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Partially purified *Glycine max* proteinase inhibitors: potential bioactive compounds against tobacco cutworm, *Spodoptera litura* (Fabricius, 1775) (Lepidoptera: Noctuidae)

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Abstract: *Spodoptera litura* (Fabricius, 1775), a polyphagous defoliator with broad host spectrum, causes significant damage to agriculturally important crops. Serine protease is primarily responsible for most of the proteolytic activity in the larval gut of lepidopteran insects. Second-instar larvae were reared on an artificial diet containing serine trypsin inhibitor partially purified from *Glycine max* seeds. Different concentrations were amended in the diet, i.e. 25, 50, 100, 200, and 400 µg/mL. The larval development period, total developmental period, longevity, fecundity, larval weight, and mean relative growth rate were decreased at the 100 µg/mL concentration. However, with further increase in concentration, the above parameters were all found to increase. Nutritional indices revealed reduction in efficiency of conversion of digested food and approximate digestibility, whereas increase in efficiency of conversion of ingested food had significant influence on food assimilation. Trypsin activity was suppressed at 100 µg/mL with increase in exposure interval. Results reveal that soybean protease inhibitor inhibited *S. litura* gut proteinase and it was more effective at lower concentrations. Thus, low levels of inhibitor would be enough to affect the growth and development of the target pest for the development of transgenic plants.

Key words: Plant protease inhibitors, lepidopteran pests, *Glycine max*, *Spodoptera litura*

1. Introduction

Plants have evolved several strategies to sustain favorable growth and survival. These strategies involve an array of compounds that constitute major components of their defense mechanism and allow them to successfully tolerate or resist insects and phytopathogenic microorganisms (Jackson and Tailor, 1996; Malek and Dietrich, 1999). One such defensive strategy includes proteinaceous molecules such as α-amylase inhibitors (α-AIs), proteinase inhibitors (PIs), lectins, and chitinases (Fritig et al., 1998). The existing methods for protecting plant crops against insect predation have heavily depended on environmentally hazardous chemicals, which has resulted in undesirable effects on beneficial organisms and has posed pollution problems. Natural plant products offer a potentially benign method for insect pest control (Andow, 2008). Thus, this kind of plant resistance can be utilized as an economic way to diminish the crop losses arising from insect pests. Digestive enzyme inhibitors that affect the growth and development of pest species have gained significant importance. There has been much work reported on the effectiveness of PIs against certain insect species in both in vitro assays against insect gut proteases (Koiwa et al., 1998) and in vivo artificial diet bioassays (Urwin et al., 1997; Vain et al., 1998).

The members of the serine class of proteinases have been the subject of more research than any other class of PIs (Ryan, 1990). The first plant proteinase inhibitor to be well characterized was the soybean trypsin inhibitor (SBTI) known as Kunitz (Kunitz, 1945), which inhibited both trypsin and chymotrypsin. Members of the Kunitz family inhibit mainly trypsin and are also capable of inhibiting other classes of serine proteinases, cysteine proteinases, aspartic proteinases or α-amylases (Pal et al., 1986; Ravichandran et al., 1999).

Numerous studies have reported the potential of PIs as effective antidigestive compounds to protect crop plants from herbivory (Michaud, 2000; Haq et al., 2004). The tobacco cutworm, *Spodoptera litura* (Fabricius, 1775) (Lepidoptera: Noctuidae) is a polyphagous pest of many important crops. With high dispersal capability, this pest has often generated high levels of agricultural losses. The host range covers over 44 families. *S. litura* has attained a major pest status on agricultural crops such as cotton, soybean, tobacco, *Colocasia*, groundnut, and cauliflower in India. Repeated exposure of *S. litura* to synthetic pesticides

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has resulted in the development of resistance, as a consequence of which an unexpected population outbreak was observed in the major cotton and groundnut growing regions of Andhra Pradesh and Tamil Nadu (Armes et al., 1997; Johny and Muralirangan, 2000; Gokulkrishnan et al., 2012).

The current study was intended to explore the antiinsect potential of partially purified protease inhibitors from soybean on the growth and development of *S. litura*. Furthermore, we investigated the effect of this inhibitor on food consumption, absorption, and utilization, as well as on the trypsin-like proteinase extracted from the larval midgut.

