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Effect of *cis*-Jasmone and Salicylic acid on the induction of defensive enzymes in Brinjal against Brinjal shoot and fruit borer (*Leucinodes orbonalis* Guenee)

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Abstract
A laboratory experiment was conducted to assess the induced resistance in brinjal (CO-2) with the exogenous application of *cis*-Jasmone (CJ) and Salicylic acid (SA). Accumulation of antioxidant enzymes such as Peroxidase (POD), Polyphenol oxidase (PPO), Super oxide dismutase (SOD) and Catalase (CAT) upon infestation by brinjal shoot and fruit borer, *Leucinodes orbonalis* was taken as measure of induced resistance in the plants. Eight treatments were set up with two replications in each and one brinjal plant of 60 day old is tested. *cis*-Jasmone and Salicylic acid of 1mM concentration were used in the treatments. The activity of the antioxidant enzymes were recorded at 4, 24, 48, and 72 hours after the infestation. The induction of enzyme activities varied among the treatments and across the time interval. The treatment with infestation followed by CJ application showed greater level of enzyme activity from rest of the treatments and the second of such treatment was pre treatment with CJ followed by infestation. Thus, the result suggest that CJ induces greater level of resistance components in the plants against borer insect such as *L. orbonalis* and hence provide greater opportunity for exploiting plant defense against herbivores.

Keywords: *cis*-Jasmone, salicylic acid, POD, PPO, SOD and CAT

Introduction
Brinjal or Egg plant (*Solanum melongena* Linn.) belonging to the family Solanaceae, is one of the most important and principal vegetable crop grown in South and South East Asia (Srinivasan, 2008, Thapa, 2010) [40, 43]. Asia alone accounts for about 94 per cent of the world area, with 92 per cent of world production (FAO 2007) [16]. The vegetable is widely grown throughout India and the statistics of 2015 revealed that the crop was grown in the area of 0.68 million hectares with the production of 12.43 million tones which accounted for 7.4 per cent of total vegetable production in the country (Horticulture-Statistical Year Book India, 2016) [22]. Under sustainable farming, the crop provides daily income because of its longer fruiting and harvesting periods which can easily enable the farmer to meet out day to day expenditure (Ghimire *et al.*, 2007) [17].

However, the cultivation of crop is not an easy job as it is reported to be attacked by plethora of insect pests and diseases. More than 36 insect pests are known to attack the crop starting from point of planting till completion of harvest (Regupathy *et al*., 1997) [31] inflicting a significant crop loss.

Brinjal shoot and fruit borer (*Leucinodes orbonalis* Gunee) is one of the most destructive key pest (Raju *et al.*, 2007, Saimandir and Gopal, 2012) [28, 33] of the crop with damage potential to the extent of 70 to 93 per cent (Eswara Reddy and Srinivasa, 2004, Dhandhapani *et al*. 2003, Chakraborti and Sarkar, 2011) [14, 13, 10]. The pest bores into the shoot during the vegetative phase causing withering and attacks flowers and fruits during the reproductive stage in a similar manner rendering the fruit unmarketable and unfit for consumption. Internal mode of feeding made this noxious pest difficult to combat even with the use of most powerful insecticides by the farmers. Apart from higher cost of production, excessive use of such toxic pesticides causes the risk of environment pollution and human health hazards.

Improving host plant resistance to insects will result in reduced losses due to herbivores, less insecticide use, better crop yields, and a safer environment to live. Plants possess an array of defensive traits to protect themselves against herbivore attack. These resistance strategies could be constitutive (present in the plant irrespective of herbivore attack) or inducible (produced upon damage by the herbivore) in nature (He *et al.*, 2011, Scott *et al.*, 2010) [18, 35].

The inducible resistance is of two types: Direct and Indirect. Direct induced resistance is mediated by the accumulation of secondary metabolites and defense-related proteins (Mithöfer and Boland 2012) [27]. Indirect induced resistance results in release of a blend of volatile organic compounds (VOCs) that specifically attract natural enemies of the herbivore species aiding in biological control of the herbivore (Bruinsma and Dicke 2008) [9]. Although, the constitutive resistance is one of the primary mechanisms to protect plant against insect pests, the inducible resistance is most effective and reliable.

