a beta-barrel domain (domain I). The carboxyl-terminal domain of about 55 to 65 kDa consists primarily of alpha-helices (domain II) and harbours the catalytic site of the enzyme. LOXs catalyze the oxygenation of polyunsaturated fatty acids (PUFAs) containing *cis*,*cis*-1, 4 pentadiene moiety such as linoleic acid and linolenic acid [14]. In tomato leaves, it has been proposed that LOX-induced synthesis of JA activates transcription of genes encoding for protease inhibitors in response to insect attack. Also, LOX activity has been observed to increase in response to mechanical wounding, treatment of plants with cell cultures and elicitors [15].

Catalases are one of several types of H_2O_2-metabolizing enzymes in plants playing potential roles in redox homeostasis and defense system of hosts [16]. Potato Class II catalase was induced in roots exposed to nematodes and bacteria and by SA in stem tissue [17]. CAT1 expression was strongly induced by treatment of *Arabidopsis* seedlings with H_2O_2 [18].

The present study thus focused at evaluating the potency of aqueous extracts from the leaves of *Tagetes erecta* on the activity and isoenzyme profiles of defense enzymes such as Peroxidase, Polyphenol Oxidase, Lipoxygenase and Catalase in *Solanum lycopersicum*. The results could provide an insight into the possibility of formulating a biopesticide from the extract.

EXPERIMENTAL SECTION

1.1. Raising of plants

Surface sterilised and aseptically dried tomato seeds (local variety) were sown in sterilized soilrite in plastic trays. The plants were raised in a sterile culture room maintained at 25 ± 1 °C with a relative humidity of 70% and a photoperiod of 12 h L/D. Trays were watered daily with autoclaved distilled water and once a week with Hoagland's solution.

1.2. Extraction of Aqueous marigold leaf extract

50 gm of mature marigold leaves were surface sterilised with 0.9% sodium hypochlorite solution and macerated in 50 ml sterile distilled water in a pre-chilled mortar and pestle under aseptic conditions. The extract obtained was filtered through 4-layered muslin cloth and the filtrate was centrifuged at 8000xg at 4°C for 30 min. The supernatant obtained was filtered through 0.45 μm membrane filter and used as for spraying on to the host plants.

1.3. Treatment of tomato plants

Eight weeks old tomato plants were chosen for the study. The plants were divided into two groups of 50 plants each. Group 1 was sprayed with autoclaved distilled water and designated as control. Group 2 was sprayed with marigold extract. The spraying was performed under aseptic conditions at third node from the base of each plant. Sampling was done for both treated (third nodal leaf) and untreated (distal leaves above the third node) leaves at 0, 24, 48, 72 and 96 hours intervals. The samples were immediately stored at -20ºC and subsequently used for analysis of various parameters under study.

1.4. Extraction of cytoplasmic enzymes

300mg of frozen leaf tissue was homogenized in 1.2 ml of ice cold Sodium-phosphate buffer (0.1M, pH 9.0) containing 10 mM β-mercaptoethanol, 1mM Phenyl methyl sulfonyl fluoride (PMSF), 0.001% Triton X-100, 1mM EDTA and 10% (w/w) Polyvinylpyrrolidone (PVP) at 4^{o}C. The homogenate was centrifuged at 10,000 rpm for 20 min at 4^{o}C. The supernatant was used as crude enzyme extract for estimation of POX, PPO, LOX and CAT and in-gel-activity-staining of their isoforms. Each enzyme estimation assay had five replicates from 5 different samplings.

1.5. Peroxidase activity assay

POX activity assay was carried out by making necessary modifications in the method earlier described by Goel and Paul [14]. The reaction mixture consisted of 0.245 ml of sodium-phosphate buffer (1M, pH 7.0), 0.25 ml of Guaiacol (0.1M), 0.05 ml Hydrogen Peroxide (H_2O_2), 0.05 ml of crude enzyme extract and 1.655 ml of Type I water. The reaction mixture was incubated at 25±1°C for 5 min and reaction was terminated by addition of 0.5 ml 10% v/v Sulphuric acid. Absorbance was recorded at 470 nm using UV-VIS spectrophotometer (Shimadzu, 1650). Reaction mixture without enzyme extract served as blank. The molar extinction coefficient taken for the calculation of enzyme activity for POX was, ε= 26.6 $mM^{-1}cm^{-1}$. Enzyme activity was expressed as $mMmin^{-1}g^{-1}$ fresh weight.

