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Protease Inhibitors in Wild Relatives of Pigeonpea against the Cotton Bollworm/Legume Pod Borer, *Helicoverpa armigera*

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ABSTRACT

Cotton bollworm/legume pod borer, *Helicoverpa armigera* is one of the most damaging pests worldwide. Because of the difficulties associated with chemical control of this pest, emphasis has been placed on developing transgenic plants with resistance to *H. armigera*. Since toxin genes from the bacterium, *Bacillus thuringiensis* (*Bt*) have been deployed on a large scale, there is need to scout for alternate genes which could be deployed alone or in combination with the *Bt* genes for pest management. Therefore, we evaluated the wild relatives of pigeonpea, which have shown high levels of resistance to this pest, for the protease inhibitors (PIs) under *in vivo* and *in vitro* inhibitions. Accessions belonging to *Cajanus albicans*, *C. cajanifolius*, *C. sericeus*, *Flemingia bracteata*, and *Rhynchosia bracteata* showed complete inhibition of *H. armigera* gut proteinases (HaGPs). Some of the *C. scarabaeoides* accessions (ICPW 116, 152, 278 and 280) exhibited partial inhibition at low concentrations of the PIs. All accessions of wild relatives of pigeonpea showed high to moderate level of inhibition at pH 7.8. Cultivated pigeonpea, ICPL 87 exhibited monomorphism in terms of trypsin inhibitor (TI) and chymotrypsin inhibitor (CTI) isoforms, contrary to the diverse inhibitory profiles of wild pigeonpeas. *Cajanus albicans*, *C. platycarpus*, *C. scarabaeoides*, and *R. bracteata* showed more number of TI and CTI bands than the cultivated pigeonpea. Protease inhibitor isoforms of wild relatives of pigeonpea showed significant variation in number, band pattern, and protein specificities towards trypsin, chymotrypsin, and *H. armigera* gut proteinases (HaGPs) as compared to the cultivated pigeonpea. The PIs from the wild relatives of pigeonpea showed considerable potential against the HaGPs, and could be considered as potential candidates for use in genetic transformation of crops for pest management, including *H. armigera*.

Keywords: Wild Relatives; Pigeonpea; Protease Inhibitors; *Helicoverpa armigera*; Transgenic Plants; Pest Management

1. Introduction

Pigeonpea [*Cajanus cajan* (L.) Millisp.] is a multipurpose grain legume grown by the resource poor farmers in the semi-arid tropics and subtropics. India produces more than 80% of the total production of pigeonpea [1]. It occupies an important position in human diet as a protein source, especially in the vegetarian population [2]. The most important constraints of pigeonpea production include *Fusarium* wilt, sterility mosaic disease, and the insect pests such as pod borer, *Helicoverpa armigera* (Hub.), spotted pod borer, *Maruca vitrata* (Geyer), pod fly, *Melanagromyza obtusa* (Malloch), pod bug, *Clavigralla* spp., Lima bean pod borer, *Etiella zinckenella* (Tr.), and the bruchids, *Callosobruchus chinensis* (F.). Amongst these, *H. armigera* is the most damaging pest of grain legumes, including pigeonpea, and causes losses valued over US $325 million annually [3,4]. Chemical control with insecticides is costly, and it has developed high levels of resistance to conventional insecticides [5]. Therefore, there is need to focus our attention on alternative methods of pest control. It is in this context that host plant resistance can play an important role in minimizing the extent of losses due to this pest. However, the levels of resistance to this pest in the cultivated germplasm are low to moderate [6], but the wild relatives of pigeonpea have shown high levels of resistance to this pest [7-9]. Transfer of insect resistance genes from the wild into cultivated species is a long term process, but certain genes that control the production and accumulation of compounds that affect the survival and development of

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insects can be utilized more effectively through gene cloning, and insertion into a desirable variety or hybrid released for cultivation by the farmers. Protease inhibitors are produced in the plants in response to biotic or abiotic stress and protect the plant against herbivores, including insect pests [10]. Their potential in the development of insect resistant plants using recombinant DNA technology has also been demonstrated [8].