The expression of herbivore induced defenses is mediated primarily by the phytohormones such as jasmonic acid (JA), ethylene, and salicylic acid (SA) (Smith *et al.*, 2009, Wu and Baldwin 2009) [39, 47]. Other related compounds are being used nowadays to deduce the induced resistance mechanism in plants is methyl jasmonate, *cis*-Jasmone and methyl salicylate etc., (Venu *et al.*, 2010, Matthes *et al.*, 2010) [45, 25]. The important oxidative enzymes induced in plants in response to insect herbivory include peroxidases (POD), polyphenol oxidase (PPO), superoxide dismutase (SOD), phenylalanine ammonia lyase (PAL), lipoxygenase (LOX), catalase (CAT) and ascorbate peroxidase (APX) (War *et al.*, 2012, Zhao *et al.*, 2009) [46, 49].

The present study was carried out to understand the induced resistance in brinjal against *Leucinodes orbonalis* by exogenous application of *cis*-Jasmone (CJ) and Salicylic Acid (SA). The study was focused on various antioxidative enzymes involved in plant resistance against insect pests.

Materials and Methods

Chemicals and Instrument

cis- Jasmone and Salicylic Acid, their analytical grade were obtained from Sigma Aldrich. Other chemicals used in the study were Ethylene diamine tetra acetic acid (EDTA), Tris-HCl, polyvinylpyrolidone (PVP), disodium hydrogen phosphate, sodium dihydrogen phosphate, guaiacol, 2-mercaptoethanol, pyrocatechol, potassium iodide (KI), sodium carbonate (Na_2CO_3), Trichloroacetic acid (TCA), dithiothretol (DTT) and nitro blue tetrazolium salt.

The spectrophotometer used for the estimation of biochemical parameters was Jasco V-730 Bio.

Plants

Seeds of brinjal genotype CO-2 were obtained from seed sale unit, Department of Vegetable Crops, TNAU, Coimbatore. The plants were grown in pots with mixture of soil, sand and vermicompost as substrate. The plants were watered regularly according to the need of the plant and care was taken to prevent the plant from other insect attack by keeping them in shade nets. Plants of 60 day old were used in the experiment

Insects and Infestation

The insect used for the study were obtained from the lab culture maintained at Department of Agricultural Entomology, TNAU, Coimbatore. Shoot and fruit borer larvae were reared on brinjal fruits in the laboratory condition with ambient temperature (27-32º C) and relative humidity (50-60 %). Two second instar larvae obtained from the stock culture were gently placed on each plant using camel hair brush.

Cis-Jasmone and Salicylic acid Application

cis-Jasmone and salicylic acid were prepared to the concentration of 1mM. To prepare 1mM of *cis*-Jasmone solution, 16.4µl of CJ was dissolved in 1ml of acetone and later dissolved in 100ml of water to make the desired concentration. To prepare, 1mM salicylic acid, 13.8mg of SA was dissolved 1ml of acetone before being dissolved in 100ml of water. Plants were sprayed sufficiently with 1mM of both the chemicals, followed by insect infestation.

Treatments

Eight treatments were set up with two replications for each treatment. The treatments are as follows. t1: Control; t2: Infested (INF); t3: Pre Treatment with *cis*-Jasmone + Infested (PT CJ+INF); t4: Pre Treatment with Salicylic acid + Infested (PT SA+INF); t5: *cis*-Jasmone alone (CJ alone); t6: Salicylic acid alone (SA alone); t7: Infested + *cis*-Jasmone (INF + CJ); t8: Infested + Salicylic acid (INF + SA).

After 0, 24, 48 and 72 h of the treatment, leaves were collected from the plants and tested for variation in defensive enzyme activities.

Enzyme Extraction

Fresh leaves (0.5 g) were collected and ground in 3 ml of ice cold 0.1 M Tris-HCl buffer (pH 7.5) containing 5 mM 2-mercaptoethanol, 1% polyvinylpyrolidone (PVP), and 0.5 mM EDTA in a chilled pestle mortar which was kept in minus 180^0C overnight. The homogenate was centrifuged at 12,000rpm for 30 min and the supernatant was used as an enzyme source.

Peroxidase (POD) Assay

POD activity was estimated as per the method of Shannon *et al.* (1966) [37] with slight modifications as followed by Barkat Hussain *et al.* (2014) [1]. The reaction mixture (2.9 ml) containing 0.1M of 2.75ml sodium phosphate buffer (pH 6.5), 0.8 mM of 0.1ml H_2O_2, and 5 mM of 0.05ml guaiacol was taken in a test tube. A total of 0.1 ml of enzyme source was added to it and the absorbance was read at 470 nm for 2 min at 15 secs intervals. Enzyme activity was expressed as ΔOD min^{-1}.

