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# *In vivo* inhibition of *Helicoverpa armigera* gut pro-proteinase activation by non-host plant protease inhibitors

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ABSTRACT

We evaluated 22 different host and non-host plant protease inhibitors (PIs) for *in vivo* inhibition of *Helicoverpa armigera* gut pro- and proteinases, and their biological activity against the pod borer, *H. armigera*, the most important pest of agriculture and horticultural crops worldwide. *In vitro* activation of *H. armigera* gut pro-proteinases (HaGPPs) in larvae fed on non-host plant PIs showed significant *in vivo* inhibition of HaGPPs activation in solution as well as in gel assays. The larvae fed on diet incorporated with *Datura alba ness* PIs showed highest inhibition of HaGPPs, followed by *Psophocarpus tetragonolobus*. Non-host plant PIs from *Pongamia pinnata*, *Mucuna pruriens*, *Capsicum annuum*, and *Nigela sativa* showed maximum inhibitory potential towards HaGPs *in vivo*, and also exhibited moderate level of inhibition of pro-proteinases. However, some of non-host plant PIs, such as those from *Penganum harmala* and *Solanum nigrum*, and the principal host plant PIs, viz., *Cicer arietinum* and *Cajanus cajan* did not inhibit HaGPP activity. Pro-proteinase level increased with the growth of the larvae, and maximum HaGPP activity was observed in the fifth-instars. Larvae fed on diets with *D. alba ness* PIs showed greater inhibition of HaGPPs as compared to the larvae fed on diets with *P. tetragonolobus*. Low concentrations of partially purified HaGPs treated with gut extract of larvae fed on *D. alba ness* showed that out of 10 proteinase isoforms, HaGPs 5 and 9 were activators of pro-proteinases. Larval growth and development were significantly reduced in the larvae fed on the non-host plant PIs, of which *D. alba ness* resulted in highest stunted growth of *H. armigera* larvae. The *in vivo* studies indicated that non-host plant PIs were good candidates as inhibitors of the HaGPs as well as HaGPPs. The PIs from the non-host plants can be expressed in genetically engineered plants to confer resistance to *H. armigera*.



## 1. Introduction

There is a large gap between potential and actual yields in chickpea, pigeonpea and cotton grown in the semi-arid tropics. While low inputs and sub-optimal crop management account for a significant share of this gap, losses due to a wide range of insect pests and diseases result in harvested yields much below than what is possible with varieties having adequate levels of resistance/tolerance to insect pests. *Helicoverpa armigera* (Hübner) is the most important constraint to increase the production and productivity of these crops. It is also a serious pest of other important crops such as tomato, sunflower, cereals and vegetables. Losses due to *H. armigera* have been estimated to be over US$2 billion annually in the semi-arid tropics (Sharma, 2005).

Chemical control of *H. armigera* is often not effective, as it has developed high levels of resistance to commonly used oragano-chlorines, organophosphates, and pyrethroids (Armes et al., 1992b). Host plant resistance, natural plant products, bio-pesticides, natural enemies, and agronomic practices offer a potentially viable option to supplement the control of insect pests, and they are relatively safe to the non-target beneficial organisms and human beings (Andow, 2008). Host plant resistance is one of the most economic means of reducing the losses due to insect pests. However, the levels of resistance in the cultivated germplasm to *H. armigera* are quite low, and therefore, plant traits contributing to *H. armigera* resistance need to be reinforced with novel genes such as δ-endotoxins from *Bacillus thuringiensis*, protease inhibitors, and lectins (Ryan, 1990; Boulter, 1993; Sharma and Ortiz, 2000).

Many biological processes are triggered and nurtured by protein–carbohydrate recognition and protein–protein interactions. Protein protease inhibitors regulate proteolytic activity through protein–protein interaction. Proteinaceous proteinase inhibitors (PIs) are ubiquitous in plant parts, and are the plant's defense compounds produced in hyper amounts in response to insect attack (Jongsma et al., 1996; Tamayo et al., 2000) and

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wounding (Pena-Cortes et al., 1995; Zhao et al., 1996). When ingested by an insect, PIs inhibit digestive proteinases, resulting in starving of the insect for amino acids and retard the growth and development (Giri et al., 2005). The insects respond by over-production of gut proteinases to compensate for the inhibited activity, but synthesis of additional proteinases further depletes the pool of essential amino acids and results in growth retardation (Broadway and Duffey, 1986). Currently, the main emphasis of plant-PI studies is on identifying potential inhibitors of digestive proteinases of the target insects, and on understanding the dynamic nature of insect midgut proteinases at the molecular level (Bown et al., 1997; Lopes et al., 2004).

A large number of enzymes are synthesized as inactive precursors that are subsequently converted to the active form by the selective cleavage (limited proteolysis) of the peptide bonds. The ultimate aim of activating enzymatic function is limited proteolysis, either in single step activation or in consecutive series (cascade). The specificity of each activation reaction is determined by the complementarities of the zymogen substrate and the active site of the attacking protease (Neurath and Walsh, 1976). A novel pro-carboxypeptidase (PCPAHa) from *H. armigera*, the first pro-enzyme of this insect, has been characterized by expressing its encoding complementary deoxyribonucleic acid (cDNA) in insect cells (Bown et al., 1998). A crystal structure form of the novel pro-carboxypeptidase has been observed in the gut extracts from *H. armigera* larvae (Estébanez-Perpiñá et al., 2001). The metalloprotease is synthesized as a zymogen of 46.6 kDa, and upon *in vitro* activation, yields a pro-segment of 91 residues and an active carboxypeptidase moiety of 318 residues. Similarly, lepidopteran insects have serine proteinases as a major component of their digestive complement, and trypsin- and/or chymotrypsin-like proteases are quite common (Purcell et al., 1992; Srinivasan et al., 2006), and its inactive isoforms are synthesized in gut, and activated by trypsins and/or other serine endoproteases (Liu et al., 2009). However, there is inadequate information on mode of synthesis of *H. armigera* gut pro-proteinases. *H. armigera* larvae have an alkaline gut, and produce nearly 10 major and several minor serine proteinases that are able to overcome the native PIs of its host plants (Johnston et al., 1991; Bown et al., 1997, 1998; Giri and Kachole, 1998). These enzymes play an important role in protein digestion by releasing amino acids from the peptides produced by endopeptidases, thus, completing the digestion process. Insects also exhibit mechanisms to produce inhibitor-insensitive (Jongsma et al., 1995; Bown et al., 1997; Brito et al., 2001; Volpicella et al., 2003), or inhibitor-degrading proteinases (Girard et al., 1998; Giri et al., 1998; Moon et al., 2004; Telang et al., 2005), or rapidly altering gut contents in response to the ingested PIs through up- and down regulation of proteinases in the midgut to overcome the effect of PIs (Hilder et al., 1987).

Non-host plant PIs such as those from *Psophocarpus tetragonolobus*, *Capsicum annuum*, and *Momordica charantia* result in growth inhibition of *H. armigera* (Harsulkar et al., 1999; Patankar et al., 2001; Telang et al., 2003; Tamhane et al., 2005). Most of the plants produce an array of protease inhibitors with overlapping specificities for several gut proteases found in insect gut (Lawrence and Koundal, 2002). The potential of PIs as a component of host plant resistance to insect pests has also been studied (Gatehouse et al., 1993). The effect of host plant PIs is of considerable significance for adaptation and survival of phytophagous insects. The adaptation of insect pests to host plant PIs might have resulted from selection pressure on the population when they encountered the PIs of their host plants. The non-host plants may be a potential source of effective PIs for the insect pests in question, as the insects are not pre-exposed to the non-host plant PIs (Jongsma et al., 1996; Harsulkar et al., 1999; Tamhane et al., 2005). The non-host plant PIs act against the proteinases of insect gut and they can also protect the host plant proteins from proteolysis, thus, giving the plant an edge over the insect pests. Engineering of PIs in plants capable of inhibiting protease(s) involved in the protease zymogen activation have the benefit of conferring resistance to insect pests. The active sites of these enzymes are highly conserved, and have shown high similarities (Srinivasan et al., 2006). The PIs also affect a number of vital processes, including proteolytic activation of enzymes and moulting (Fan and Wu, 2005). Therefore, present investigations were aimed at evaluating *in vivo* effects of host and non-host plant PIs on *H. armigera*, and to identify promising PIs for inhibition of zymogen activation in *H. armigera*.