1.6. Polyphenol Oxidase activity assay

PPO activity assay was carried out by the method earlier described by Goel and Paul [11]. The reaction mixture consisted of 0.5 ml of sodium phosphate buffer (1M, pH 9.0), 1.25 ml of catechol (0.2M), 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 addition of 0.5 ml 10% v/v Sulphuric acid. Absorbance was recorded at 420 nm using UV-VIS spectrophotometer (Shimadzu, 1650). Reaction mixture without enzyme extract served as blank. Enzyme activity was expressed as units g^{-1} min^{-1} fresh weight.

1.7. Lipoxygenase activity assay

LOX activity assay was carried out by the modifications in the method earlier described by Goel and Paul [14]. The modified reaction mixture consisted of 1.955 ml of sodium phosphate buffer (0.1M, pH 7.0), 0.025 ml of Linoleic Acid (5 mM), 0.02 ml of crude enzyme extract. The reaction mixture was incubated at 25±1°C for 2 min and terminated by addition of 0.5 ml 5% v/v Sulphuric acid. Absorbance was recorded at 234 nm using UV-VIS spectrophotometer (Shimadzu, 1650). The molar extinction coefficient for LOX used was,ε=25 $mM^{-1}cm^{-1}$. Reaction mixture without enzyme extract served as blank. Enzyme activity was expressed in mM min^{-1} g^{-1}fresh weight.

1.8. Catalase activity assay

Catalase assay was carried out by method described by Gayatridevi *et al.* [19] with certain modifications. The reaction mixture consisted of 2 ml phosphate buffer (0.05M, pH-7.8), 0.5 ml of hydrogen peroxide (H_2O_2) and 0.05 ml of enzyme extract. The reaction mixture was incubated for 2 minutes at room temperature and the reaction was terminated by addition of 0.5mL10% (v/v) sulphuric acid. Absorbance was recorded at 240 nm using UV-VIS spectrophotometer (Shimadzu 1650). Reaction mixture without enzyme extract served as blank. Enzyme activity was expressed μ mol min^{-1} g^{-1}fresh weight.

The total proteins of the samples were estimated by Bradford's method. Five replicates were taken for each sample.

1.9. Native-Basic PAGE and in-gel-activity-staining

The isozyme profiles of acidic POX, PPO, LOX and CAT located in the cytoplasm were analysed by native basic PAGE, without SDS. Electrolyte for electrode reservoirs was Tris-glycine (pH 8.3). Bromophenol blue (0.01%) was used as tracking dye. For each sample75 μg proteins were loaded onto the native basic polyacrylamide gel for isoform analysis. The native gel consisted of 10% resolving gel and 4% stacking gel. Electrophoresis was carried out at 70mA/gel for 3 hours at 4°C. After electrophoresis, the gels were stained for iso-POX by incubating in 0.1 M Sodium-phosphate buffer (pH 7.0) containing 10 mM Guaiacol and 0.75% H_2O_2 [13]. PPO isoforms were visualized by the modified method of Goel *et al*. [20] by equilibrating the gel in 0.1% p-phenylenediamine followed by addition of 50 mM catechol in 0.1M Sodium-phosphate buffer (pH 7.0). LOX isoforms were stained by incubating the gel in 50 mM Potassium phosphate buffer (pH 6.0) containing 0.1% linoleic acid and 0.02% o-dianisidine [14]. CAT isoforms were visualized by the method described by Gayatridevi *et al.* [19]. After the completion of the electrophoretic run, the gels were placed in a substrate solution 0.003% H_2O_2 solution (30% solution, v/v) for 10 min at room temperature. The gel was rinsed off this solution and was washed with distilled water twice. After washing, the gel was stained in 2% ferric chloride (w/v) and 2% potassium ferricyanide (w/v) solution.

