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Spatial pattern and longevity of induction of plant defense enzymes in cotton

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Abstract
Plants often increase their resistance to herbivores by locally increasing their production of defensive compounds at the site of damage, as well as systemically on undamaged leaves. The strength of these plant responses can vary depending on the amount, concentration, and location of damage. The spatial pattern/mapping of induction of defense enzymes *viz.,* polyphenol oxidase (PPO), peroxidase (POD), lipoxygenase (LOX) and protease inhibitor (PI) in the damaged plants of cotton varied with the type of damage employed have been studied. The results of the spatial pattern of expression of defense proteins in cotton in relation to different induction regimes at four leaf positions of cotton plant showed that plant systemic induction by *Spodoptera litura* with a 3.27 fold increase in PPO activity in terminal leaf (plant systemic) followed by a 2.43 fold increase in upper leaf and the induction was localized to damaged leaf and the leaf adjacent to the damaged leaf with a 2.08 fold increase and 2.04 fold increase respectively due to damage by *Aphis gossypii.* However induction of POD and LOX was localized irrespective of the type of inducing agent. Maximum and significant increase in PI activity against *S. litura* damage was observed in damaged leaf position (54.66%) compared to that of control plants (14.33%), followed by adjacent leaf, terminal and lower leaf. For *A. gossypii* feeding damage, high PI activity was recorded only at damaged and adjacent leaf (57.66 and 31% respectively) indicating induction only up to leaf systemic position. The PPO activity and levels of chymotrypsin inhibitors expressed in *Gossypium hirsutum* peaked on the fifth day of the treatment and they persisted at significantly higher levels even on the seventh day after induction by *S. litura.* Damage by *A. gossypii* showed highest PI activity on 3rd DAT and no significant increase was observed thereafter but the activity was significantly different from the control. The longevity of the induced response offers longer protection against herbivory is of great significance in pest management.

Keywords: Cotton, induced plant defense, spatial pattern and longevity

Introduction
Plants express constitutive defenses and serve as a first line of defense against an insect attack. Insects overcome these damages and in turn results in the plants responding by eliciting the induced resistances. Induced responses following insect damage enable a better assessment of defense systems of the plants which tend to restrict the host plant preference, insect survival and reproductive efficiency. Induced defenses tend to be more effective against unpredictable herbivores than constitutive defenses (Faeth, 1994) [12]. Plants have a generalized defensive response to wounding that can be divided into two phases- activation and induction. Activation represents the immediate response to cellular damage wherein cell integrity is lost and a variety of hydrolytic and oxidative enzymes are released from compartmentalization. This release results in the generation of chemical signals that trigger the systemic and /or local induction of defenses, and in the generation of chemically reactive products that lead to cell death through destruction of membranes and polymerization of cellular components (Ryan, 2000; Engelberth *et al.*,, 2000; Leon *et al.,* 2001; Sanjayan, 2005, Usha Rani, 2006) [22, 11, 19, 23, 29]. This polymerization is mediated by polyphenol oxidase, peroxidase and lipoxygenase. The activity of these enzymes can therefore be used to quantify the induction of defense response and serve as markers of induced response. Mechanical damage and damage by specific insect herbivores generally elicit unique molecular, biochemical, and morphological responses. As the study on induction of resistance is gaining importance, it is necessary to understand how plants respond to different inducers including insect with biting and chewing and piercing type of mouth parts. The longevity of such induced response offers longer protection against herbivory and is of great significance in pest management. The presence of induced resistance for a longer time extends the possibility of any direct or indirect effects on natural enemies

and other non-target arthropods/ herbivores. Hence it was proposed to study the spatial pattern and longevity of induced defense reposes upon different damage treatments in cotton.

Materials and Methods

Cotton seeds (var. MCU 7) were soaked in water for six hrs and then incubated at 28 °C for 24 hr. Germinated seeds were sown in 4l pots in a green house. *Spodopters litura*, were reared on cotton leaves in the laboratory and *Aphis gossypii* were reared on cotton plants in green house under constant light and temperature.