2. Materials and methods

2.1. Insect culture and diets

Rearing of *S. litura* was done on both natural and artificial diets. The culture of *S. litura* was maintained in a biochemical oxygen demand (B.O.D.) incubator at a temperature of 27 ± 2 °C, relative humidity of 60%, and photoperiod of L16:D8 on castor (*Ricinus communis*) leaves. Rearing on artificial diet was done as suggested by Koul et al. (1997).

2.2. Partial purification of PIs

Plant specimens, i.e. soybean seeds, *Glycine max* (Family: Fabaceae), were identified with voucher number 0405/HRB from the Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar. The method given by Duranti et al. (2003) was followed for extraction of the inhibitor from soybean seeds. For this, soybean seeds were dipped overnight in 10 mM potassium phosphate buffer (pH 7.2) at 4 °C and were then ground the next day. They were then filtered with double-layered muslin cloth to obtain crude extract, which was centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatant was then collected and precipitated with 0%–80% ammonium sulfate and was kept overnight at 4 °C. The mixture was centrifuged at 10,000 rpm for 15 min. The supernatant was discarded, and the precipitates were dissolved in a minimum amount of distilled water and then dialyzed against distilled water for 48 h. The dialysate was centrifuged and the supernatant was pooled and stored at –20 °C for the detection of inhibitory activity.

2.3. Proteinase inhibitory activity

Proteinase inhibitory activity was determined according to Paulino da Silva et al. (2001) using N-α-benzoyl-DL-arginine *p*-nitroanilide (BApNA) as a substrate. First, 20 µL of trypsin and 30 µL of inhibitor were preincubated in 50 mM Tris-HCl buffer (pH 8.2) for 10 min at room temperature in a total volume of 200 µL and the reaction was initiated with the addition of 100 µL of 1 mM BApNA. The liberation of *p*-nitroaniline was measured at 410 nm. The inhibitory activity was calculated as the difference between proteolytic activity with and without inhibitor. One unit of trypsin activity (TA) is defined as the enzyme activity that gives an increase in 0.01 OD at 410 nm under the said experimental conditions. One unit of trypsin inhibitory activity (TIA) is defined as the activity that inhibits or reduces one unit of enzyme activity.

2.4. Bioassay

The second-instar (5 to 6 days old) larvae of *S. litura* were treated with 1% sodium hypochlorite solution and shifted to sterilized empty plastic cups (vol. 25 mL) containing cubical pieces of control diet and diet supplemented with partially purified PIs from soybean. The experimental plastic cups were kept in the B.O.D incubator and observed daily for various developmental parameters. There were 6 replications with 5 larvae in each replication for each concentration.

2.5. Nutritional indices

A number of nutritional parameters were compared among second-instar larvae exposed to different concentrations of untreated diet and diet treated with soybean PIs. The larvae, feces, and remaining uneaten diet were separated under a microscope, dried, and weighed. Nutritional indices of consumption, digestion, and utilization of food were calculated as described by Waldbauer (1968) and Koul et al. (2005). The experiment was planned for 3-day intervals with 5 larvae in 6 replications for each concentration. The nutritional indices, specifically the relative growth rate (RGR), relative consumption rate (RCR), efficiency of conversion of ingested food (ECI), efficiency of conversion of digested food (ECD), and approximate digestibility (AD), were calculated. RGR and RCR were calculated on a dry weight basis after 3 days of feeding as G/I (G = change in larval dry weight/day and I = starting larval dry weight) and C/I (C = change in diet dry weight/day and I = starting larval dry weight), respectively. ECI was calculated as 100 × G/C, where G = dry weight gain of the insect and C = dry weight of food consumed; ECD as [weight gained/(food ingested – frass weight) × 100]; and AD as [(food ingested – frass weight)/food ingested × 100]. Larval weight was taken into consideration starting from the second instar to the final instar after a 24-h interval for determining mean RGR (Martinez and Emden, 2001) and food assimilated (Khan and Saxena, 1985).