The stained isoforms were distinguished by calculating the relative distance (Rf value) [14] of each isozyme band from each zymogram using the following equation: Rf value = Distance migrated by the isoenzyme band from the start of the resolving gel/Distance migrated by tracking dye from the start of the resolving gel.

1.10. 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 with treatment and time as the two factor with replications. The corrections used for multiple comparisons were Tukey's honest significantly differences test (HSD) procedure. Data for disease 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 AND DISCUSSION

The results of present study demonstrate the potential of an aqueous extract from *Tagetes erecta* in inducing the activity and additional isoforms of defense enzymes of *Solanum lycopersicum*, not only at the site of application but also away from it (in the distal untreated leaves). This could possibly be brought about by the induction of a signal transduction mechanism mediated by secondary messengers which eventually results into the *de novo* expression of such defense enzymes in the host. The induction of these enzymes could be instrumental in enhancement of host resistance against a large array of pests and pathogens.

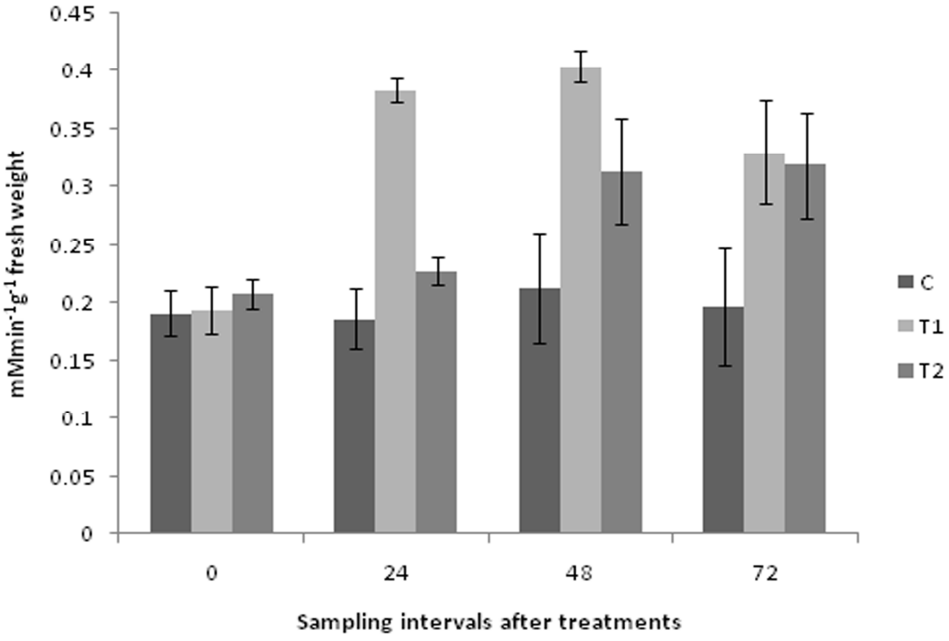

Figure 1. POX activity in treated plants

C= control, T1= Third node leaves of plants treated with marigold extract, T2= Distal untreated leaves of plants treated with marigold extract. Note: The bars represent average POX activity ± S.E (n=5).

Extracts from different parts of *Tagetes erecta* are known to possess defense inducing properties. Franzener *et al*. [21] demonstrated that aqueous extracts from leaves, flowers and roots of *Tagetes* sps. could protect tomato plants against *Meloidogyne incognita* infection. Marigold leaf extracts were found effective against brinjal pests [22]. The leaf extract of Indian neem followed by Mexican marigold plants have been proved the best spray for managing *L. erysimi* population and achieving high yield of Indian mustard [23]

The activities of POX, PPO, LOX and CAT were significantly enhanced and additional isoforms of POX, PPO, LOX were induced by the marigold extract. The involvement of these enzymes in the defense mechanism of plants is well known. The appearance of additional isoforms after marigold treatment suggested that either the already expressed but inactive isoforms were activated or new ones were expressed as a result of extract-elicited reactions.