Polyphenol oxidase (PPO) assay

PPO activity was estimated as per the method of Mayer and Harel (1979) [26] with some modifications followed by Hussain *et al.* (2014) [1]. To 2.9 ml of 0.1 M of sodium phosphate buffer (pH 6.8), 0.1 ml of enzyme source and 0.1 ml of substrate (0.05 M catechol) were added. Absorbance was read at 420 nm for 3 min at 30-s intervals. Enzyme activity was expressed as ΔOD min^{-1}.

Superoxide dismutase (SOD) assay

The activity of SOD was assayed by the method of Beauchamp & Fridovich (1971) [2]. 3 ml of 0.05 M sodium phosphate buffer with 0.1% NaCl (pH 7.8) was taken in a test tube to which 0.3 ml of 0.1 mM EDTA, 0.3 ml of 0.13mM methionine, 0.1 ml of 0.02mM KCN, 0.3 ml of 0.75 mM NBT, 0.3 ml of 0.02 mM riboflavin and 0.1 ml of enzyme extract were added. The reaction mixture was illuminated in glass test tubes for 1 h. Identical solutions that were kept in the dark served as blanks. Absorbance was read at 560 nm against the blank and the activity was expressed as per cent NBT reduction. (Per cent inhibition of NBT reduction by SOD = control OD- treatment OD/ control x 100).

Catalase (CAT) assay

Catalase activity was assayed as described by Zhang *et al.* (2008) [48]. The reaction mixture consisted of 1 ml of Tris-HCl buffer (pH 7.0), 0.1 ml of partially purified enzyme extract

and 0.2 ml of H_2O_2. Absorbance was read at 240 nm for 2 min and the enzyme activity was expressed as ΔOD min^{-1}.

Statistical analysis

The replication data were pooled together and mean and standard error were calculated. The data were analyzed by analysis of variance (ANOVA) using Minitab software. Tukey's HSD test was applied to separate the means.

Results

POD Activity

At 4 hour after the treatment, CJ alone and SA alone treatments are found to be on par with each other and statistically significant from rest of the treatments. The next best treatments that are on par are INF, PT CJ+INF and PT SA+INF. The other three treatments (Control, INF+CJ, INF+SA) are in next levels and on par with each other. After 72 hours after the treatment, POD activity in CJ+INF shot up and proved to be statistically different from rest of the treatments. The next best treatment is PT CJ followed by PT SA+INF and SA+INF which are at same statistical level of significance. The control showed least activity of POD along with SA alone treatment (Fig. 1, a).

Overall, POD activity is higher in INF+CJ plants followed by PT CJ+INF, PT SA+INF, CJ alone and SA+INF treatments at same level of significance. Control, Infested and SA alone treatments showed least activity of POD and are on par with each other (Fig. 1.b).

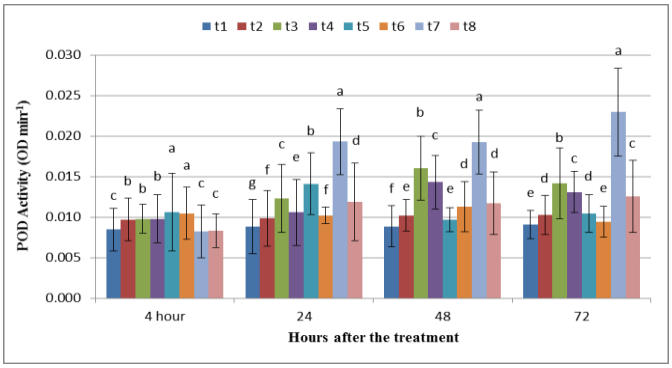

Fig 1(a)

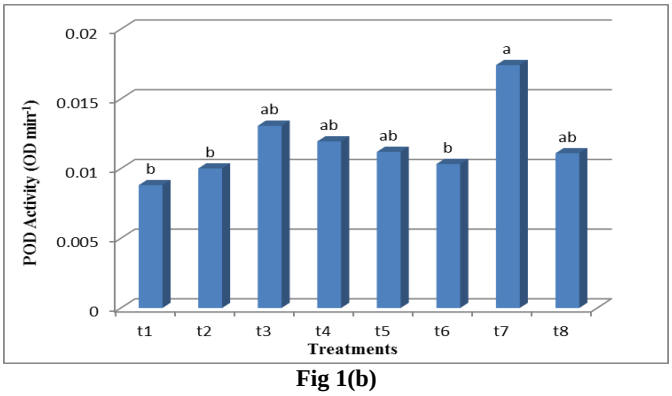

Fig 1(b)