## 2. Materials and methods

### 2.1. Materials

Bovine trypsin, chymotrypsin, *N*-α-benzoyl-DL-arginyl-*p*-nitroanilide (BA*p*NA), *N*-α-benzoyl-L-arginine ethyl ester (BAEE), *N*-α-benzoyl-L-tyrosine ethyl ester (BTEE), polyvinylpyrrolidon (PVP), azocasein, and bovine serum albumin (BSA) were procured from Sigma Chemicals, USA. Seed samples used for bioassay and inhibition studies were purchased from Davasaj, Aurangabad, India. Acrylamide, *N*,*N*′-methylene bisacrylamide, Tris–Cl, and glycine were of analytical grade, and obtained from Sisco Research Laboratory (SRL), Mumbai, India. X-ray films were purchased from AGFA, Selvas Photographics Limited, Silvassa, India.

### 2.2. Rearing of H. armigera larvae in the laboratory

The *H. armigera* larvae were reared on an artificial diet (Armes et al., 1992a) under controlled laboratory conditions [26 ± 1 °C temperature, 60–70% relative humidity (RH) and 16 h daylight] at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India. The laboratory culture was regularly supplemented with field-collected larvae. To ensure greater genetic homogeneity among the test populations, the insects were reared on a control chickpea based artificial diet for maximum of three generations, after which they were used for *in vivo* assays.

### 2.3. Extraction of PIs from host and non-host plant seeds

Dry mature seeds of host and non-host plants were ground in pestle-mortar, and/or mixer-blender to make a fine powder. The powder was dehydrated, depigmented, and defatted by several washes of acetone, followed by hexane, and Folch's mixture (chloroform:methanol, 2:1). The solvents were removed by filtration and the powders air-dried. The powders were mixed with six volumes of distilled water containing 1% PVP and kept overnight at 4 °C for extraction, with intermittent shaking. The suspension was centrifuged at 10,000 rpm for 20 min at 4 °C and the supernatant was stored in aliquots at −20 °C. Protein concentration of the host and non-host seed extracts was quantified by Lowry's method (Lowry et al., 1951).

### 2.4. Bioassay

Studies on the effects of diet incorporated with host and/or non-host plant PIs on growth and development were carried on third-instar larvae of *H. armigera*. For this, newly moulted second-instar larvae were taken from the culture, and examined at 9:00 and 18:00 h daily. Larvae moulting between the observation times were placed in labeled vials. The larvae were weighed, and divided into control and experimental groups. The same numbers of larvae from each chronological age were placed on the test diets, ensuring that variation between populations fed on each diet was minimized. The required amount of PIs from host and/or non-

host plant was calculated for maximum inhibition of total proteolytic activity of single larval gut tissue, and such amount was incorporated per gram of artificial diet. The experiment was terminated on 5th day, and the larval weights were recorded for computing weight gain and growth rate. Thirty larvae were placed on each diet, and each feeding assay was carried out in triplicates. In order to examine the inhibitory potential of non-host plant PIs against gut pro- and proteinases in different instars, two non-host plants, *Datura alba ness* and *P. tetragonolobus* were selected, and artificial diet fed larval *H. armigera* gut proteinases (HaGPs) and *H. armigera* gut pro-proteinases (HaGPPs) were analyzed.

### 2.5. Extraction of gut enzymes

Fourth- and fifth-instar *H. armigera* larvae reared on host and non-host plant PIs in the laboratory were used for the enzyme extraction. The larvae were starved for 5 h, and killed by decapitation to collect the midguts. The midgut contents were extracted in 0.2 M glycine–NaOH buffer, pH 10.0. The extraction was allowed to stand for 2 h at 4 °C. The suspension was then centrifuged at 14,000 rpm for 30 min at 4 °C. The resulting supernatant was collected, frozen in aliquots, and used for analysis of pro- and proteinases. Protein contents of enzyme solutions were determined using the Lowry's method (Lowry et al., 1951), with BSA fraction V as a standard.

### 2.6. Trypsinogen, chymotrypsinogen and proteinases assay

Trypsinogen, chymotrypsinogen and/or *H. armigera* gut trypsinogen, and chymotrypsinogen activation, total gut pro- and proteinases, and trypsin activities were measured by trypsinogen assay (Perlmann and Lorand, 1970; Bergmeyer et al., 1974), chymotrypsinogen assay (Rick, 1974), azo-caseinolytic (Brock et al., 1982) assay, and BA*p*NA assay (Erlanger et al., 1964), respectively. Activating reaction mixtures were prepared in 2 mL of 1 M calcium chloride, 38 mL of 400 mM Tris–Cl buffer (pH 8) at 37 °C, and 2 mL of trypsin enzyme solution, and mixed gently by swirling. Total trypsin activity of trypsinogen and/or HaGPP were measured at zero time, and then 0.1 mL of 5 mg/mL of trypsinogen and/or HaGPP extract was added to 1 mL of the activating mixture, and incubated at 37 °C for 30 min, and trypsin assay carried out. Suitable blanks were kept and equilibrated at 37 °C. Observations were recorded at $A_{253\ nm/min}$ using maximum linear rate for both the test and blank. One trypsinogen unit was defined as one BAEE unit that produces $A_{253\ nm}$ of 0.001 per minute, with BAEE as a substrate at 37 °C (pH 8) in a reaction volume of 3.2 mL (1 cm light path). The *H. armigera* gut chymotrypsinogen isoforms activity was measured by the chromogenic substrate BTEE at 37 °C, pH 7.8 using continuous spectrophotometer rate determination method. In a 3 mL reaction mixture, the final assay concentrations were 38 mM Tris, 0.55 mM BTEE, 30% (v/v) methanol, 53 mM calcium chloride, 0.03 mM HCl, 0.48 μg trypsin, and 50 μg of chymotrypsinogen and/or HaGPP. Tris buffer, substrate BTEE, and $CaCl_2$, were pipetted into two tubes separately. For the test, 0.1 mL activating mixture was added in one tube, while for the blank, 0.1 mL of HCl solution was added in another tube. The increase in absorbance maxima at $A_{256\ nm/min}$ was recorded for 5 min to obtain maximum linear rate for both, the test and the blank. One chymotrypsinogen activity unit was defined as one unit of enzyme that hydrolyzed 1.0 μmol of BTEE per minute at 37 °C (pH 7.8).