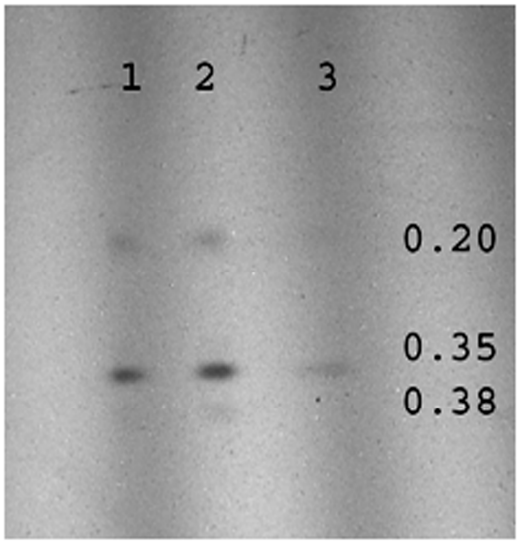

Figure 2. POX isoforms in treated samples after 48 h
Lane 1= 3rd nodal treated leaves, Lane 2= distal untreated leaves, Lane 3= control.
Note: The numbers on the right are the Rf values of the corresponding POX isoform.

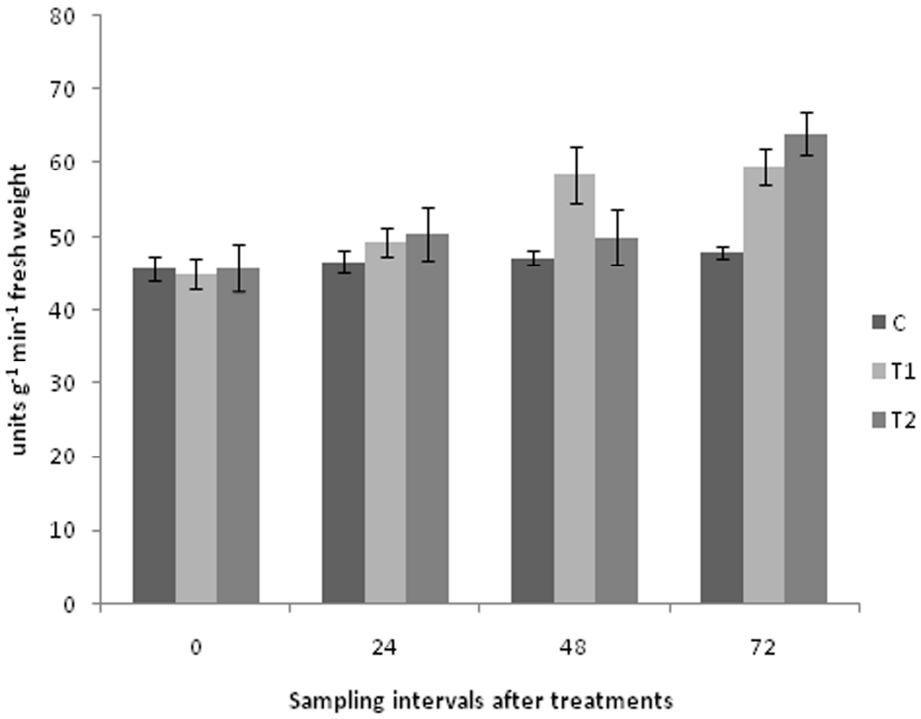

Figure 3. PPO activity in treated plants
C= control, T1= Third node leaves of plants treated with marigold extract, T2= Distal untreated leaves of plants treated with marigold extract.
Note: The bars represent average PPO activity ± S.E (n=5).