Spatial mapping of induced response: Plants used in experiments were at the 6-8 true-leaf stages (one month to six weeks after planting). Plants at this stage of development were large enough to permit spatial mapping of induction but small enough to make such studies traceable in terms of sampling and replication (Stout *et al.,* 1996a). Experiments were started by subjecting a group of 5 plants to one of the four following types of damages: i) feeding by *Spodoptera litura* (biting and chewing type); ii) feeding by *Aphis gossypii* (piercing and sucking type); iii) crushing of leaf tissue with a pair of forceps (mechanical) and iv) immersion of leaf in an insecticidal soap solution (I-Soap ™). Another group of 5 plants, similar size and age was left undamaged as control. After two days, the plants were transferred to the laboratory and the leaves from several positions on control and experimental plants were excised at the petiole with a razor blade and assayed for PPO, POD, LOX and PI activities. The activities of these proteins in leaves from damaged plants were compared to activities in corresponding leaves from the control plants. Leaves from four positions, relative to the damaged leaf, were sampled - Damaged leaf, (designated as leaf position D), undamaged leaf adjacent to the damaged leaf (leaf position Y), terminal leaf which is above the leaf position Y (leaf position T), leaf immediately below the damaged leaf (Leaf position L). This procedure allowed providing biotic, mechanical or chemical treatments. The treatments are

***S. litura* feeding**: Third instar larvae were used in all the experiments. They were starved for 6-8 hrs prior to the start of experiments to encourage immediate feeding. Larvae were used only once in a bioassay and were then discarded. One third instar larva was confined to the third leaf (leaf position D) using a clip cage (perforated plastic zip cover) and allowed to feed for 24 hr period in greenhouse. Larvae usually consumed 10-50% of the leaf approximately during this time. A clip cage with no larva was applied to plants in the control group. After 24 hr, cages and insects were removed and the plants kept in green house for additional 48hr. Whole plants were taken to the laboratory and assayed for protein activities at three leaf positions. Because the assays used were destructive, it was not possible to assay enzyme activities and Proteinase inhibitor activities from the same leaf. Enzyme activities and PI activities at positions D, Y, T and L were determined from different leaves. Samples were taken from four sets of plants (5 each) at 48 hrs after the removal of larvae (Stout *et al.,* 1996a**)**. PPO, POD, LOX & PI analysis was carried out and compared with that of the control. The difference between the control and treated samples were taken for analysis. This experiment was repeated for two times with 5 plants per treatment per trial.

***A. gossypii* feeding**: Approximately 50 nymphs and adults were transferred from heavily infested plants to the third leaf of the experimental plant and confined to that leaf by using clip cage around the petiole of the leaf. Aphids were allowed to feed on the plant for 48 hr after which the clip cages and insects were removed and the plants kept in green house for another 48 hr. Subsequently, the plants were taken to laboratory and enzyme activity was estimated on the leaves from different positions. The experiment was repeated twice with five replications per trial. Enzymes were assayed from leaf positions D, Y, T and L.

Mechanical damage: Plants were wounded by crushing leaf tissue between a pair of forceps. Two wounds were made perpendicular to the midvein on the third leaf of the plants. The size of the wound was approximately 2 cm long and 0.5 cm wide, and the wounding did not sever the leaf. Plants were assayed after 48 hr wounding for enzyme activities. Plants of similar size and age were used as control but were not wounded. The experiment was repeated for two times with five replications per trial. Enzymes were assayed from leaf positions D, Y, T and L.

Insecticidal soap immersion treatment: The entire third leaf was immersed for approximately five seconds in a 5% (v : v) solution of safer insecticidal soap (Safer, Inc. USA). Plants were maintained in a greenhouse for 48 hr after dipping and were then taken to the laboratory and assayed for protein activities. Control leaves of plants of similar size and age were dipped in distilled water.