For the azo-caseinolytic assay, 60 μL (diluted *H. armigera* gut enzyme) was added to 200 μL of 1% azocasein (in 0.2 M glycine–NaOH buffer, pH 10.0), and incubated at 37 °C for 30 min. The reaction was terminated by the addition of 300 μL of 5% TCA. After incubation at room temperature for 30 min, the tubes were centrifuged at 10,000 rpm for 10 min and an equal volume of 1 N NaOH was added to the supernatant. The activity was estimated by measuring the OD at 450 nm. One proteinase unit was defined as the amount of enzyme that increased the absorbance by 1 OD under the given assay conditions. The *H. armigera* gut extracts were treated with trypsin (0.0001%), followed by azo-caseinolytic assay for determining the activation of pro-proteinase isoforms. Activities of trypsin isoforms of HaGP were estimated using the chromogenic substrate *N*-α-benzoyl-DL-arginyl-*p*-nitroanilide (BA*p*NA). For trypsin assay, 150 μL of diluted *H. armigera* gut extract enzyme was added to 1 mL of 1 mM BA*p*NA (in 0.2 M glycine–NaOH, pH 10.0) and incubated at 37 °C for 10 min. The reaction was terminated by the addition of 200 μL of 30% acetic acid; the OD was measured at 410 nm. One unit of proteinase activity was defined as the amount of enzyme that caused an increase of 1 unit of optical density at 410 nm due to the release of *p*-nitroaniline.

### 2.7. Electrophoretic determination of activation of gut pro-proteinases

Midgut pro-proteinases inhibition was determined on non-denaturing polyacrylamide (pH 8.8) (Laemmli, 1970). The gut contents of each group of larvae were homogenized separately in 500 μL of 0.2 M glycine–NaOH buffer, pH 10.0. The proteins in these homogenates were precipitated with 1 mL cold acetone and stored for 2 h at −20 °C. The acetone-precipitated proteins were centrifuged at 10,000 rpm for 30 min at 4 °C, and then redissolved in 100 μL of buffer, and the protein solution was used for electrophoretic separation.

Visualization of HaGP isoforms and activation of HaGPPs after 10% native-PAGE was carried out using the gel X-ray film contact print technique (Pichare and Kachole, 1994). Electrophoresis was performed at room temperature at a constant current of 30 mA till the tracking dye front reached at the bottom of the resolving gel (in approximately 4–6 h). The gel was removed and gently shaken at 37 °C for 10 min in 0.2 M glycine–NaOH buffer, pH 10.0. For proteinase activity visualization, the gel was overlaid on X-ray film for 30 min (Pichare and Kachole, 1994; Harsulkar et al., 1998). Three prints were taken subsequently and the results compared by visualization of proteinase activity. Then, HaGPPs isoforms were activated by immersing the gel in 0.0001% trypsin solution (0.2 M glycine–NaOH, pH 10.0). After activation, excess trypsin was removed. The gel was equilibrated in the same buffer solution, and then overlaid on unprocessed X-ray film. After 30 min (the same exposer time period was maintained for visualization of HaGP and activation of HaGPPs), the gel was removed and the X-ray film washed with tap water to observe the proteinase activity and activated proteinase bands as hydrolyzed gelatin. The X-ray film was developed and then contact printed.

### 2.8. Electrophoretic detection of HaGPPs activation on treatment of HaGP

The *H. armigera* larvae fed on non-host plant PIs incorporated in chickpea (PIs removed) diet were analyzed for the activation of HaGPPs by treating individual partially purified HaGPs. Approximately 0.02 U activity of partially purified individual HaGPs was used for analysis of solution assay as well as *in-gel* activation of HaGPPs. Gut extract of the larvae fed on *D. alba ness* PIs was separated on 10% native-PAGE. After electrophoresis, the gel was incubated in activation buffer, followed by 0.2 M glycine–NaOH buffer, pH 10.0, and placed on an undeveloped X-ray film. The gel and the film were incubated at 37 °C in a water bath. The appearance of activated proteinase bands on X-ray film was monitored visually. The film was then rinsed with tap water or placed in a water tray and shaken gently to remove the hydrolyzed

**Table 1**
*In vivo* inhibition of HaGPs and further *in vitro* activation of HaGPPs fed on host and non-host plant PIs. Azocasein and BA*p*NA were used as substrates to measure the *in vivo* inhibition of total proteinase and trypsin isoforms activities, respectively. *In vitro* activation of pro-proteinases and trypsinogen isoforms were measured using azocasein and BAEE as substrates, respectively, in activation buffer as described in Section 2.

| Host and non-host plant | Inhibition of HaGPs (%) mean ± SE ($n = 3$) | | Activation of HaGPPs (%) mean ± SE ($n = 3$) | |
|---|---|---|---|---|
| | Proteinase | Trypsin | Pro-proteinase | Trypsinogen |
| Host plant | | | | |
| *Cicer arietinum* | 16.02 ± 1.00 | 11.48 ± 2.61 | ND | ND |
| *Cajanus cajan* | 8.02 ± 4.35 | 10.62 ± 1.20 | ND | ND |
| Non-host plant | | | | |
| Group I | | | | |
| *Datura alba ness* | 91.33 ± 0.87 | 80.02 ± 0.31 | 26.04 ± 1.48 | 42.97 ± 1.31 |
| *Psophocarpus tetragonolobus* | 87.98 ± 1.43 | 88.61 ± 1.85 | 24.17 ± 1.62 | 34.92 ± 1.58 |
| *Pongamia pinnata* | 85.03 ± 1.35 | 83.94 ± 0.83 | 20.25 ± 1.99 | 25.16 ± 1.93 |
| *Mucuna pruriens* | 83.69 ± 0.58 | 84.23 ± 0.64 | 21.77 ± 1.28 | 21.34 ± 1.80 |
| *Capsicum annuum* | 83.56 ± 1.43 | 65.68 ± 3.26 | 23.21 ± 1.16 | 33.29 ± 1.07 |
| *Nigela sativa* | 81.84 ± 2.17 | 86.28 ± 0.33 | 19.95 ± 1.14 | 22.55 ± 3.81 |
| Group II | | | | |
| *Murraya koenigii* | 73.23 ± 2.80 | 73.89 ± 3.01 | 14.44 ± 0.42 | 15.01 ± 0.51 |
| *Acacia nilotica* | 71.53 ± 1.87 | 53.78 ± 2.38 | 11.90 ± 0.65 | 27.81 ± 0.95 |
| *Vigna unguiculata* | 70.94 ± 1.62 | 74.97 ± 2.46 | 13.19 ± 0.34 | 13.57 ± 0.58 |
| *Hordeum vulgare* | 70.01 ± 1.70 | 67.02 ± 2.94 | 14.58 ± 0.61 | 12.37 ± 1.27 |
| *Hygrophila schulli* | 65.75 ± 1.56 | 69.90 ± 0.55 | 14.23 ± 0.51 | 11.50 ± 0.24 |
| *Glycine max* | 61.54 ± 2.38 | 70.60 ± 1.13 | 18.78 ± 3.71 | 30.83 ± 2.30 |
| *Trigonella foenum-graecum* | 61.05 ± 1.74 | 62.96 ± 1.80 | 10.81 ± 0.20 | 12.47 ± 0.44 |
| *Momordica charantia* | 53.48 ± 1.90 | 58.98 ± 0.74 | 12.62 ± 1.29 | 12.37 ± 1.19 |
| *Blepharis edulis* | 50.96 ± 2.50 | 44.51 ± 1.47 | 11.68 ± 0.47 | 19.43 ± 0.31 |
| *Psoralea corywfolia* | 42.68 ± 2.67 | 43.61 ± 3.18 | 11.61 ± 0.57 | 12.94 ± 0.86 |
| *Achyranthes aspera* | 38.59 ± 2.48 | 34.83 ± 7.42 | 16.77 ± 1.61 | 23.96 ± 2.08 |
| *Foeniculum vulgare* | 29.43 ± 5.29 | 40.08 ± 3.02 | 19.44 ± 0.59 | 18.59 ± 0.31 |
| Group III | | | | |
| *Solanum nigrum* | 50.65 ± 4.19 | 52.92 ± 2.47 | ND | ND |
| *Penganum harmala* | 24.85 ± 0.42 | 33.43 ± 1.67 | ND | ND |

ND: not detectable.

gelatin. The gel was rinsed in 0.2 M glycine–NaOH, pH 10.0, and placed on another film, with opposite side of the gel in contact with the film. For comparison of sensitivity of detection of HaGPPs activation using X-ray film, a gel containing triplicate samples was cut into three pieces after electrophoresis and processed under similar conditions.