Fig 1(a&b): POD activity (OD min^{-1}) in brinjal genotype (CO-2) at various time interval (Fig.1.a) and overall comparison of different treatments (Fig.1.b). Different letters on the bars (mean +SE) of different colours within time interval are statistically different at $P < 0.05$ (Fig.1a). Similar letters on the bars of are not statistically different at $P < 0.05$ (Fig.1.b). t1: Control; t2: Infested (INF); t3: Pre Treatment with *cis*-Jasmone + Infested (PT *cis*-J+INF); t4: Pre Treatment with Salicylic acid + Infested (PT SA+INF); t5: *cis*-Jasmone alone (*cis*-J alone); t6: Salicylic acid alone (SA alone); t7: Infested + *cis*-Jasmone (INF + *cis*-J); t8: Infested + Salicylic acid (INF + SA)

Polyphenol oxidase activity

PT CJ treated plants showed highest activity of PPO followed by INF+CJ and INF+SA treated plants after 4 hours of treatment. There is significant difference between control and infested plants. The CJ alone and SA alone treatments were on par with each other. After 48 hours after the treatment, INF+CJ treated plants recorded highest PPO activity over other treatments and the activity was slowly decreased after 72 hours of treatment although found statistically significant level with rest of the treatments. PT CJ treated plants showed better activities of PPO across the time interval with the peak of the activity at 24 to 48 hours after the treatment which slightly reduced after 72 hours. The INF+SA and PT SA+INF treated plants also performed well showing same kind of pattern of PPO activities (Fig. 2.a)

Overall, plants treated with INF+CJ found to show elevated levels of PPO activity and found to be highly significant from rest of the treatments at 5 per cent level. The next best treatments which are on par were PT CJ+INF and INF+SA. Control plants showed least PPO activity, whereas INF, PT SA+INF, SA alone and CJ alone treated plants showed no significant differences (Fig. 2.b).

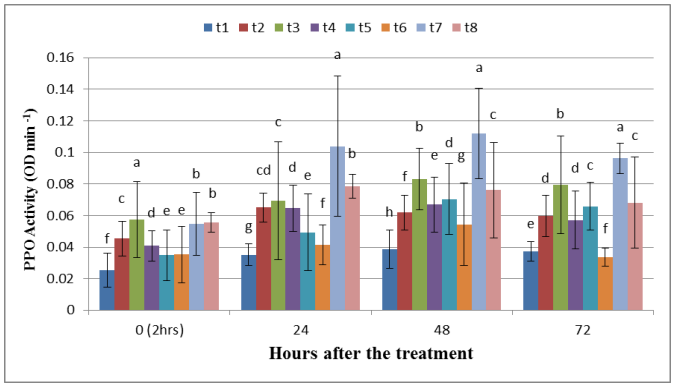

Fig 2(a)

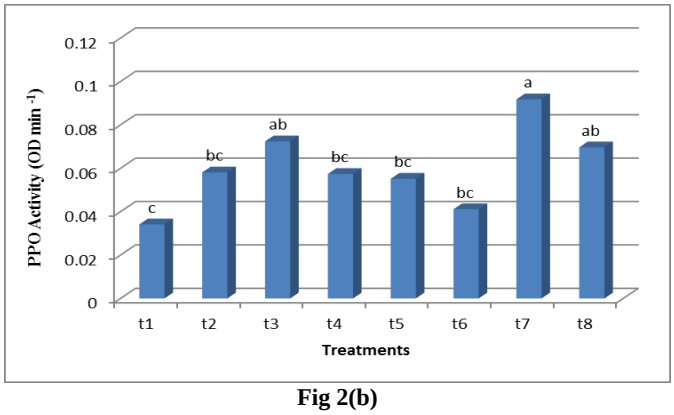

Fig 2(b)