Longevity of induced response: To study the longevity of induced response, defoliation (25-50%) by *S. litura* was produced by placing two IIIrd instar larvae on the third leaf for 24 h (Srinivas *et al.,* 2001) [25]. Approximately 50 nymphs and adults *A. gossypii* were used for aphid feeding treatment. After 24 hr of insect feeding, cages and insects were removed and plants kept in green house. Leaf samples from five plants each were taken on 0, 1, 3, 5, and 7 days after herbivory (DAH). Similarly leaves from control plants of the same age were also taken. The terminal leaves were excised and chemical assays for PPO and PI activities were carried out. The difference between the control and treated samples was taken for analysis. The experiment was repeated two times with five replications per trial. Similar studies on the longevity of induction were carried out where insect feeding was replaced by either mechanical wounding or chemical (insecticidal soap) treatment.

Data analysis: All enzyme activity data were transformed using a $\log_{10}(x+1)$ transformation and PI data were transformed using square root (x + 1) transformation in an attempt to correct unequal variances between treatments. Data on different damage and control groups were analysed using one-way ANOVA with LSD using the AGRES statistical software 3.01.

Results and Discussion

The concept of induced resistance is gaining importance in insect pest management programs in recent years. Understanding of the quantitative and qualitative basis of induction is fundamental in these programs. In this section the results of the spatial pattern of expression of defense proteins *viz.*, PPO, POD, LOX and PI in cotton in relation to different induction regimes is presented.

The enzyme activity at four leaf positions of cotton (var. MCU 7) plant namely D, U, T and L was recorded. The PPO activity of undamaged control leaves indicate higher activity in the terminal leaf than the mature leaf (Table 1). Damage by *S. litura* feeding resulted in an increase in PPO activity at all the leaf positions, the induction of enzyme activity being significantly higher compared to control plants at <0.01 level at all the leaf positions except position L The results indicate a plant systemic induction by *S. litura* with a 3.27 fold increase in PPO activity in terminal leaf (Position T- plant systemic) followed by a 2.43 fold increase in upper leaf (Position-U) and 2.05 and 1.44 fold increase in damaged leaf and lower leaf (Position D &L). However, the induction was localized to damaged leaf (D) and the leaf adjacent to the damaged leaf (U) with a 2.08 fold increase and 2.04 fold increase respectively due to damage by *A. gossypii.* PPO induction in mechanically damaged treatment was significantly high only in damaged leaflet (D) and insecticidal soap solution treatment showed no significant difference from the control in all the leaf positions. The results are substantiated with similar findings of Stout *et al.,* (1996a) where a nearly 5.0 fold increase in PPO activity in damaged leaflets of tomato plant upon *Heliothis zea* damage and a 2.0 fold increase upon russet mite damage were reported. Similarly tomato plants with system in through cut stems induced PPO activity in leaves, and wounding lower leaves of young tomato plants induced PPO activity in both wounded and unwounded leaves to levels equal to those induced by systemin (Constabel *et al.,* 2004; Stout *et al.,* 1999) [7, 14].