2.6. Digestive protease assay

The whole alimentary canal was dissected out from late instars fed on control and PI-incorporated diets. Trypsin-like enzyme from the midgut of *S. litura* was assayed using the synthetic substrate BApNA as described by Christeller et al. (1990, 1992) for three time intervals (24 h, 48 h, and 72 h) with three different concentrations (50, 100, and 200 µg/mL). All incubations were performed in triplicates and appropriate controls were included.

3. Results

3.1. Effect of PIs on growth and development

PIs partially purified from soybean exhibited 28 trypsin inhibitory units (TIUs) per milligram of protein with 71.72% antitryptic activity (Table 1). Bioassay results showed significant reduction in growth and development among larvae fed with inhibitors at all stages of the larval growth period. A concentration-dependent decline by 7.17 days in the larval period was observed up to 100 µg/mL; it thereafter increased with increase in concentration of PI (Table 2). Significant reduction in the total development period was recorded up to 100 µg/mL ($F = 7.07$, $df = 5$, $P \leq 0.01$). Total life period did not show any regular trend with partially purified soybean PI treatment (Table 2). However, reduction in percentage pupation and emergence was observed up to 100 µg/mL (data not shown). Adults with 57.20% deformities were noticed at 100 µg/mL, whereas at higher concentrations this rate was the same as that in untreated larvae (Table 3). The deformed adults had crumpled and underdeveloped wings as well as being half emerged from pupae, as shown in Figures 1A–1D. The life span of adults that emerged at 200 µg/mL was maximum, whereas at 100 µg/mL it was less than in the control and other treatments ($F = 4.72$, $df = 5$, $P \leq 0.01$). The percentage of surviving adults was 24% less at 100 µg/mL in comparison to the control (Figure 2). On the other

Table 1. Partial purification of protease inhibitors from soybean.

Purification step	Volume (mL)	Protein (mg/mL)	TIU/mL	Total IU	TIU/mg protein	Total activity (mg/mL)	Specific activity	Recovery (%)	Purification fold increase
Crude soybean sample	250	9.263	33.44	8360.00	3.61	0.623	0.067	100	1
Partially purified sample	31	1.190	33.33	1033.23	28.00	0.520	0.436	71.72	7.75

TIU: Trypsin inhibitory units.

Table 2. Larval period, total developmental period, and total life period of *S. litura* when the larvae were fed on artificial diets incorporated with different concentrations of soybean PIs.

Concentrations (soybean PIs in µg/mL)	Larval period (in days) (mean ± SE)	Total developmental period (in days) (mean ± SE)	Total life period (in days) (mean ± SE)
Control	25.28 ± 0.534^{ab}	35.93 ± 0.465^{abc}	42.36 ± 0.63^{ab}
25	20.32 ± 0.399^{cd}	34.88 ± 0.429^{bcd}	44.80 ± 0.53^{a}
50	18.38 ± 0.303^{d}	34.31 ± 0.468^{cd}	42.80 ± 0.66^{ab}
100	18.11 ± 0.289^{d}	33.90 ± 0.377^{d}	41.90 ± 0.76^{b}
200	23.79 ± 1.73^{bc}	36.59 ± 0.460^{ab}	44.92 ± 0.70^{a}
400	28.38 ± 1.19^{a}	36.95 ± 0.595^{a}	44.90 ± 0.51^{a}
F-value	20.12**	7.07**	4.72**

The averages followed by the same letter do not differ statistically between themselves, Tukey's test ($P \leq 0.05$). **: Significant at 1% level.

Table 3. Percentage of abnormality in emerged adults and fecundity of *S. litura* when the larvae were fed on artificial diets incorporated with different concentrations of soybean PIs.

Concentrations (soybean PIs in µg/mL)	Percentage abnormality in emerged adults (w.r.c.) (mean ± SE)	Fecundity (No. of eggs laid/female) (mean ± SE)
Control	-	583.3 ± 60.1^{a}
25	16.7 ± 10.50^{a}	291.7 ± 46.8^{bc}
50	19.4 ± 12.50^{a}	451.7 ± 62.5^{ab}
100	57.20 ± 3.37^{a}	165.0 ± 18.8^{c}
200	29.43 ± 9.90^{a}	550.0 ± 29.6^{a}
400	20.00 ± 10.00^{a}	476.6 ± 30.0^{ab}
F-value	2.94*	13.12**

The averages followed by the same letter do not differ statistically between themselves, Tukey's test ($P \leq 0.05$). **: Significant at 1% level; *: significant at 5% level; w.r.c.: with respect to control.