Pigeonpea seeds contain proteinaceous inhibitors (PIs) of trypsin, chymotrypsin, and amylases [11-13] as well as phytolectins, and secondary metabolites [14-16], which serve as a defense mechanism against the herbivores. Pigeonpea PIs are Kunitz type PIs, having inhibitory activity against trypsin and chymotrypsin [16,17]. However, *H. armigera* has developed the ability to overcome the effect of host plant PIs either by producing a different suite of proteases or overproduction of certain proteases to overcome the adverse effects of host plant PIs [18]. However, PIs from the non-host plants have been found to be more effective against this pest [19]. It is likely the PIs from the wild relatives of pigeonpea, that have shown high levels of resistance to this pest, will be more effective as inhibitors of proteases in the insect gut. Therefore, the present studies were undertaken to assess the potential of PIs from the wild relatives of pigeonpea to identify species/accession with high PI activity for possible use in genetic transformation of crops for resistance to *H. armigera*.

2. Materials and Methods

2.1. Seeds of Wild Relatives of Pigeonpea

Twenty-nine accessions (germplasm lines kept in the genebank) belonging to 13 species (*Cajanus scarabaeoides*, *C. cajanifolius*, *C. sericeus* , *C. albicans*, *C. acutifolius*, *C. lineatus*, *C. platycarpus*, *Rhynchosia bracteata*, *R. aurea*, *Dunbaria ferruginea*, *Flemingia bracteata*, *F. stricta*, and *Paracalyx scariosa*) of wild relatives of pigeonpea were evaluated for their PI activity against the larvae of pod borer, *H. armigera*, along with two genotypes of cultivated pigeonpea, *Cajanus cajan* (ICPL 87—susceptible check, and ICPL 332—resistant check) [8,9]. The test material was planted under field conditions at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh, India. The seeds were sown on ridges 75 cm apart, and thinned to a spacing of 30 cm between the plants at 15 days after seedling emergence. Standard agronomic practices were followed for raising the crop (basal fertilizer—N:P:K: 100:60:40 kg·ha^{-1}). A fungicide spray (metalaxyl @ 1.0 kg·*ai*·ha^{-1}) was applied to control *Fusarium* wilt during the seedling stage. The crop was raised under rainfed conditions between June to October, but irrigated at monthly intervals between November to February during the postrainy period. Wooden pegs (1.5 m high) were used to provide support for *C. scarabaeoides* and *C. platycarpus* accessions, which have a creeping habit. The pods were collected from different accessions during December-January, thrashed, and the seeds used for assessing the PI activity under laboratory conditions.

2.2. Extraction of Seed Proteins

Mature seeds were washed with water, dried, and ground to a fine powder in a pestle and mortar. The seed powder was defatted with hexane and depigmented with acetone. Defatted seed powder was suspended in six volumes of distilled water containing 1% PVP and kept at 15˚C for 12 h for extraction of seed proteins. The suspension was centrifuged at 10,000 rpm for 30 min at 4˚C, and the supernatant was used for analysis of inhibitors of bovine trypsin and chymotrypsin. Protein content in the seed extracts was estimated by the method of Lowry *et al.* (1951) [20] using bovine serum albumin as a standard.

2.3. Detection of Protease Inhibitors by Dot-Blot Method

We used X-ray film method for the estimating serine protease inhibitor activity [21]. Trypsin and chymotrypsin solutions were prepared in 0.1 M Tris-HCl buffer, pH 7.8, to obtain a final concentration of 0.1 mg·ml^{-1}. Three varying concentrations of the enzyme and inhibitor 3:1, 1:1, and 1:3 (v/v), were prepared. The volume of the reaction mixture was adjusted with Tris-HCl buffer for trypsin and chymotrypsin, and glycine-NaOH buffer for *H. armigera* gut proteinases (HaGPs). The final volume was made upto 20 µl, and then spotted onto a strip of X-ray film. Spots were incubated for 20 min on X-ray film depending on the extent of gelatin hydrolysis. The film was washed with warm water. When the inhibitor is present, the trypsin/chymotrypsin did not degrade the gelatin on the X-ray film. When the inhibitor was absent, a clear zone formed at the site of sample application on the X-ray film. The reverse side of the film was cleared with trypsin/chymotrypsin, and the film scanned.

2.4. Proteinase Assay

The residual proteinase activity was estimated using casein as a substrate [22]. The proteinase-inhibitor mixture was added to 0.5 ml of 0.5% casein (in 0.2 M glycine-NaOH, pH 10.0) and kept at 37˚C for 20 min. The reaction was terminated by the addition of 750 µL of 5% trichloroacetic acid. After centrifugation at 10,000 rpm for 10 min, absorbance of the supernatant was checked at 280 nm. For every assay, suitable controls were co-in-

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cubated with the test samples. Trypsin-like activities were estimated using chromogenic substrate, benzoyl-arginyl *p*-nitroanilide (BA*p*NA) [23]. For trypsin assay, diluted enzyme (150 µL) 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, and the absorbance measured at 410 nm. One proteinase unit was defined as the amount of enzyme that increased absorbance by 1 OD under the given assay conditions.