Peroxidase enzymes are involved in the oxidation of compounds at the expense of H_20_2 and play a key role in several aspects of plant physiology and development, such as lignification and suberization of ce11 walls and also directly involved in defense mechanisms. In the present study, POD activity in control undamaged plants indicated high activity in terminal young leaves of cotton. Damage by *S. litura* showed a significant increase in induction of POD at upper leaf (U) with a 2.77 fold increase over control followed by the damaged leaf (D) with an increase of 2.49 fold and no significant increase in activity at lower mature leaf (L). However, the damage by *A. gossypii* showed a significant increase in POD activity only at damaged leaf (D). For the other treatments *viz.*, mechanical damage and insecticidal soap solution, no significant increase was recorded for POD induction when compared to undamaged control plants at corresponding leaf positions (Table 2). These results are in agreement with the perception of Hildebrand *et al.,* (1986) [18] who reported increase in POD induction in soybean foliage damaged by two-spotted spider mite. Larval *H. zea* feeding increased the foliar POD activity 1.6 times (Bi and Felton, 1995). In the cotton-aphid system, the activity of most foliar antioxidant enzymes was not increased, unlike the response in barley and wheat (Argandoña 1994; Argandoña *et al.,* 2001) [2, 3], alfalfa (Dillwith *et al.,* 1991) [8], and *Arabidopsis* (Moran *et al.,* 2002) [20]. The levels of POD were similar in aphid-infested and non-infested leaves (Gomez *et al*., 2004) [16]. In tomato Stout *et al.,* (1996) [27] reported localized induction of POD (only in the damaged leaflets) upon *H. zea* feeding with 0.6 fold increase and upon russet mite damage a higher degree of induction was recorded.

Similar to POD induction, LOX activity was higher and significant in the *S. litura* damaged plants at position D with a 1.39 fold increase and position U with 1.27 fold increase at < 0.01 level. A slight increase in induction was recorded at position D. No significant increase was observed for the remaining damage types in all the leaf positions (Table 3). Lipoxygenases are considered to be ubiquitous in plant tissues, yet are found at widely varying levels in different plant organs. Jasmonic acid (JA) is a product of the lipoxygenase pathway, also called the octadecanoid pathway (Sembdner and Parthier, 1993) [24]. Activation of the jasmonate pathway leads to production of jasmonic acid (JA), a central signaling molecule in plant defense (Wasternack and Parthier, 1997) [30].

Expression of PI genes appears to be a major component in induced plant resistance upon herbivory. Inhibition of chymotrypsin activity upon induction treatment was used as an index for analyzing PI activity. The present study has demonstrated that the induction of PIs is the maximum at the damaged leaf position and the induction was plant systemic (Table 4). The induction again was maximum for *S. litura* treatment, followed by *A. gosyypii.* However, the insecticidal soap treatment did not induce PIs in cotton. The results showed a maximum and significant increase in PI activity for damage by *S. litura* at all the four leaf positions at <0.01 level compared to other damage treatments. Maximum inhibition was observed for the damaged leaf position (D) (54.66%) compared to that of control plants (14.33%), followed by position U, T and L. The results are in agreement with the previous reports in tomato plants with *H. zae* damage and mechanical damage showing induction of PI activity up to plant systemic level (Stout *et al.,* 1996a). Herbivore feeding or mechanical wounding of potato and tomato plants result in the systemic expression of genes encoding PI proteins (Green and Ryan, 1972; Doares *et al.,* 1995; Farmer and Ryan, 1990) [17, 9, 13]. Systemic induction of PIs has also been demonstrated in rice (Xu *et al*., 1993) [31] and maize (Eckelkamp *et al.,* 1993) [10]. In tomato and other solanaceous plants proteinase inhibitors are expressed rapidly and systemically in response to wounding (Ryan, 1990; Gatehouse, 2002) [21, 15].

For *A. gossypii* treatment, high and significant inhibition was recorded only at positions D and U (57.66 and 31% respectively) and no significant influence was observed for the remaining leaf positions T and L, which corroborates with the findings of Stout *et al.,* (1999) [26] in tomato plants. Aphid feeding damage by either of two species of aphid (*Macrosiphum euphorbia* and *Myzus persicae*) induced host responses that were similar to those observed with pathogens i.e. the aphids were potent inducers of pathogen related proteins but did not elicit proteinase inhibitors (Fidantsef *et al.,* 1999) [14]. Increased PI activities were not detected systemically, but only in infested leaves (Casaretto and Corcuera 1998) [5]. Similarly the lack of systemic induction of PI activity upon aphid infestation or mechanical wounding was reported by Casaretto *et al.,* (2004) [6]. Mechanical damage treatment influenced significantly the inhibition of chymotrypsin activity at all the leaf positions when compared to the corresponding controls at <0.01 level. Insecticidal soap solution showed non-significant results in the induction of PIs in all the leaf positions of cotton. The difference in values of enzyme activity in the induced plants over the control plants were subjected to 2-way analysis of variance to study the induction in totality with different treatments (Table 5) indicated that the leaf positions and damage treatments significantly influence the induction of defense enzymes in cotton and the values had significant within variation.