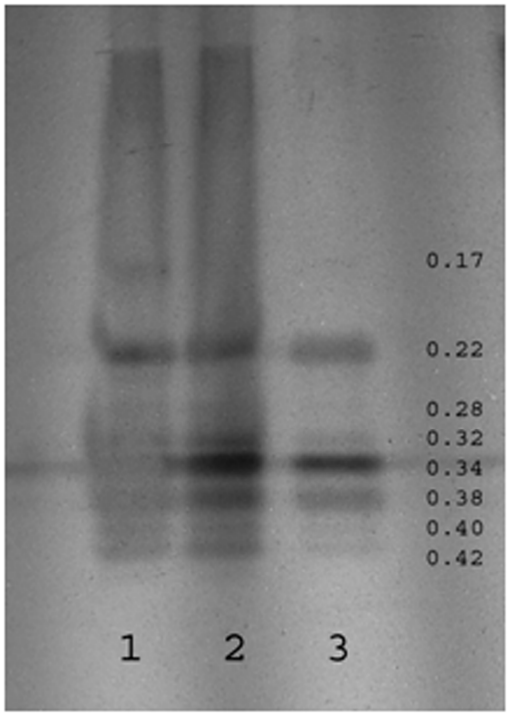

Figure 4. PPO isoforms in treated samples after 72 h
Lane 1= 3rd nodal treated leaves, Lane 2= distal untreated leaves, Lane 3= control.
Note: The numbers on the right are the Rf values of the corresponding PPO isoform.

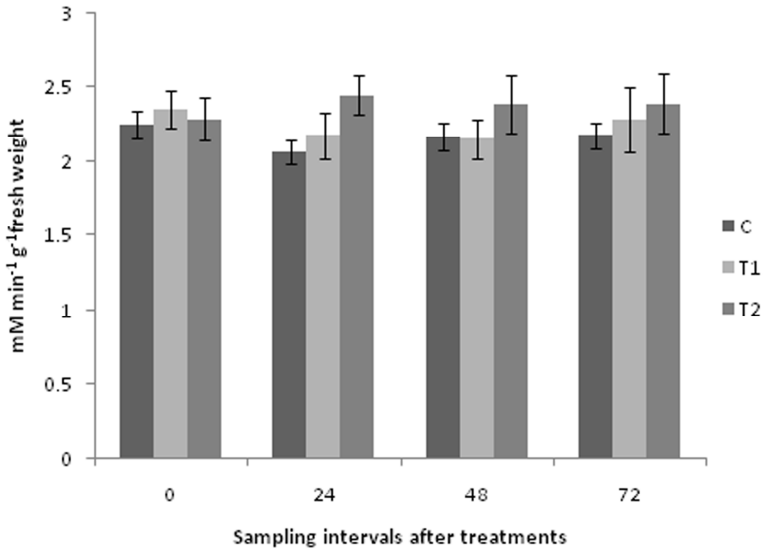

Figure 5. LOX activity in treated plants
C= control, T1= Third node leaves of plants treated with marigold extract, T2= Distal untreated leaves of plants treated with marigold extract.
Note: The bars represent average LOX activity ±S.E (n=5).