The data were subjected to analysis of variance to compare the significance of differences between the treatments using *F*-test at $P \leq 0.01$.

## 3. Results

### 3.1. In vivo inhibition of HaGP isoforms by host and non-host plant PIs

Inhibitory potential of host and non-host plant PIs was analyzed against HaGP and trypsin isoforms by solution assay (Table 1). Among the non-host plant group (I), *D. alba ness* exhibited the highest inhibition of HaGP (91.33%), while *P. tetragonolobus* showed the highest inhibition of trypsin isoforms (88.61%). In the same group, other non-host plant species, viz., *Pongamia pinnata*, *Mucuna pruriens*, *C. annuum*, and *Nigela sativa* also exhibited maximum inhibition of HaGP and trypsin isoforms. In group II, all non-host plant species showed high to moderate levels of inhibition of gut proteinase and trypsin activity, except those of *Psoralea corywfolia*, *Achyranthes aspera*, and *Foeniculum vulgare*, which resulted in comparatively low inhibition (Table 1). The PIs from the host plants, viz., *Cicer arietinum* and *Cajanus cajan* showed very low inhibition of HaGP (16.02% and 8.02%), and trypsin isoforms (11.48% and 10.62%) of *H. armigera*, respectively.

The total *H. armigera* gut proteinase activity was recorded in at least 10 isoforms, of which four were the major proteinases (HaGPs 2, 5, 7, and 9), four were relatively important, while the remaining two were minor [Fig. 1(A)]. HaGPs 5 and 7 showed the highest activity, while HaGPs 4 and 8 exhibited moderate level of activity. HaGP 10 showed the lowest activity. Proteinase isoforms recorded in the resolving gel were categorized into three groups on the basis of mobility. HaGPs 9 and 10 were closer and fast-moving bands, while HaGPs 1 and 2 exhibited very low mobility. HaGPs 8 and 10 could be detected by increasing the overlay time of gel on X-ray film (35–40 min at 37 °C). No proteinase activity band was observed in stacking gel when the total *H. armigera* gut proteinase activity was resolved. Electrophoretic visualization of *in vivo* inhibition of HaGP isoforms by host and non-host plant PIs is shown in Fig. 1(B). Larvae fed on non-host plant PIs showed stunted growth, and the HaGPs activity was also inhibited significantly. Among the non-host plants, *D. alba ness* showed total inhibition of HaGPs [Fig. 1(B); lane 7]. *Glycine max*, *P. tetragonolobus*, and *Vigna unguiculata* PIs also inhibited the gut proteinase activity, except that of HaGP 5 [Fig. 1(B); lanes 9, 19, and 22]. Non-host plants such as *A. aspera* and *F. vulgare* showed low inhibition in azocasein assay, but high inhibition of HaGP activity was detected on X-ray film [Fig. 1(B); lanes 4 and 8]. *M. charantia*, *Murraya koenigii*, and *P. corywfolia* resulted in increased expression of HaGPs 4, 5, 6, 7, and 8 [Fig. 1(B); lanes 14, 15, and 16]. *Blepharis edulis* showed partial inhibition, but low- and fast-moving proteinase bands were not observed on the X-ray film [Fig. 1(B); lane 5]. However, *Penganum harmala* did not show much inhibitory activity in solution assay (Table 1), but slow-moving bands (HaGPs 6, 7, 8 bands) were not visualized on the X-ray film. *Acacia nilotica*, *M. pruriens*, and *P. pinnata* showed maximum inhibition in azocaseinase and BA*p*NAase assay, and HaGPs 5 and 6 were observed on X-ray film [Fig. 1(B); lanes 1, 13, and 18]. *Hordeum vulgare*, *Solanum nigrum*, and *Trigonella foenum-graecum* inhibited slow-moving bands, but did not affect the fast-moving bands [Fig. 1(B); lanes 10, 20, and 21]. Non-host PIs showed high inhibitory potential towards the HaGPs activity, while host plant PIs were weak inhibitors of HaGPs.