Table 1: Spatial pattern of PPO induction upon different treatments in cotton (var MCU 7)

Treatment/ Leaf position	*S. litura*			*A. gossypii*			Mechanical			Insecticidal Soap		
	Control	Damaged	P	Control	Damaged	P	Control	Damaged	P	Control	Damaged	P
D- damage	3.462	7.083	<0.001	3.462	7.210	<0.001	3.462	5.733	0.025	3.462	4.333	0.030
T- terminal	5.100	16.687	<0.001	5.100	6.163	0.008	5.100	5.330	0.160	5.100	5.067	0.795
U- upper	3.88	9.433	<0.001	3.88	7.903	<0.001	3.88	5.733	0.010	3.88	3.964	0.182
L- lower	2.633	3.800	0.053	2.633	3.433	0.078	2.633	2.700	0.768	2.633	2.567	0.770

Values represent mean enzyme activity measured as increase in OD/unit time ± SD, P= Probability level

Table 2: Spatial pattern of POD induction upon different treatments in cotton (var MCU 7)

Treatment/ Leaf position	*S. litura*			*A. gossypii*			Mechanical			Insecticidal Soap		
	Control	Damaged	P	Control	Damaged	P	Control	Damaged	P	Control	Damaged	P
D- damage	2.20±0.11	5.467±0.05	<0.001	2.20±0.17	3.296±0.03	<0.001	2.20±0.13	2.567±0.15	0.025	2.20±0.10	2.263±0.11	0.481
T- terminal	3.20±0.20	5.267±0.15	<0.001	3.20±0.20	3.40±0.10	0.196	3.20±0.2	3.33±0.15	0.411	3.20±0.20	3.207±0.11	0.962
U- upper	1.997±0.10	5.533±0.05	<0.001	1.997±0.10	2.619±0.23	0.013	1.997±0.10	2.20±0.13	0.068	1.997±0.10	2.063±0.05	0.370
L- lower	1.113±0.11	1.339±0.16	0.120	1.113±0.11	1.275±0.11	0.153	1.20±0.12	1.293±0.05	0.222	1.122±0.08	1.100±0.10	0.780

Values represent mean enzyme activity measured as increase in OD/unit time ± SD, P= Probability level

Table 3: Spatial pattern of LOX induction upon different treatments in cotton (var MCU 7)

Treatment/ Leaf position	*S. litura*			*A. gossypii*			Mechanical			Insecticidal Soap		
	Control	Damaged	P	Control	Damaged	P	Control	Damaged	P	Control	Damaged	P
D- damage	5.100±0.20	7.067±0.25	<0.001	5.100±0.20	5.567±0.41	0.155	5.100±0.20	5.227±0.11	0.391	5.100±0.20	5.133±0.15	0.830
T- terminal	8.300±0.20	8.327±0.26	0.895	8.300±0.20	8.333±0.35	0.893	8.300±0.20	8.317±0.26	0.935	8.300±0.20	8.307±0.29	0.976
U- upper	6.557±0.14	8.367±0.15	<0.001	6.557±0.14	6.600±0.10	0.685	6.557±0.14	6.570±0.08	0.894	6.557±0.14	6.630±0.12	0.531
L- lower	2.623±0.75	3.767±1.10	0.220	2.623±0.75	2.660±0.65	0.952	2.623±0.75	3.163±0.33	0.920	2.623±0.75	2.817±0.73	0.979

Values represent mean enzyme activity measured as increase in OD/unit time ± SD, P= Probability level