Fig 2(a&b): PPO activity (OD min^{-1}) in brinjal genotype (CO-2) at various time interval (Fig.2.a) and overall comparison of different treatments (Fig.2.b). Different letters on the bars (mean +SE) of different colours within time interval are statistically different at $P < 0.05$ (Fig.2a). Similar letters on the bars are not statistically different at $P < 0.05$ (Fig.2.b). t1: Control; t2: Infested (INF); t3: Pre Treatment with *cis*-Jasmone + Infested (PT *cis*-J+INF); t4: Pre Treatment with Salicylic acid + Infested (PT SA+INF); t5: *cis*-Jasmone alone (*cis*-J alone); t6: Salicylic acid alone (SA alone); t7: Infested + *cis*-Jasmone (INF + *cis*-J); t8: Infested + Salicylic acid (INF + SA)

SOD activity

The spectrophotometric analysis of SOD activity expressed in terms of per cent NBT reduction showed that all treatments

were found to be above 30 percent and below 40 per cent NBT reduction at 4 hours of recording. Over the next three days, the control plants showed less than 30 per cent NBT reduction whereas treatments like PT CJ+INF and INF+CJ crossed above 45 percent reduction at first and second days and PT CJ+INF in third day. The INF+CJ treated plants recorded a slight reduction in SOD activity in the third day of the treatment.

After 48 hours of treatment, PT CJ+INF and INF+CJ treated plants recorded almost equal amount of NBT reduction followed by PT SA+INF and INF+SA treated plants. The percent NBT reduction was found to be least in SA alone treated plants which is just above the control plants. The INF treated plants showed a variation in NBT reduction in which highest was recorded in 1 day after the treatment and it reduced in next two days (Fig. 3.a).

Overall, PT CJ+INF treated plants were significantly different from rest of the treatments with the next best treatment in terms of per cent NBT reduction is being INF+CJ. There is no significant difference between PT SA+INF and CJ alone treatment. The next treatments in their order of significance are INF+SA, INF, SA alone and control (Fig. 3.b).

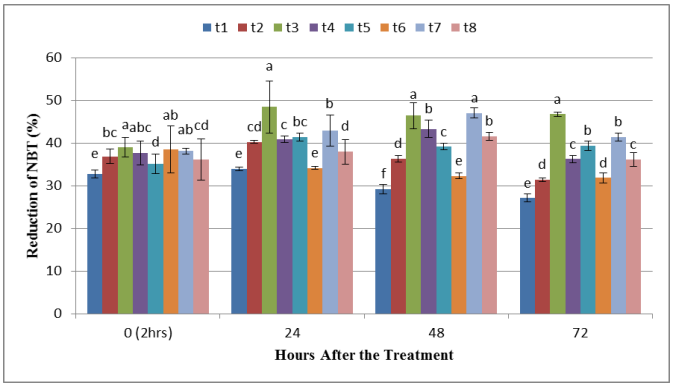

Fig 3(a)

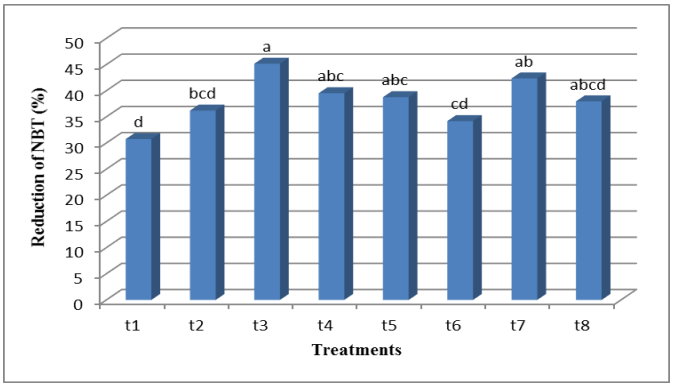

Fig 3(b)

Fig 3(a&b): SOD activity (expressed as percent NBT reduction) in brinjal genotype (CO-2) at various time interval (Fig.3.a) and overall comparison of different treatments (Fig.3.b). Different letters on the bars (mean +SE) of different colours within time interval are statistically different at $P < 0.05$ (Fig.3a). Similar letters on the bars are not statistically different at $P < 0.05$ (Fig.3.b). t1: Control; t2: Infested (INF); t3: Pre Treatment with *cis*-Jasmone + Infested (PT *cis*-J+INF); t4: Pre Treatment with Salicylic acid + Infested (PT SA+INF); t5: *cis*-Jasmone alone (*cis*-J alone); t6: Salicylic acid alone (SA alone); t7: Infested + *cis*-Jasmone (INF + *cis*-J); t8: Infested + Salicylic acid (INF + SA)