(A) (B) (C) (D)

Figure 1. (A) Normal *S. litura* adult, (B–D) abnormality in adults observed at 100 µg/mL concentration of soybean PIs.

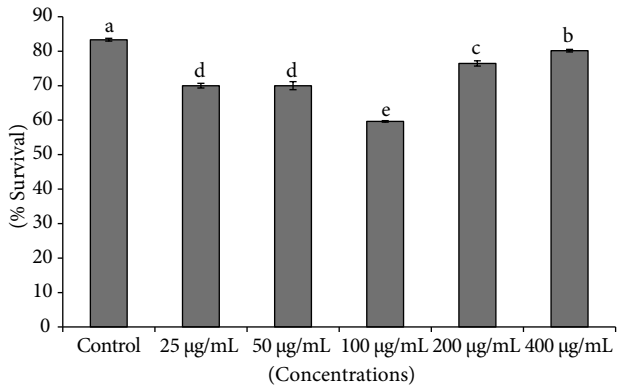

Figure 2. Percentage survival of adults when second-instar larvae of *S. litura* were given different concentrations of soybean PIs. Columns and bars represent the mean ± SE. Different letters above the columns representing each concentration indicate significant differences with Tukey's test at $P \leq 0.05$.

hand, the egg-laying capacity of adult female moths that emerged from treated second-instar larvae was adversely affected as a decline in fecundity was observed at all concentrations. At 100 µg/mL, the fecundity decreased to a maximum of 28.29% of the control (Table 3). Observations made on larval weight for different time intervals showed a significant increase initially at 2 and 4 days, but on day 6 it was 10.7 mg less than in the control (Table 4). The mean RGR calculated from the fresh weight of the larvae recorded on different days was not significantly different from the control at lower concentrations, but it increased significantly at the higher concentrations of 200 and 400 µg/mL (Table 5).

3.2. Effect of PIs from soybean on nutritional parameters

Decrease in RCR showed no correlation with increase in concentration. Maximum decrease was observed at 50 µg/mL, where the RCR was reduced to 27.15% of that of the control. ECI was increased significantly and this increase was more noticeable at 50 µg/mL, where a 2.9-fold increase was observed. ECD was reduced significantly in the treated larvae. At the highest concentration of 400 µg/mL it was reduced to almost half of that in the control ($F = 3.39$, $df = 5$, $P \leq 0.05$). A significant decrease in AD in all the treatments was observed. This decrease was greater at 100 µg/mL, where the AD was 13.02 as compared to 64.67 in the control (Table 6). A significant influence was seen on food assimilation, which decreased considerably at 100 µg/mL (Figure 3).

The activity of trypsin in the larvae fed at 100 µg/mL revealed a noticeable suppression in enzyme activity with increase in exposure interval. At the 72-h feeding interval the enzyme activity decreased to 94.61% of that of the control. Although at higher treatment concentrations the activity of trypsin increased at the 24-h treatment interval, with prolonged feeding, it decreased (Figure 4).

4. Discussion

To successfully ascertain a novel insect control strategy based on plant secondary metabolites, it is essential to know the metabolite systems and in vivo assays using purified compounds (Silva et al., 2006). In this study, different concentrations of partially purified SBTI were used against second-instar larvae of *S. litura*. The significant decrease in growth and development of larvae at lower concentrations clearly suggests the antinutritional nature of this extract, as it decreased the total developmental period, particularly at lower concentrations, and increased the total life period at some concentrations. Shukle and Murdock (1983) also had similar results with *Manduca sexta* when given an artificial diet supplemented with different amounts of soybean lipoxygenase and Kunitz trypsin inhibitor. Similarly, a significant reduction in growth and development of *Helicoverpa zea* and *Spodoptera exigua* larvae was noticed when given a diet incorporated with SBTI (Broadway and Duffey, 1986). McManus and Burgess (1995) found that

Table 4. Larval weight (in mg) of *S. litura* when the larvae were fed on artificial diets incorporated with different concentrations of soybean PIs.