Table 4: Spatial pattern of PI induction upon different treatments in cotton (var MCU 7)

Treatment/ Leaf position	*S. litura*			*A. gossypii*			Mechanical Damage			Insecticidal Soap		
	Control	Damaged	P	Control	Damaged	P	Control	Damaged	P	Control	Damaged	P
D- damage	14.33±1.1	54.66±2.0	<0.001	14.33±1.1	57.66±1.5	<0.001	14.33±1.1	48.67±3.5	<0.001	14.33±1.1	19.00±3.0	0.066
T- terminal	22.00±2.0	70.33±2.5	<0.001	22.00±2.0	33.66±5.8	0.031	22.00±2.0	34.33±3.0	<0.001	22.00±2.0	23.33±2.0	0.411
U- upper	17.00±2.2	63.33±1.5	<0.001	17.00±2.2	31.00±4.58	0.008	17.00±2.2	38.33±1.5	<0.001	17.00±2.2	17.00±2.1	0.391
L- lower	14.33±2.0	35.33±4.7	0.002	14.33±2.0	22.33±4.5	0.049	14.33±2.0	27.33±1.6	<0.001	14.33±2.0	17.00±1.8	0.186

Values represent % Inhibition of chymotrypsin activity with SD, P= Probability level

Table 5: Two-way Anova summary of enzyme activity and damage treatments in cotton

Source of Variation	PPO		POD		LOX		PI	
	F	P-level	F	P-level	F	P-level	F	P-level
Damage treatment	61.294	<0.001	62.125	<0.001	66.506	<0.01	192.35	<0.001
Leaf Position	18.078	<0.001	30.089	<0.001	40.745	<0.001	42.321	<0.001
Damage treatment x Leaf Position (Interaction)	8.191	<0.001	11.586	<0.001	22.311	<0.001	11.757	<0.001

Difference between the values of control and treated plants were taken for analysis; P= Probability level

Longevity of induced response

The spatial pattern of induction of plant defense enzymes revealed PPO and PI induction to be plant systemic. Therefore these two components were selected to study the longevity of induction in cotton (var. MCU 7). The induction of PPO activity in *G. hirsutum* lasts up to 7 days after treatment (DAT) upon *S. litura* damage. The highest activity was observed on the fifth day after induction and the induction persisted at levels significantly higher than the control even on the seventh day. Also the induction by *S. litura* was quantitatively more than *A. gossypii* (Figure 1). Highest induction of PPO activity was observed on third DAT and then diminished on 5th and 7th day but with significant difference from control plants. The longevity of the PI activity (chymotrypsin inhibition) upon induction by *S. litura* and *A. gossypii* feeding damage expressed in *G. hirsutum* steadily increased and peaked on the 5th DAT for *S. litura* damage type and it persisted at significantly higher levels even on the 7th DAT (Figure 2). Damage by *A. gossypii* showed highest PI activity on 3rd DAT and no significant increase was observed thereafter but the activity was significantly different from the control. However, Underwood (1998) [28] demonstrated that induced resistance against *E. varivestis* lasted three days after damage in soybean. Stout *et al.,* (1996b) reported persistence of proteinase inhibitors and oxidative enzymes like polyphenol oxidase, peroxidase and lipoxygenase for at least 21 days after induction in damaged tomato leaflets. Previously Casaretto and Corcuera (1998) [5] observed the peak activity of chymotrypsin inhibitor between 48 and 72h of infestation with aphids which is in support of the present findings. Leaflets from soyabean plants treated by bean leaf beetle herbivory revealed that induced responses were highest on 14th and lowest on 25 days after initiation of feeding (Srinivas *et al.,* 2001) [25]. The induced response may be either short term and rapid or long term and delayed, the former tending to be more effective against generalist insect and the latter against both generalist and specialist insects (Agarwal and Karban, 1999) [1].