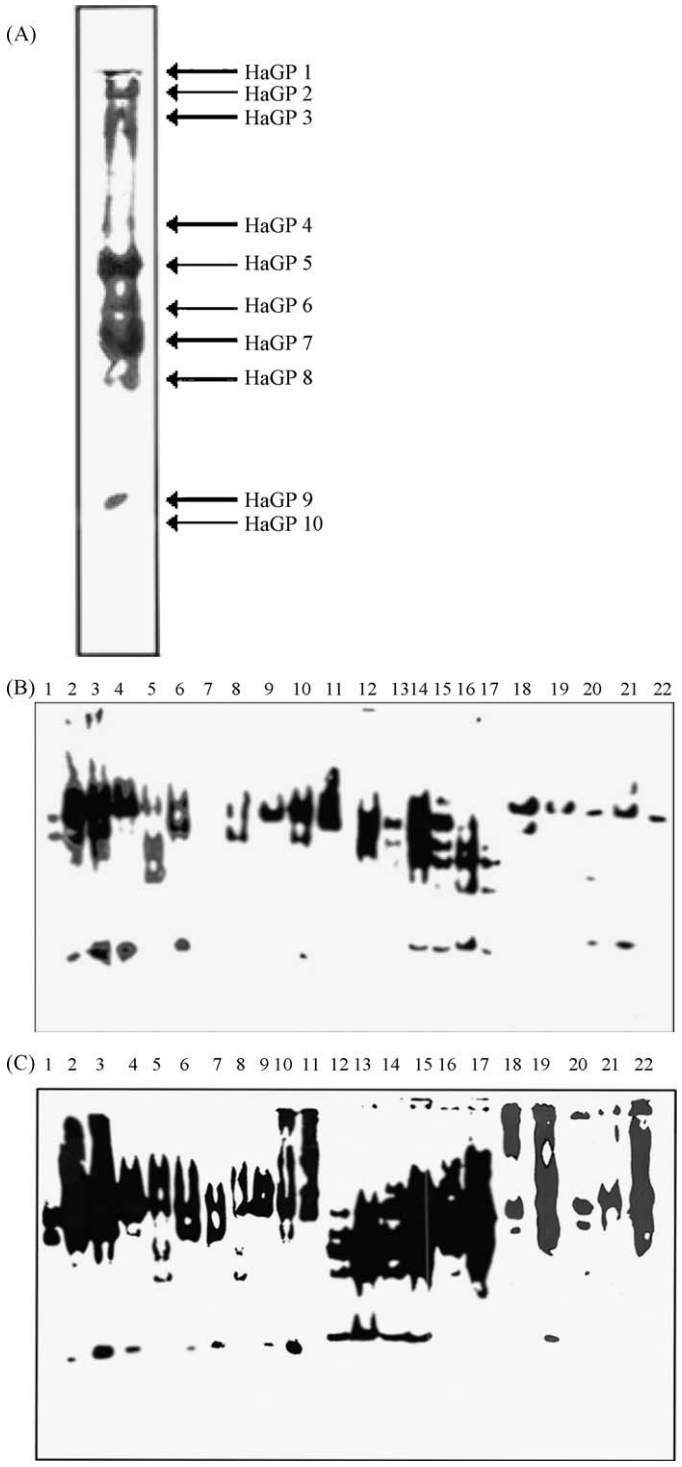


**Fig. 1.** Gut proteinase profile of *H. armigera* fed on chickpea (PIs removed) based artificial diet (A). Approximately 0.02 U activity of HaGP was used for visualization of gut proteinase profile. *In vivo* inhibition of HaGPs isoforms (B) and *in vitro* activation of HaGPP isoforms (C) visualized by gel X-ray film contact print method. High activity units of the *H. armigera* larvae fed on host and non-host plant PIs gut extracts were loaded on 10% native-PAGE and the gel processed for *in-gel* activation as described in Section 2. Lane 1, *Acacia nilotica*; lane 2, *Cajanus cajan*; lane 3, *Cicer arietinum*; lane 4, *Achyranthes aspera*; lane 5, *Blepharis edulis*; lane 6, *Capsicum annuum*; lane 7, *Datura alba ness*; lane 8, *Foeniculum vulgare*; lane 9, *Glycine max*; lane 10, *Hordeum vulgare*; lane 11, *Penganum harmala*; lane 12, *Hygrophila schulli*; lane 13, *Mucuna pruriens*; lane 14, *Momordica charantia*; lane 15, *Murraya koenigii*; lane 16, *Psoralea corywfolia*; lane 17, *Nigela sativa*; lane 18, *Pongamia pinnata*; lane 19, *Psophocarpus tetragonolobus*; lane 20, *Solanum nigrum*; lane 21, *Trigonella foenum-graecum*; and lane 22, *Vigna unguiculata*.

### 3.2. Activation and visualization of HaGPPs in insects fed on host and non-host plant PIs

The effect of host and non-host plant PIs on *in vivo* inhibition of HaGPP was studied by activation of pro-proteinase and trypsinogen isoforms by azocasein and BAEE assay, respectively (Table 1). The larvae fed on diets with *D. alba ness* PIs showed highest inhibitory activity against HaGPs, and also resulted in *in vivo* inhibition of HaGPPs activation. Activation of HaGPPs by trypsin resulted in 26.04% activation of HaGPPs and 42.97% activation of trypsinogen isoforms. *P. tetragonolobus* also exhibited high inhibitory potential towards HaGPs, and resulted in 24.17% inhibition of HaGPPs activation and 34.92% inhibition of trypsinogen isoforms activation of *H. armigera* based on azocasein and BAEE assays, respectively (Table 1). Larvae fed on *C. annuum*, *M. pruriens*, *P. pinnata*, and *N. sativa* showed high inhibitory potential towards HaGPs, and also resulted in 23.21%, 21.77%, 20.25%, and 19.95% activation of HaGPPs in *in vitro* assay; and 33.29%, 21.34%, 25.16%, and 22.55% activation of trypsinogen isoforms, respectively (Table 1). Larvae fed on non-host plant PIs from *P. harmala* and *S. nigrum* did not result in inhibition of HaGPP and trypsinogen isoform. Interestingly, *F. vulgare* showed low inhibitory effect on HaGPs, but inhibited the HaGPPs (19.44%) and trypsinogen isoforms (18.59%).

Activation of *H. armigera* gut pro-proteinases after feeding on host and non-host plant PIs revealed that most of proteinases were inhibited and, further activation of pro-proteinases showed either increased activity or activated proteinase bands on X-ray film [Fig. 1(C)]. *In vitro* activation of HaGPPs in insects fed on *D. alba ness* showed that four proteinases were activated (HaGPs 4, 5, 6, and 9) as visualized on X-ray film [Fig. 1(C); lane 7]. Larvae fed on *P. tetragonolobus* and *M. pruriens* showed high activation of HaGPPs, but the activated proteinases merged and formed a smear [Fig. 1(C); lanes 19 and 13]. *Capsicum annum* fed larvae showed activation of major proteinase bands, and HaGPs 4 and 5 were activated [Fig. 1(C); lane 6], but no activation was observed in slow- or fast-moving bands. Larvae fed on *A. nilotica* showed increased activity of HaGPs 5 and 6 [Fig. 1(C); lane 1], while *A. aspera* showed activated isoforms of HaGPs 4, 5, and 6 [Fig. 1(C); lane 4]. *F. vulgare* fed larvae showed activation of HaGPs 7 and 8, while *G. max* exhibited activation HaGPs 4 and 9 [Fig. 1(C); lanes 8 and 9]. *H. vulgare* fed larvae showed activation of slow- and fast-moving HaGPP bands [Fig. 1(C); lane 10]. Interestingly, larvae fed on *P. harmala* and *S. nigrum* resulted in activation of slow-moving bands [Fig. 1(C); lanes 11 and 20], but no activation was observed in substrate assays (Table 1). *Hygrophila schulli* fed larvae showed activation of fast-moving bands, while *M. charantia*, *M. koenigii*, *P. corywfolia*, *N. sativa*, *P. pinnata*, *T. foenum-graecum*, and *V. unguiculata* fed larvae showed activation of major pro-proteinase bands as well as slow-moving HaGPPs [Fig. 1(B); lanes 12, 14, 15, 16, 17, 18, 21, and 22]. The results were also corroborated by substrate assays. The studies indicated that non-host plant PIs are good candidates as inhibitors of the HaGPs as well as HaGPPs, however, *H. armigera* larvae fed on host plant PIs did not show the activation of HaGPPs and trypsinogen isoforms, because they were weak inhibitors of HaGPs.

### 3.3. Activation of gut pro-proteinases during the development of H. armigera

Azocaseinase (HaGPPs), BAEEase, and BTEEase activities were greater in *H. armigera* larvae fed on diets with PIs from *D. alba ness* than that on *P. tetragonolobus* PIs (Table 2). Proteolytic activities were barely detected in first-instar larvae, and it was difficult to measure inhibition and activation of HaGPPs (data not shown). In second-instar larvae, low total pro-proteinase activation was

**Table 2**
*In vitro* activation from HaGPPs in different instars of *H. armigera* larvae fed on non-host plant PIs of *Datura alba ness* and *Psophocarpus tetragonolobus*. Pro-proteinases, trypsinogen, and chymotrypsinogen isoforms activation were measured using azocasein, BAEE, and BTEE as substrate, respectively.

| Larval instar | Activation of pro-proteinases (U/g of gut tissue) mean ± SE ($n$ = 3) | | |
|---|---|---|---|
| | Azocaseinase | BAEEase | BTEEase |
| Second | | | |
| A | 5.66 ± 0.52 | ND | ND |
| B | 4.71 ± 0.86 | ND | ND |
| Third | | | |
| A | 12.51 ± 0.14 | 48.07 ± 1.29 | 0.371 ± 0.010 |
| B | 8.09 ± 0.68 | 38.45 ± 1.08 | 0.306 ± 0.015 |
| Fourth | | | |
| A | 16.85 ± 0.55 | 71.54 ± 1.29 | 0.355 ± 0.010 |
| B | 11.80 ± 0.35 | 52.66 ± 0.17 | 0.277 ± 0.029 |
| Fifth | | | |
| A | 20.52 ± 0.40 | 76.40 ± 2.50 | 0.394 ± 0.006 |
| B | 15.18 ± 0.17 | 59.84 ± 2.35 | 0.343 ± 0.018 |

ND: not detectable; A: *Datura alba ness*; and B: *Psophocarpus tetragonolobus*.

detected in larvae fed on non-host plant PIs, but BAEEase and BTEEase activations were not detected. Activation of HaGPPs increased significantly in the third-instar larvae, while maximum activation was observed in fifth-instar larvae. Activity declined drastically in the sixth-instars (data not shown). Total pro-proteinase (azo-caseinolytic) and trypsinogen isoforms (BAEEase) showed significantly greater activation in diets with non-host plant PIs from *D. alba ness* and *P. tetragonolobus*. However, very low chymotrypsinogen isoform (BTEEase) activation was detected in third-, fourth-, and fifth-instars (Table 2). The gut extract of different instars of *H. armigera* fed on non-host plant PIs from *D. alba ness* and *P. tetragonolobus*, and loaded on 10% native-PAGE showed differential *in vivo* inhibition of HaGPs, followed by *in vitro* activation of HaGPPs [Fig. 1(A and B)]. Third-instar larvae showed the presence of HaGPs 2, 4, 5, 6, 7, 8, and 9, which were inhibited by *D. alba ness* PIs, except HaGP 5 (visualized on X-ray film) [Fig. 2(A); lane 1; *in vivo* inhibition].