Concentrations (in µg/mL)	2 days (mean ± SE)	4 days (mean ± SE)	6 days (mean ± SE)
Control	63.05 ± 2.11^{a}	103.10 ± 6.14^{c}	193.00 ± 5.49^{ab}
25	60.25 ± 3.34^{a}	106.55 ± 5.84^{bc}	180.03 ± 9.14^{b}
50	60.72 ± 2.12^{a}	130.45 ± 3.42^{b}	198.95 ± 6.41^{ab}
100	59.05 ± 1.61^{a}	105.63 ± 7.29^{bc}	182.30 ± 10.90^{b}
200	84.90 ± 4.39^{a}	161.78 ± 6.57^{a}	238.00 ± 17.40^{a}
400	86.65 ± 7.18^{a}	166.02 ± 5.80^{a}	241.30 ± 14.20^{a}
F-value	10.83**	23.42**	5.75**

The averages followed by the same letter do not differ statistically between themselves, Tukey's test ($P \leq 0.05$). **: Significant at 1% level.

Table 5. Mean relative growth rate of *S. litura* when the larvae were fed on artificial diets incorporated with different concentrations of soybean PIs.

Concentrations (in µg/mL)	2 days (mean ± SE)	4 days (mean ± SE)	6 days (mean ± SE)	8 days (mean ± SE)
Control	13.20 ± 0.74^{b}	18.55 ± 0.95^{b}	26.17 ± 1.87^{bc}	28.74 ± 2.27^{c}
25	12.18 ± 1.32^{b}	18.48 ± 1.80^{b}	22.54 ± 0.91^{c}	33.39 ± 5.71^{abc}
50	12.59 ± 0.952^{b}	21.27 ± 1.21^{b}	26.90 ± 0.76^{abc}	35.82 ± 2.41^{abc}
100	11.70 ± 0.425^{b}	20.27 ± 2.08^{b}	25.12 ± 1.52^{c}	30.34 ± 2.60^{bc}
200	22.88 ± 2.05^{a}	31.80 ± 1.39^{a}	34.27 ± 2.68^{a}	46.87 ± 2.73^{a}
400	23.65 ± 3.35^{a}	31.51 ± 1.57^{a}	34.41 ± 2.75^{ab}	42.89 ± 2.77^{ab}
F-value	10.08**	16.68**	6.70**	4.70**

The averages followed by the same letter do not differ statistically between themselves Tukey's test ($P \leq 0.05$). **: Significant at 1% level.

Table 6. Nutritional indices of *S. litura* when the larvae were given artificial diets incorporated with different concentrations of soybean PIs.

Concentrations (in µg/mL)	RCR (mean ± SE)	ECI (mean ± SE)	ECD (mean ± SE)	AD (mean ± SE)
Control	5.34 ± 0.45^{a}	25.50 ± 4.61^{b}	50.46 ± 72.6^{a}	64.67 ± 6.06^{a}
25	5.94 ± 0.64^{a}	24.90 ± 4.25^{b}	30.66 ± 19.9^{ab}	79.78 ± 6.99^{a}
50	1.45 ± 0.08^{b}	73.54 ± 8.92^{a}	33.86 ± 49.5^{ab}	13.90 ± 3.59^{b}
100	2.57 ± 0.13^{b}	56.83 ± 9.97^{ab}	28.52 ± 48.5^{b}	13.02 ± 1.74^{b}
200	2.03 ± 0.12^{b}	64.21 ± 7.27^{a}	27.42 ± 39.3^{b}	19.26 ± 5.60^{b}
400	1.74 ± 0.14^{b}	61.81 ± 9.83^{a}	26.35 ± 48.8^{b}	21.70 ± 1.40^{b}
F-value	33.2**	7.06**	3.39*	37.74**

RCR: Relative consumption rate, ECI: efficiency of conversion of ingested food, ECD: efficiency of conversion of digested food, AD: approximate digestibility. The averages followed by the same letter do not differ statistically between themselves, Tukey's test ($P \leq 0.05$). *: Significant at 5% level; **: significant at 1% level.