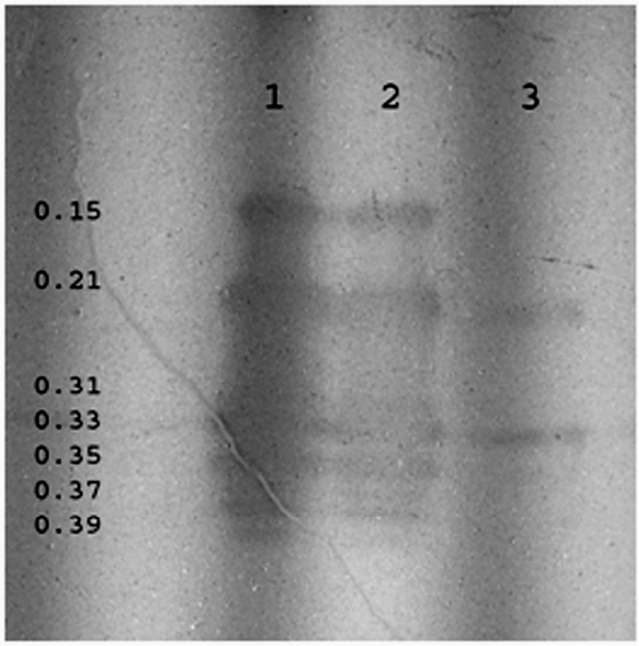

Figure 6. LOX isoforms in treated samples after 24 h
Lane 1= 3rd nodal treated leaves, Lane 2= distal untreated leaves, Lane 3= control.
Note: The numbers on the left are the Rf values of the corresponding LOX isoform.

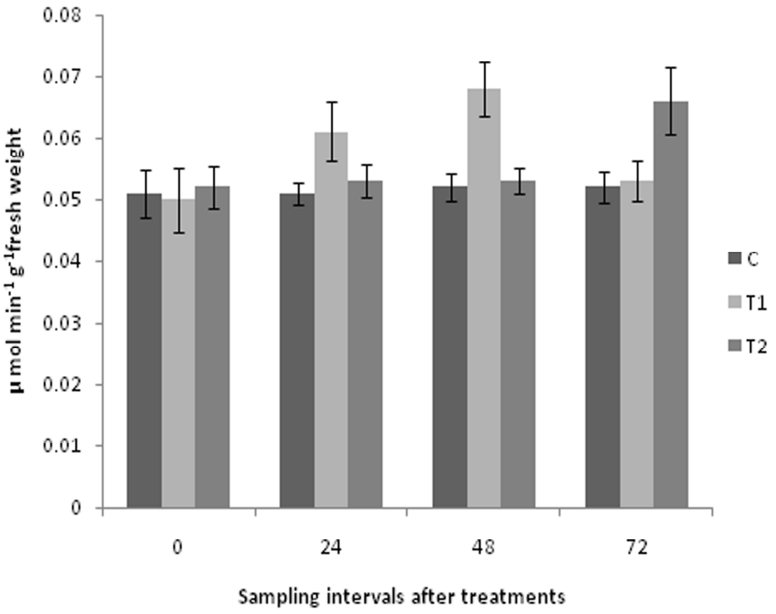

Figure 7. CAT activity in treated plants
C= control, T1= Third node leaves of plants treated with marigold extract, T2= Distal untreated leaves of plants treated with marigold extract.
Note: The bars represent average CAT activity ±S.E (n=5).

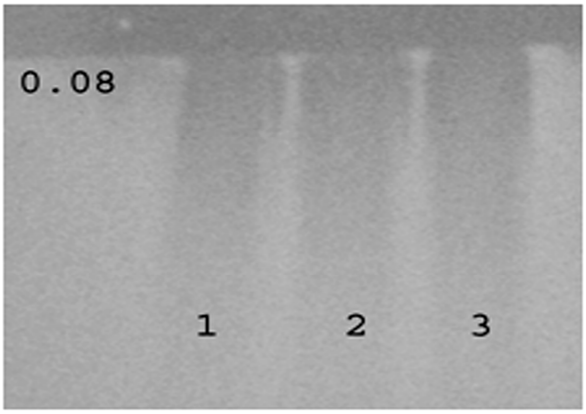

Figure 8. CAT isoforms in treated samples after 24 h
Lane 1= 3rd nodal treated leaves, Lane 2= distal untreated leaves, Lane 3= control.
Note: The numbers on the left are the Rf values of the corresponding CAT isoform.

Therefore, it can be inferred that application of aqueous extract of *Tagetes erecta* was able to increase resistance in the tomato plants by inducing the expression defense proteins.

Significant (p = 0.34) increase in POX activity was observed in the leaves sprayed with marigold extract within 24 h of treatment which continued up to 72 h. The distal untreated leaves had significantly (p = 0.229) increased POX activity after 48 h (Figure 1). The in gel-activity results demonstrated the constitutive expression of a single POX isoform (Rf = 0.35) in all the samples including control. However, additional POX isoforms were observed in the treated leaves (Rf = 0.20) and the distal untreated leaves (Rf = 0.20, 0.38) after 48 h of treatment (Figure 2).