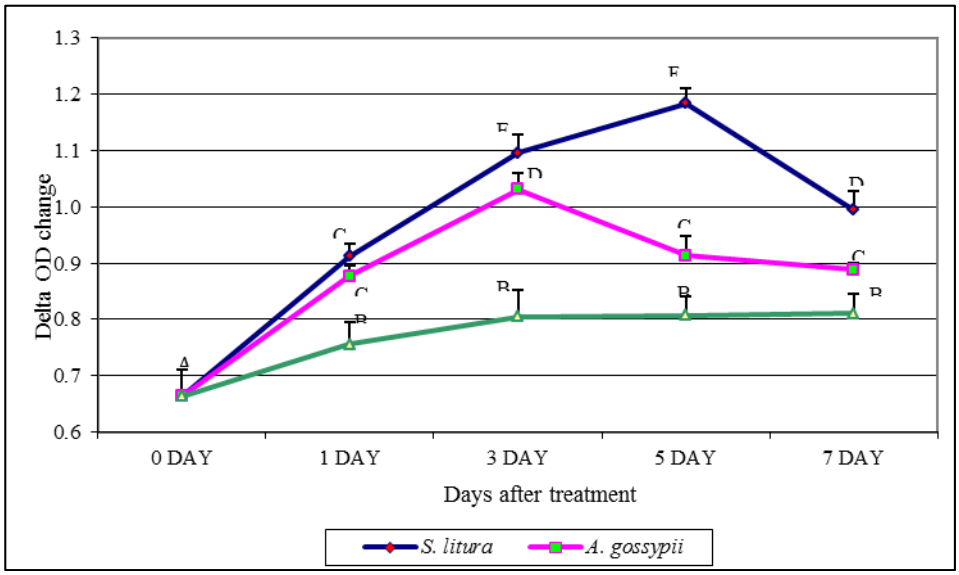

Fig. 1: Persistence in PPO activity upon *S. litura* and *A. gossypii* feeding damage

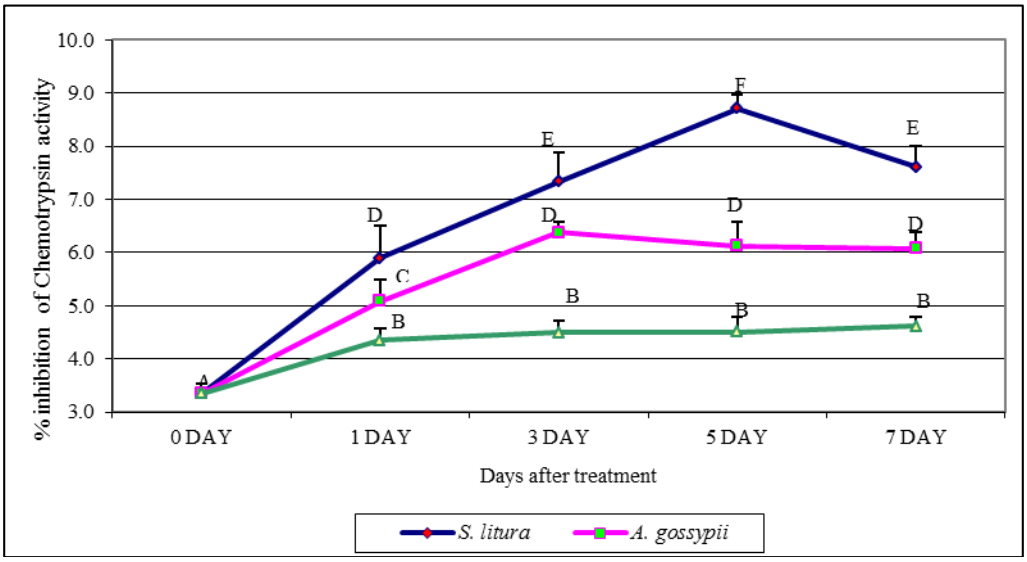

Fig. 2: Persistence in PI activity upon *S.litura* and *A. gossypii* feeding damage

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