High proteinase activity units were loaded for visualization of major and minor bands of third-, fourth-, and fifth-instar larvae. The HaGPs 5 and 9 were not inhibited. HaGP 6 was observed on X-ray film in the fifth-instar larvae [Fig. 2(A); lanes 2 and 3; *in vivo* inhibition]. Significant increase in activation of HaGPs in third-, fourth-, and fifth-instar larvae was observed *in gel* X-ray film contact print technique [Fig. 2(A); lanes 1, 2, and 3; *in vitro* activation]. In third-instar larvae, HaGPs 3, 4, 6, and 9 were activated; while in the fourth-instars, HaGPs 3, 4, 6, and 7 were activated. Fifth-instar larvae showed maximum activation of all HaGPPs, but HaGPs 8 and 10 bands were not detected. Larvae fed on *P. tetragonolobus* were also assayed for *in vivo* inhibition of HaGPs and *in vitro* activation of HaGPPs [Fig. 2(B)]. When high activity units were loaded on 10% native-PAGE, *P. tetragonolobus* showed considerable specificity for inhibition of HaGPs as compared to *D. alba ness*. *In vivo* inhibition results showed that third-instar larvae exhibited inhibition of HaGPs 4, 6, 7, and 9, but not of HaGPs 2 and 5. In fourth-instars, HaGPs 2, 3, and 5 were inhibited, while in fifth-instars, HaGP 6 was not inhibited by plant PIs [Fig. 2(B); lanes 2 and 3; *in vivo* inhibition]. *In vitro* activation of HaGPPs on X-ray film visualized HaGPs 2, 4, and 5 in third-instar larvae. Fourth- and fifth-instar larvae showed similar profiles, but HaGPs 4 and 6 were activated [Fig. 2(B); lanes 1, 2, and 3; *in vitro* activation].

### 3.4. Activation of HaGPPs on treatment of partially purified HaGPs

To assess activation of pro-proteinase in crude *H. armigera* gut extract in larvae fed on non-host plant PIs, individual electrophoretically purified HaGPs from the fifth-instar were determined by azo-caseinolytic and BAEE assays (Fig. 3). Approximately 0.02 U activity of each proteinase was individually treated with *H. armigera* gut pro-proteinases of larvae fed on *D. alba ness*. Of all HaGP isoforms, HaGP 5 showed 19% and 32% activation; while HaGP 9 showed 17% and 27% activation of total pro-proteinase and trypsinogen isoforms, respectively (Fig. 3). HaGPs 5 and 9 are trypsin-like major proteinase isoforms, which showed specificity towards BA*p*NA (data not shown).

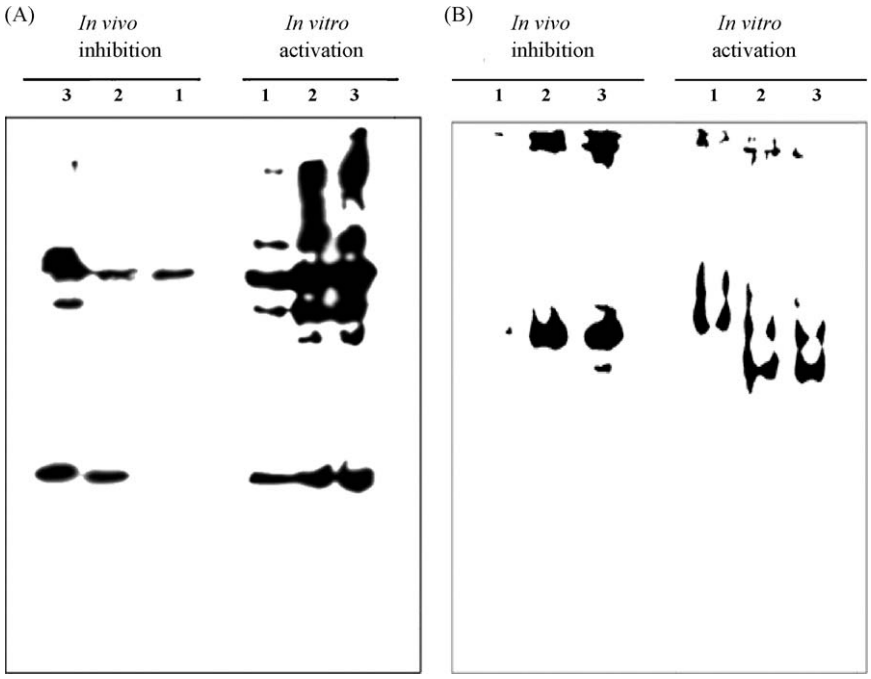


**Fig. 2.** *In vivo* inhibition of HaGPs and *in vitro* activation of HaGPPs of *H. armigera* larvae fed on non-host plant PIs; *Datura alba ness* (A), *Psophocarpus tetragonolobus* (B). High amounts of proteinase and pro-proteinase activity units were loaded to visualize the maximum inhibition and activation bands on X-ray film during larval development. Third-instar (lane 1), fourth-instar (lane 2), and fifth-instar (lane 3).

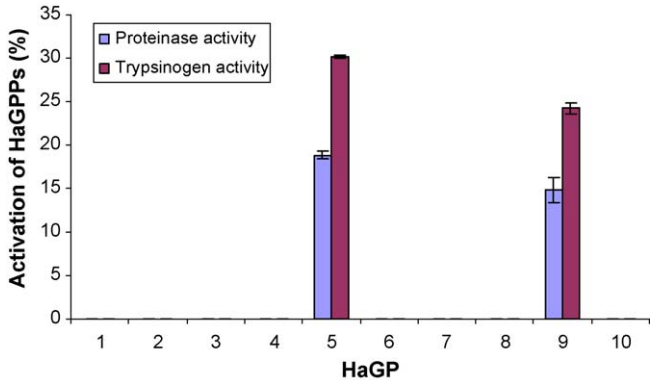


**Fig. 3.** Effect of partially purified individual *H. armigera* gut proteinase(s) on activation of HaGPPs. Total proteolytic activation and trypsinogen isoforms activation of HaGPPs were determined by azocasein and BAEE, respectively.