2.5. Inhibition Potential of Wild Pigeonpea PIs against Gut the Proteinases of *H. armigera* at Different pH and Temperature Conditions

Inhibition potential of different PIs against gut proteinase activity of *H. armigera* was determined using BA*p*NA as a substrate. Activity assays were performed at pH 7.8 and pH 10.0. Double concentration of the gut extract was required to obtain equivalent units of BA*p*NAase activity at pH 7.8 than at pH 10.0. Five different concentrations of inhibitors were used to assess the potential of inhibitor for inhibiting HaGP. *In vitro* stability of wild pigeonpea PIs against HaGP was determined by pre-incubating the inhibitors with HaGP for 30 min, and for 3 h at 37˚C, and then assayed for their inhibitory activity towards HaGP.

2.6. Effect of Synthetic Protease Inhibitors on Gut Proteinase Activity

Nine chemical inhibitors viz., antipain, leupeptin, pefabloc, aprotinin, chymostatin, E-64, pepstatin, EDTA, and soybean trypsin inhibitor were used in the range of 1.8 µM to 10 mM concentrations for maximum inhibition of the enzyme in assays. Inhibitors were dissolved in water (antipain, pefabloc, EDTA, soybean trypsin inhibitor, and aprotinin) or DMSO (chymostatin) or methanol (pepstatin) or water/ethanol (1:1) (E-64) as per the manufacturer's instructions. For the inhibitor assay, suitable volume of seed extract of chemical inhibitor required for maximum inhibition of the enzyme was added to the gut proteinase extract and incubated at room temperature (27˚C) for 15 min. The residual proteinase activity was then estimated using casein as a substrate [22].

2.7. Electrophoretic Analysis of Wild Pigeonpea PIs

Pigeonpea seed extracts were analyzed by non-denaturing acidic and basic, and denaturing sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) in a vertical slab gel electrophoresis system. Acidic gel electrophoresis was carried out in 10% polyacrylamide gel using a cathodic discontinuous buffer system. Basic gel electrophoresis was carried out in 10% or 12% polyacrylamide gel by using Davis buffer system.

After electrophoresis, non-SDS gel was placed in 0.1 M Tris-HCl (pH 7.8) for 5 - 10 min for equilibration. The SDS-gel was washed three times (30 min each) with 2.5% Triton X-100 in 0.1 M Tris-HCl (pH 7.8) for a few minutes. After equilibration, the gel placed in 0.1 mg/ ml trypsin or in chymotrypsin in 0.1 M Tris-HCl (pH 7.8) for 5 - 10 min, rinsed briefly in the Tris-HCl buffer (pH 7.8), and placed on an exposed undeveloped X-ray film, or exposed and developed photographic paper. The gel and the film were placed in a tray and incubated at 37˚C in a water bath. The appearance of inhibitor bands on X-ray film was monitored visually. The X-ray film was then rinsed under tap water or placed in a water tray and shaken gently to remove hydrolyzed gelatin. The gel was rinsed in 0.1 M Tris-HCl (pH 7.8) briefly, and placed on another film with second side of the gel in contact with the film. For comparison of sensitivity of detection of inhibitory activity using film, a gel containing duplicate samples was cut into two pieces after electrophoresis and processed under similar conditions.

2.8. Statistical Analysis

The data were subjected to analysis of variance to compute the standard deviations from the mean for each assay.

3. Results and Discussion

At three concentrations of HaGPs and the inhibitor (3:1, 1:1 and 1:3), 18 accessions (ICPW nos 13, 14, 28, 29, 160, 68, 83, 90, 94, 125, 130, 137, 141, 281, 192, 202 and 214, and ICPL332) resulted in total inhibition of HaGP (**Table 1**). Accessions belonging to *C. albicans*, *C. cajanifolius*, *C. sericeus*, *F. bracteata*, and *R. bracteata* showed complete inhibition of HaGPs. Some of the accessions belonging to *C. scarabaeoides* (ICPW nos 116, 152, 278 and 280) also exhibited partial inhibition at low concentrations of PIs (3:1; enzyme:inhibitor).