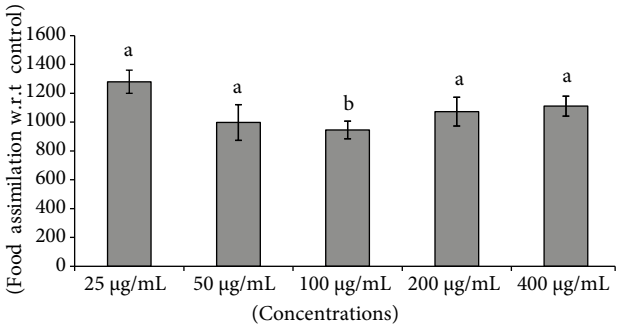

Figure 3. Food assimilation (in mg) with respect to control when second-instar larvae of *S. litura* were given different concentrations of soybean PIs. Columns and bars represent the mean ± SE. Different letters above the columns representing each concentration indicate significant differences with Tukey's test at $P \leq 0.05$.

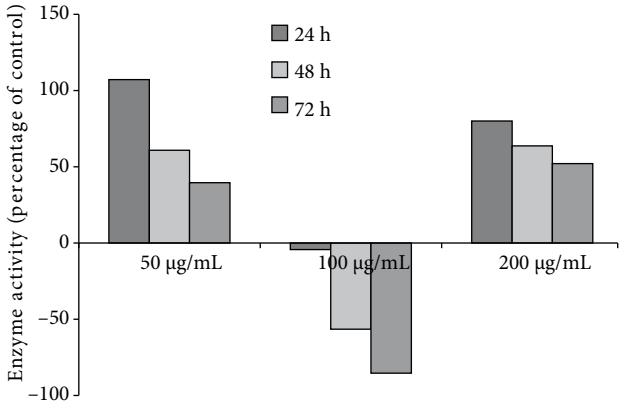

Figure 4. Trypsin activity in larvae of *S. litura* at different time intervals under the influence of partially purified soybean PIs.

the neonate larvae of *S. litura* showed more reduction in growth than late instars when they were given Kunitz trypsin inhibitor in their diets. On the other hand, the consumption of soybean protease inhibitor by *Diatraea saccharalis* delayed the developmental time to pupation as well as pupal duration significantly (Pompermayer et al., 2001). Moreover, a maximum number of adults that emerged from treated larvae had morphological deformities. Gomes et al. (2005) observed several deformities in *Abies grandis* treated with chickpea PIs. Macedo et al. (2004) also found severe deformities in adults that emerged from *Callosobruchus maculatus* larvae treated with the Kunitz type inhibitor from *Adenanthera pavonina* seeds. Vasudev and Sohal (2015) also noticed adult deformities after feeding larvae with partially purified protease inhibitors from *Leucaena leucocephala*.

The mean life span of emerged adults was not significantly influenced, whereas the egg-laying capacity declined considerably in the female adults that emerged from treated larvae. Reduction in egg-laying capacity was noticed by Telang et al. (2003) in *S. litura* and another polyphagous lepidopteran, *Helicoverpa armigera*, fed on bitter gourd protease inhibitors. Reduced fecundity and fertility was also observed in *H. armigera* fed on serine protease inhibitors from *Capsicum annum* (Tamhane et al., 2005), in *D. saccharalis* treated with soybean protease inhibitor (Pompermayer et al., 2001), and in larvae of a coleopteran insect, *C. maculatus*, when treated with a Kunitz type inhibitor (Macedo et al., 2004). The weight of the larvae was significantly affected. Although larval mortality was not observed, reduction in larval weight demonstrated the efficiency of PIs in interfering with the insect's physiological system. Such reduction in larval weight in lepidopteran insects was reported earlier in bioassay studies conducted with PIs (Nandeesha and Prasad, 2001; Franco et al., 2003; Dorrah, 2004; Vaijayanti et al., 2005; Bhavani et al., 2007; Vasudev and Sohal, 2013). Reduced insect growth was observed in *M. sexta* larvae when reared on transgenic tobacco plants expressing SBTI (McManus et al., 1999). This suggests the usefulness of PIs as a resistance factor in transgenic plants. Transgenic plants expressing protease inhibitors are often effective against one pest but not others (McManus et al., 1999).