A significant (p = 0.46) increase in PPO activity was observed at 48 h in the leaves treated with the extract. The increase (p = 0.21) in the PPO activity of distal untreated leaves was observed at 72 h of treatment (Figure 3). The zymogram of PPO demonstrated the constitutive expression of four PPO isoforms (Rf = 0.22, 0.0.32, 0.34, 0.38) in all the samples including control. In the 72 h zymogram, the 3rd node treated samples expressed four additional PPO isoforms (Rf = 0.17, 0.28, 0.40, 0.42) and the distal untreated leaves expressed three additional PPO isoforms (Rf = 0.28, 0.40, 0.42) (Figure 4).

Spraying of marigold extract on the leaves of tomato plant didn't had significant ($p \leq 0.5$) effect on the activity of Lipoxygenase (Figure 5). However, drastic difference in the expression of its isoforms was observed in both the treated and untreated leaves. The LOX zymogram demonstrated the constitutive expression of only two isoforms (Rf = 0.21, 0.33) in all the samples including control. But, in the 24 h zymogram, additional expression of five LOX isoforms was observed in all the test samples (Rf = 0.15, 0.31, 0.35, 0.37, 0.39) (Figure 6).

Catalase activity was significantly (p = 0.25) increased in the 3rd nodal treated samples after 24 h of marigold extract application which continued up to 48 h. Significant (p = 0.46) increase in CAT activity of the distal untreated leaves was observed at 72 h (Figure 7). The lysozyme zymogram demonstrated the expression of a single isoform (Rf = 0.08) in all the samples including control. No additional CAT isoform was induced by the extract in any of the test samples (Figure 8).

The seeds treated with *Pseudomonas fluorescens* lead to accumulation of higher phenolic compounds and higher activities of POX and PPO which may play a role in defense mechanism of maize plants against *R. solani* f. sp. *Sasakii* [24]. The activities of POX and PPO were positively correlated to the enhanced disease resistance against bacterial wilt in *Eucalyptus urophylla* [25]. POX and PPO have been reportedly involved in defense response of tomato [26]. Role of LOX for plant defense under different stress conditions have been enumerated by Nemchenko et al. [27]. LOX activity showed a positive relationship with resistance in Brazilian rice cultivars [28]. Spraying of cacao plants with a heterogeneous chitosan suspension (MCp) from *Crinipellis perniciosa* mycelium showed a significant increase of oxidative POX and PPO activities [29]. El-Khallal [30] reported that bio-elicitation of tomato plants by arbuscular myccorhiza induced LOX-mediated synthesis of phytolexins and increased accumulation of salicylic acid which led to increased resistance of the plants against *F. oxysporum.* The pepper 9-Lipoxygenase

Gene *CaLOX1* functions in defense and cell death responses to microbial pathogens [31]. Willekens *et al.* [32] demonstrated that catalase-deficient plants show nectrotic patterns in presence of high light. Verslues *et al*. [33] reported the involvement of catalases during kinases-mediated induction of resistance in *Arabidopsis* against salt stress. Catalases are the main route of H_2O_2 degradation and hence inhibition of catalase activity results in the activation of SAR [19].

CONCLUSION

The study here reports the capability of an aqueous extract from *Tagetes erecta* leaves in induction of POX, PPO, LOX and CAT in tomato. However, the compounds present in the extract which interacted with the cells of target plants leading to the induction of the activities and higher expression of these defense related enzymes, need to be identified. Also, the molecular events following this interaction should be traced in order to identify the target genes of these compounds. This would allow engineering of these genes in disease-susceptible crops and investigating their responses towards the application of marigold leaf extract, and their subsequent defense against pests and pathogens. All these informations may lead to the possibility of preparation of an eco-friendly biocide from marigold leaves.

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