Catalase activity

Plants treated with CJ followed by infestation (INF+CJ) showed highest catalase activity with highest activity after one day of the treatment. The next two days, the activity was found to be less and on par with PT CJ+INF (during second day) and INF (during third day) treated plants. Other treatments such as PT SA+INF, CJ alone and SA alone were on par with each other at one day after the treatment and after three days, the catalase activity in those treatments are in decreasing levels in the treatments as PT SA+INF > CJ alone > SA alone. INF+SA treated plants showed significant difference from rest of the treatments after CJ+INF treated plants, however, their activity was came down after three days and was on par with PT SA+INF, CJ alone treatments (Fig. 4.a)

Overall, catalase activity was highest in CJ treated plants and lowest in case of control. INF and PT CJ+INF treatments are not significantly different, likewise the next treatments such as PT SA+INF and INF +SA are on par with each other, CJ alone and SA alone were on par with each other (Fig. 4.b).

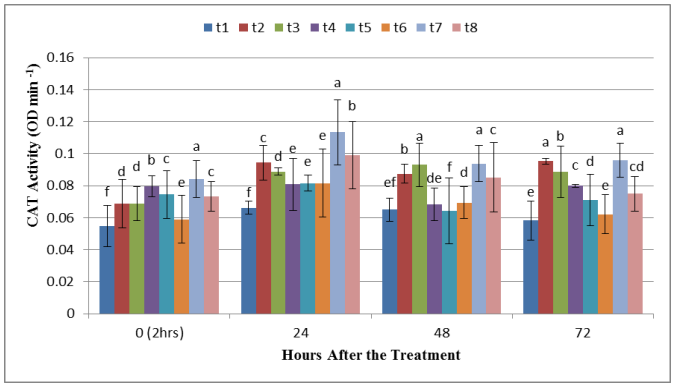

Fig 4(a)

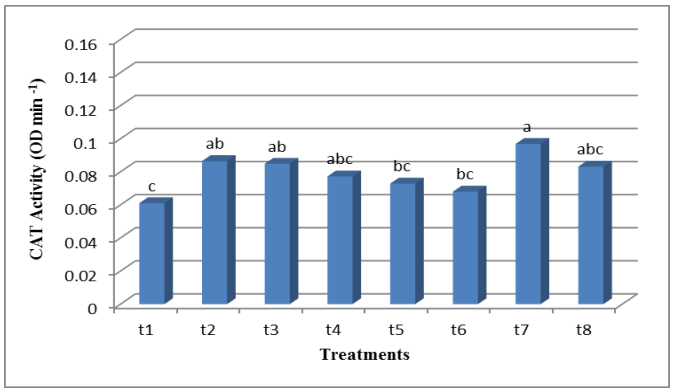

Fig 4(b)

Fig 4(a&b): Catalase activity (OD min^{-1}) in brinjal genotype (CO-2) at various time interval (Fig.4.a) and overall comparison of different treatments (Fig.4.b). Different letters on the bars (mean +SE) of different colours within time interval are statistically different at $P < 0.05$ (Fig.4.a). Similar letters on the bars are not statistically different at $P < 0.05$ (Fig.4.b). t1: Control; t2: Infested (INF); t3: Pre Treatment with *cis*-Jasmone + Infested (PT *cis*-J+INF); t4: Pre Treatment with Salicylic acid + Infested (PT SA+INF); t5: *cis*-Jasmone alone (*cis*-J alone); t6: Salicylic acid alone (SA alone); t7: Infested + *cis*-Jasmone (INF + *cis*-J); t8: Infested + Salicylic acid (INF + SA)

Discussion

The immunity offered to the plant via induced resistance is effective and benignly useful to reduce the pest attack. Such induced resistance makes the plant dynamic with increased level of defensive enzyme titre or by alerting the natural enemies thereby keeping the pest population at check. Secondary metabolites and defensive proteins accumulate in plant tissues as a result of insect damage or pathogen

infestation and defend the plants against further damage by insects and/or pathogens (Torres *et al.,* 2006) [44].