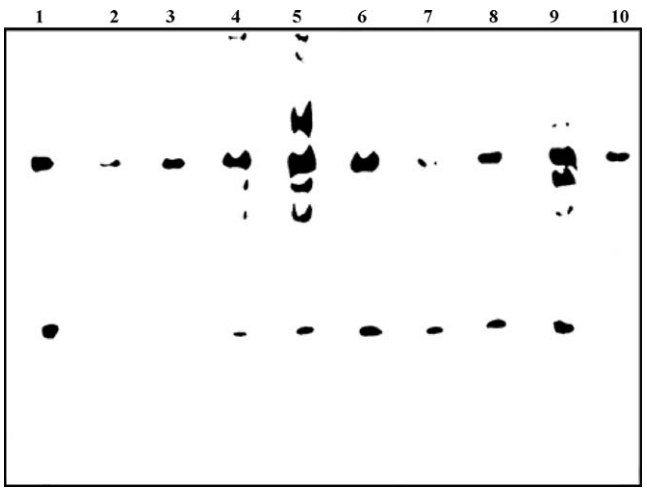


**Fig. 4.** Effect of individual partially purified HaGP on gut extract of *H. armigera* larvae fed on non-host plant PIs from *Datura alba ness*. High BAEE activity units of larvae fed on PIs gut extract was loaded on 10% native-PAGE, and *in-gel* activation of HaGPPs was checked on the treatment of electrophoretically purified HaGPs, separately. The activation profile of HaGPPs was visualized by gel X-ray film contact print technique. Lanes 1–10 represent treatments of individual HaGPs 1–10, respectively.

To observe activation of pro-proteinase(s) of *H. armigera* fed on non-host plant, PIs from *D. alba ness* fed larval gut extract was used. The experiment was carried out by the treating individual electrophoretically purified HaGP on HaGPPs, and activated gut proteinases visualized by *in-gel* activation method on X-ray film (Fig. 4). HaGPs 5 and 9 showed activation of pro-proteinases, however, HaGP 5 activated the inactive isoforms of HaGPs 1, 2, 4, 6, and 7; while HaGP 9 activated inactive isoforms of HaGPs 4, 6, and 7 (Fig. 4; lanes 5 and 9). Interestingly, HaGP 4 did not activate HaGPP in azocaseinase or BAEEase assay, but activation was observed on X-ray film (activated HaGP 1, 6, and 7 isoforms). HaGPs 1, 6, 7, and 8 showed active proteinase isoforms of 5 and 9, but did not exhibit the activation of HaGPP. Similarly, HaGPs 2, 3, and 10 showed active isoform of HaGP 5, but no activation band was observed on X-ray film (Fig. 4; lanes 2, 3, and 10, respectively).

### 3.5. Effect of host and non-host plant PIs on growth and development of H. armigera

Development of *H. armigera* larvae fed on host and non-host plant PIs incorporated into artificial diet was also evaluated for activity under *in vivo* conditions (Table 3). The standard artificial diet used in the feeding assay was chickpea (PIs removed) flour based diet, supplemented with vitamins and salts. Larvae were

**Table 3**
*Helicoverpa armigera* larvae fed on host and non-host plant PIs for evaluation of weight gain and growth rate. The experiment was carried out in three replications and each replication contains thirty larvae.

| Sample name | Initial weight (mg) | Final weight (mg) | Weight gain (mg) | Growth rate (%) |
|---|---|---|---|---|
| Host plant | | | | |
| *Cicer arietinum*[*] | 22.24 | 426.80 | 404.60 | 1902.50 |
| *Cicer arietinum* | 25.50 | 377.40 | 351.90 | 1436.50 |
| *Cajanus cajan* | 51.27 | 369.20 | 317.90 | 723.90 |
| Non-host plant | | | | |
| Group I | | | | |
| *Datura alba ness* | 25.18 | 39.10 | 13.70 | 53.80 |
| *Psophocarpus tetragonolobus* | 23.32 | 94.00 | 71.40 | 334.30 |
| *Pongamia pinnata* | 22.57 | 73.20 | 50.20 | 254.70 |
| *Mucuna pruriens* | 26.26 | 70.60 | 44.30 | 181.00 |
| *Capsicum annuum* | 26.60 | 56.70 | 29.70 | 102.70 |
| *Nigela sativa* | 24.98 | 97.10 | 72.20 | 294.60 |
| Group II | | | | |
| *Murraya koenigii* | 20.59 | 72.80 | 52.20 | 275.30 |
| *Acacia nilotica* | 30.72 | 143.10 | 112.50 | 381.90 |
| *Momordica charantia* | 96.11 | 389.00 | 292.90 | 326.80 |
| *Vigna unguiculata* | 38.63 | 301.50 | 262.90 | 696.40 |
| *Hordeum vulgare* | 23.65 | 92.10 | 68.30 | 308.00 |
| *Hygrophila schulli* | 24.74 | 85.80 | 61.10 | 274.30 |
| *Glycine max* | 60.95 | 330.90 | 269.40 | 1296.50 |
| *Trigonella foenum-graecum* | 30.34 | 298.00 | 267.90 | 933.70 |
| *Momordica charntia* | 96.11 | 389.00 | 292.90 | 326.80 |
| *Blepharis edulis* | 49.00 | 280.70 | 231.70 | 469.00 |
| *Psoralea corywfolia* | 38.51 | 373.50 | 334.50 | 876.60 |
| *Achyranthes aspera* | 19.93 | 319.30 | 299.30 | 1047.30 |
| *Foeniculum vulgare* | 28.98 | 316.60 | 287.40 | 976.00 |
| Group III | | | | |
| *Solanum nigrum* | 20.27 | 126.30 | 106.00 | 513.90 |
| *Penganum harmala* | 24.51 | 356.70 | 331.80 | 1352.40 |
| SE± | 1.74 | 10.73 | 10.34 | 43.14 |
| LSD at *P* 0.05 | 4.97[**] | 29.77[**] | 28.71[**] | 119.67[**] |

[*] PIs removed from the sample.
[**] Significant at $P \leq 0.01$.

**Fig. 5.** Development of *H. armigera* fed on diets with and without plant PIs. Larvae fed on control diet-containing chickpea (PIs removed) showing normal growth (upper row), while larvae fed on test diet-containing *Datura alba ness* PIs showing retarded growth (lower row).

released on diet ± inhibitors and on the control (chickpea, PIs removed) diet, and larval weights recorded at the time of releasing the larvae on the diet (Table 3). The *H. armigera* larvae reared on host plant, standard artificial diet, and non-host plants, e.g. *D. alba ness* PIs showed the normal and stunted growth of larvae, respectively (Fig. 5). Larval growth was significantly reduced by non-host plant PIs compared to the larvae fed on control diet (significant at $P < 0.01$) from the 7th or 8th day of larval feeding onwards (Table 3). As compared to control (*C. arietinum**), the host plant PIs from *C. cajan* and *C. arietinum* also showed reduction in weight gain.

Among the non-host plant PIs, *D. alba ness* resulted in highest stunted growth of *H. armigera* larvae, followed by group I and II plant species, viz., *C. annuum*, *M. pruriens*, *H. schulli*, *P. pinnata*, *M. koenigii*, *N. sativa*, *H. vulgare*, *P. tetragonolobus*, *A. nilotica*, and *B. edulis* (Table 3). *Solanum nigrum*, *V. unguiculata*, *P. corywfolia*, *T. foenum-graecum* showed moderate reduction in larval weights. Larvae fed on diets with non-host plant PIs such as *A. aspera*, *G. max*, and *P. harmala* resulted in low reduction in growth of *H. armigera* larvae.