Quality of diet influences the growth of larvae directly and the inhibitor supplementation adds to the effect in retarding the larval growth and development. However, at higher concentrations the effect was reversed, which could be associated with the adaptation of larvae to the soybean PI, but prolonged feeding might have interfered with the digestion process, which was further manifested in the form of increased abnormalities observed in adults that emerged from treated larvae.

Dietary utilization experiments showed that the consumption rate was decreased. It is likely that the decrease in consumption rate could be due to the antifeedant or antinutritive nature of the soybean extract and this could have accounted for a decrease in growth rate. ECD values decreased in all treatments in comparison to the control. House (1974) and Scriber and Slansky (1981) reported that low ECD values usually result from the presence of a toxin or from nutrient imbalance. Decrease in ECD accounts for the compensation for the deficiency in foodstuff conversion, perhaps by diverting energy from biomass production into detoxification (Nathan and Kalaivani, 2005), which could be related to the reduction in growth. Food assimilation by second-instar larvae of *S. litura* declined at 100 µg/mL but increased with increase in concentration. This could be accredited to the low nutrient value of food ingested, which might have resulted in decreased food assimilation efficiency (Lindroth, 1993).

After an initial increase at 50 µg/mL, a significant suppression in enzyme activity was noticed at 100 µg/mL. Johnston et al. (1993) also reported a significant reduction in trypsin-like enzyme activities in the gut contents of larvae of *H. armigera* fed on a diet containing SBTI. In 1995, Johnston et al. further observed an inhibitory effect of cowpea trypsin inhibitor, SBTI, and soybean Bowman-Birk trypsin-chymotrypsin inhibitor on trypsin from *Heliothis virescens* larval gut extracts. Prior investigation by Hegedus et al. (2003) also proved that SBTI reduced midgut protease activity by 80% in *Mamestra configurata* larvae. Reduction of trypsin activity in *Eurygasta integriceps* fed with low doses of SBTI was noticed by Saadati and Bandani (2011). Dorrah (2004) also reported a significant inhibition of serine proteases in vitro by soybean protease inhibitor in *S. littoralis*. Inhibition of trypsin activity could be related to the blockage of enzyme active sites, thus inhibiting the formation of enzyme–substrate complexes, which could be attributed to impairment in digestion (De Leo et al., 2001; Wheeler and Isman, 2001). The serine protease inhibitors affect not only digestive enzymes, but also water balance, the development of the insect, and enzymatic regulation (Boulter, 1993). However, increase in enzyme activity at the 72-h time interval with 200 µg/mL PI concentration in the diet suggests that either the insect has adapted to this concentration or has produced a new iso/enzyme that is insensitive to the inhibitor. These results agree with the findings of McManus and Burgess (1995), where significant stimulation of tryptic activities in *S. litura* larvae was found with an artificial diet containing SBTI. The presence of dietary plant protease inhibitors strongly modulates protease activities in the insect gut (Erlandson et al., 2010), whereby the insect can switch over to production of inhibitor-insensitive proteases. This could be correlated with an increase in larval growth and development at higher concentrations.

The adverse effects of soybean inhibitor on *S. litura* larvae were more pronounced at lower concentrations, which could be attributed to the lesser availability of amino acids required for normal growth and development at these concentrations. However, at higher concentrations, the effect was reversed. Above the concentration of 100 µg/mL, insects might have produced inhibitor-insensitive proteases for attaining maximum growth.

In conclusion, the results from the present study suggest the effectiveness of inhibitor at lower concentrations and adaptation to higher concentrations. These findings signify that insect digestive tracts can adapt to these challenges and competently deal with different toxins as well as antimetabolites in their diets. Thus, it is essential to assay PIs against the larval gut enzymes of the target insect pest before considering them as candidates for development of transgenic plants for insect resistance.

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