Jasmonic acid and Salicylic acid are the two important phytohormones actively involved in defense mechanisms of plants by mediating octadecanoid and phenylpropanoid pathways, respectively (Scott *et al.*, 2010, Shivaji *et al.,* 2010) [35, 38]. The biosynthetically related *cis*-jasmone is also a catabolite of the stress-produced jasmonic acid, but had previously been considered as only a biological sink for the jasmonate pathway. There are evidences that role of CJ in defensive functioning of plant against herbivores. To mention few examples here, lettuce aphid, *Nasonovia ribis-nigri* Mosh (Birkett *et al.,* 2000) [5] and the damson-hop aphid, *Phorodon humuli* Schrank, (Bruce *et al.,* 2008) [8] showed repellency against *cis*-Jasmone application in olfactometer studies and in the field respectively. In addition, *cis*-jasmone was directly attractive to the predatory ladybird beetle *Coccinella septempunctata* L, in an olfactometer, and to the aphid parasitoid, *Aphidius ervi* Haliday, in wind tunnel studies (Birkett *et al.,* 2000) [5]. The earlier and immediate response of plants to insect infestation results in the induced expression of plant metabolites and defensive enzymes. In this study we examined the defensive biochemical response of to feeding by *Leucinodes orbonalis* and CJ and SA treatment.

Our results revealed that treatment with CJ followed by infestation with *Leucinodes orbonalis* resulted in greater POD activity in Brinjal genotype (CO-2) followed by PT CJ + INF treated plants. This could be attributed to the prior insect infestation resulted in higher accumulation of the compound *cis*-Jasmone which in turn boosted by exogenous application of the compound. Hence, the increased level of CJ in the plant may be the reason for higher levels of defensive enzymes like peroxidases. However, the lower POD activity in SA applied treatments like PT SA +INF and INF + SA could be because of the cross talk between JA and SA (Cipollini *et al.,* 2004, Koornneef & Pieterse 2008) [12, 24]. Higher levels of POD activity in response to JA and SA application and/or insect attack will defend plants from the insects, pathogens and other stresses through cell lignifications, wound healing, and the production of secondary metabolites (Heng-Moss *et al.,* 2004, Rangasamy *et al.,* 2009) [21, 29]. Previous reports have shown that cis-Jasmonetriggers defense signaling pathways distinct from JA (Bruce *et al.,* 2008, Matthes *et al.,* 2010) [8, 25], leading to reduced aphid (sucking pest) survival and increased repellency as a consequence of elevated defense metabolite production. For example, the effects observed in wheat cultivars were increased levels of benzoxazinoids and phenolic acids (Bruce *et al.,* 2003) [7] and, for cotton, increased levels of homoterpenes (Hegde *et al.,* 2012) [19]. In addition to this, in our study cis-Jasmone was found effective in increasing the levels of defensive enzymes.

The plants showed differential expression of PPO activity upon treatment with CJ, SA and Insect infestation. This might be due to the difference in sensitive up-regulation response to the biotic stress. An increase in PPO activity in response to stresses is a common phenomenon (Zhao *et al.,* 2009) [49]. The quinines formed from the oxidation of phenols interact with the nucleophilic side chain of amino acids and cause protein cross-linking and, thereby, reducing their availability to insect pests (Bhonwong *et al.* 2009, Zhang *et al.,* 2008) [3, 58]. PPO is also involved in the melanin formation that increases the cell wall resistance to insects and pathogens (Zhao *et al.,* 2009) [49].

The differential activity of SOD might be due to the difference in plant response across the treatments. SOD is involved in the removal of highly toxic and unstable ROS (Raychaudhuri & Deng 2000) [30]. Saruhan *et al.* (2012) [34] reported the induction of SOD activity by SA and its relation to the reduced oxidative damage. It has been further reported that *Helicoverpa zea* infestation produced higher levels of SOD activity in tomato and soybean (Bi and Felton 1995, Felton *et al.,* 1994) [4, 15]. It reduces the toxicity of ROS by converting them into less toxic and more stable components such as H_2O_2 and water (Heidari 2009, Khattab & Khattab 2005) [20, 23]. Higher activity of CAT in plants plays a leading role in cell wall resistance, besides signaling the expression of various plant defensive genes (Chen *et al.,* 1993) [11].

Conclusion

Brinjal responded differentially to the infestation *L. orbonalis* and treatment with CJ and SA in terms of the defensive enzyme activities such as POD, PPO, SOD and CAT. Since these enzymes and other defensive components are responsible for the plant defense against biotic and abiotic stresses, brinjal with higher activity of these enzymes could acquire more resistant characters. The induced resistance could play an important role in pest management and defense mechanism against insect pests. A detailed understanding of plant immunity to arthropod herbivores will provide new insights into basic mechanisms of chemical communication and plant– animal co-evolution and may also facilitate new approaches to crop protection and improvement.

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