## 4. Discussion

Although serine proteinase inhibitors may contribute to the defense of plants against invading organisms, the efficacy of a specific inhibitor depends upon the structural compatibility of the reactive site of the plant proteinase inhibitor with the structure-binding site of the proteinases in the target organism. However, trypsin-like isoforms present in the midgut of *H. armigera* may have undergone minor substitution at the binding site, resulting in moderate inhibitory interactions with PIs from the host and non-host plants. In the present investigation, we evaluated the physiological response of *H. armigera* larvae to the PIs from host and non-host plants under *in vivo* conditions. The PIs from the two host plants, viz., *C. arietinum* and *C. annum* were totally degraded by *H. armigera* gut proteinases. Giri et al. (1998) observed that *C. arietinum* defensive trypsin inhibitors (TIs) are degraded by *H. armigera* gut proteinases. Ability to overcome the effect of host plant PIs is of great significance for adaptation and survival of phytophagous insects. Jongsma et al. (1996) suggested that the non-host plants could be a potential source of effective inhibitors for the target insect pests in question, as the insect is not pre-exposed to the inhibitors. Transgenetically expressed PIs of non-host plants have been found to be effective against many insect species (Lawrence and Koundal, 2002). The non-host plant PIs are of dual benefit; they act against the proteinases of insect gut, and they also protect the host plant's defense proteins from proteolysis, thus giving the plants an edge over the insect pests. Harsulkar et al. (1999) reported several non-host plants of *H. armigera* as new sources of potent PIs. Continuous exposure of insects to different PIs might result in insect adaptation to any or all of the available defense mechanisms. Earlier studies have demonstrated that precursor proteins of Pin-II type inhibitors in various plants consist of 1–8 inhibitory repeat domains (IRD), which upon cleavage by endogenous proteinases release single inhibitor proteins that are active against one or multiple serine proteinases (Heath et al., 1995; Horn et al., 2005).

Non-host plant PIs have been extensively studied and used as a model to obtain insight of plant defenses against herbivore attack (Ryan, 1990; Jongsma et al., 1996; Ussaf et al., 2001). The present studies demonstrated the efficacy of non-host plant PIs against *H. armigera* larvae in feeding assays, which corresponds to their effectiveness as inhibitors of gut pro- and proteinases, as estimated by *in vivo* inhibition assays. Feeding bioassays with the non-host plant PIs provided valuable information about the role played by the activator enzymes in the digestive system. In the present study, non-host PIs from *D. alba ness*, *P. tetragonolobus*, *P. pinnata*, *M. pruriens*, *C. annuum*, and *N. sativa* inhibited more than 80% of the total proteolytic (azo-caseinolytic) activity of *H. armigera* larvae *in vivo*, while *in vitro* activation showed more than 20% pro-proteinase activity (Table 1). Many insect species have adapted to host plant PIs by synthesizing proteinases that are either insensitive to PIs (Broadway, 1995; Jongsma et al., 1995; Lawrence and Koundal, 2002) or have the capacity to degrade them (Michaud, 1997; Girard et al., 1998). Therefore, it is necessary to study the non-host plant PIs as potential sources to overcome the problem of insect adaptation to the defense mechanisms of the host plants. Non-host plant PIs showed high inhibitory potential against the HaGP activity, while host plant PIs were found to be weak inhibitors of HaGPs as well as HaGPPs.

Non-host plant, *D. alba ness* PIs showed highest inhibition of HaGPs as well as HaGPPs, followed by *P. tetragonolobus*. Giri et al. (2003) observed that *P. tetragonolobus* PIs, especially TIs have different binding potentials towards HaGP, although HaGP activity is trypsin-like; and *P. tetragonolobus* PIs might be good candidates for engineering resistance to *H. armigera* in host plants (Harsulkar et al., 1999). Mature seeds of *P. tetragonolobus* are known to contain several PIs, some of which are inhibitors of only trypsin and chymotrypsin, while others inhibit both types of proteinases (Shibata et al., 1986). *P. harmala* and *S. nigrum* failed to inhibit the activation of HaGPPs, while *F. vulgare* inhibited the activity of HaGPs, but showed low level of inhibition of HaGPPs activation. Activation of pro-proteinases resulted in high concentration of proteinases, and the isoforms moving closely merged and formed a smear. This is one of the disadvantages of GXCP. In the present studies, larvae fed on artificial diet without non-host PIs showed normal growth in contrast to the inhibited growth of the larvae fed on artificial diet impregnated with non-host plant PIs. The larvae fed on diets with non-host PIs showed a decrease in gut proteinase activity. *In vivo* studies on the fate of non-host plant PIs, viz., *D. alba ness* and *P. tetragonolobus* in *H. armigera* guts indicated that these inhibitors reduced the larval growth efficiently. Quantitative analysis of *in vitro* activation of pro-proteinases of *H. armigera* revealed that *D. alba ness* possessed greater capability to inhibit pro-proteinases as compared to *P. tetragonolobus*. However, in second-instar larvae, pro- and proteinase activity was quite low, while fifth-instars showed the highest activity.

The columnar cells in the midgut epithelium are involved in absorption of digested food as well as secretion of enzymes. Digestive enzymes are secreted in secretory vesicles, and may become partly or entirely trapped in the intermicrovillar glycocalyx. These enzymes are initially an integral protein of the

membrane of small vesicles, which migrates to the cell microvilli, and at the microvilli, the digestive enzymes may be partly or entirely processed to become soluble inside the small vesicles, which bud laterally from the microvilli. The vesicles become solubilized, and release digestive enzymes into the lumen (Terra and Ferreira, 1994). However, the present studies indicated that the active isoforms present in the midgut lumen were involved in activation of its inactive isoforms and/or other digestive enzyme precursors. Recently, Liu et al. (2009) reported that trypsins and other serine endoproteases are the most important proteases in *H. armigera*, because of their key roles in food digestion and zymogen activation. A serine protease is present in the gut lumen, which activated zymogen phenol oxidase (PPO) of *Spodoptera litura* (F.) (Arora et al., 2009). Though *H. armigera* pro-proteinases were activated upon treatment of its active isoforms, the other biochemical parameters ($Ca^{2+}$, pH, and temperature) were also important for activation of zymogen proteinases of *H. armigera* (unpublished data). Partially purified trypsin isoforms of *H. armigera* activated its inactive isoforms as well as other pro-proteinases, and they were also responsible for autoactivation. The non-host plant, *D. alba ness* PIs resulted in greater activation of *H. armigera* gut pro-proteinase(s) as compared to *P. tetragonolobus*.

Strong inhibitors of gut proteinases *in vitro* do not necessarily retard larval growth and development (Edmonds et al., 1996). Insect feeding assays were therefore performed to assess the antibiosis exerted on *H. armigera* by the host and non-host plant PIs. Development of *H. armigera* larvae fed on plant PIs incorporated into chickpea (PI removed) based artificial diet was evaluated *in vivo*. Chickpea seeds contain Bowmann-Birk (BBI) and Kunitz (CaKPI) type proteinase inhibitors, in which BBIs are ineffective against the digestive proteinases, but CaKTI causes antagonistic effects on developing *H. armigera* larvae (Srinivasan et al., 2005a,b). The present studies indicated that larval growth and development were significantly reduced when the larvae were fed on diet with PIs from non-hosts. Reduced feeding of larvae was observed in case of PI-incorporated diet as compared to those fed on control diet. Ashouri et al. (1998) reported that oryzacystatin-I affected fertility and fecundity of *Perillus bioculatus* (F.). Ingestion of potent PIs adversely affected the protein intake at the larval stage, which caused developmental abnormalities and also reduced fertility and fecundity of the adults. However, starvation and added stress on gut proteinase expression system resulted in synthesis of new and/or higher amounts of proteinases and this could be one of the possible reasons for arrested growth and mortality of *H. armigera*. Several researchers have observed growth retardation and mortality with high PI doses in various insects (Jongsma et al., 1996; Stotz et al., 1999; Murdock and Shade, 2002; Tamhane et al., 2005). In the present studies, it was observed that the ingested PIs exerted physiological stress on the larvae and resulted in retarded growth. Amongst the non-host plant PIs, *D. alba ness* resulted in highest stunted growth and therefore, non-host plant PIs could be deployed in transgenic plants for enhancing the resistance to *H. armigera*.

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