Maximum HaGP inhibition by PIs of pigeonpea and its wild relatives was observed when different concentrations of protein (10 - 550 µg) were independently tested against the HaGP. Among the cultivated pigeonpea cultivars, the inhibition of HaGP was greater in the *H. armigera*-resistant cultivar—ICPL 332 (65%) than in the susceptible check, ICPL 87 (38%) (**Table 2**). Among the wild relatives of pigeonpea, highest inhibition of HaGP (85%) was observed in *C. albicans* (ICPW 14) and *R. bracteata* (ICPW 214), and lowest (63%) in *C. cajanifolius* (ICPW 28) (**Table 2**). *Cajanus scarabaeoides* accessions exhibited 65% to 74% inhibition of HaGP. Low inhibition potential of HaGPs explained the susceptibility

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Table 1. *In vitro* screening of *Helicoverpa armigera* gut proteases inhibition using spot test.

Species	Accession	Concentration of HaGP:Seed extract		
		3:1	1:1	1:3
Cajanus acutifolius	ICPW 1	P	T	T
	ICPW 2	P	T	T
C. albicans	ICPW 13	T	T	T
	ICPW 14	T	T	T
C. cajanifolius	ICPW 28	T	T	T
	ICPW 29	T	T	T
C. lineatus	ICPW 40	N	P	T
C. sericeus	ICPW 159	P	P	T
	ICPW 160	T	T	T
C. platycarpus	ICPW 68	T	T	T
C. scarabaeoides	ICPW 83	T	T	T
	ICPW 90	T	T	T
	ICPW 94	T	T	T
	ICPW 116	P	T	T
	ICPW 125	T	T	T
	ICPW 130	T	T	T
	ICPW 137	T	T	T
	ICPW 141	T	T	T
	ICPW 152	P	T	T
	ICPW 278	P	T	T
	ICPW 280	P	T	T
	ICPW 281	T	T	T
Flemingia bracteata	ICPW 192	T	T	T
F. stricta	ICPW 202	T	T	T
Paracalyx scariosa	ICPW 207	P	T	T
Rhynchosia bracteata	ICPW 214	T	T	T
R. aurea	ICPW 210	P	T	T
C. cajan	ICPL 87	N	P	T
	ICPL 332	T	T	T

N = No inhibition. The HaGPs activity is not inhibited by the protease inhibitors. P = Partial inhibition. The HaGPs activity is moderately inhibited due to some protease inhibitors belonging to serine class, but are not present in enough concentrations to inhibit the total activity. T = Total inhibition. The HaGPs activity is totally inhibited by the protease inhibitors.

of pigeonpea to *H. armigera* larvae. Pigeonpea PIs and amylase inhibitors accumulate during seed development, and host defense is inadequate during the early stages of grain development [24,25]. Insects also inactivate host plant defenses by expressing inhibitor resistant or inhibitor degrading proteinases [12,26-30]. Therefore, screening of non-host plants and/or wild relatives for identification of strong insect gut PIs is a prerequisite for application of PI-based strategy for developing insect-resistant transgenic plants [12,19,31]. Screening of several wild relatives of chickpea did not lead to the identification of strong inhibitors of HaGP [29] as *H. armigera* is a polyphagous pest. This certainly makes it difficult to find potential PIs for HaGP, even from the non-host plants. In this connection, the wild relatives of pigeonpea could serve as good sources of powerful HaGP inhibitors.

To examine the specificity of HaGP of *H. armigera* larvae feeding on chickpea and pigeonpea, the gut extracts were also assayed for inhibition by chemical inhibitors of different specificities (**Table 3**). The HaGPs' of larvae fed on chickpea were inhibited strongly by serine PIs, antipain (83%), leupeptin (84%), pefabloc (85%), aprotinin (65%), soybean trypsin inhibitor (54%), and chymostatin (50%), but not by E-64, pepstatin, and EDTA, suggesting the absence of cysteine proteinases, aspartic proteinases, and metalloproteinases, respectively. Similarly, the HaGPs' of the larvae fed on pigeonpea showed inhibition of serine PIs, antipain (65%), leupeptin (75%), pefabloc (92%), aprotinin (87%), and soybean trypsin inhibitor (72%) (**Table 3**). However, the proteinase activity was significantly inhibited by chymostatin (80%) and EDTA (58%), and to a lesser extent by E-64 (28%) and pepstatin (28%), indicating the existence of proteinases with highly complex specificities in *H. armigera* fed on pigeonpea.

The gut proteinase complement of *H. armigera* exhibits predominately trypsin-like proteinase activity [32]. Although, the insect trypsins are similar to bovine trypsin in their catalytic properties, they differ in their pH optima [32,33], and sensitivity towards inhibitors of plant or chemical origin [34,35]. The differences in the extent of inhibition by the five serine PIs towards HaGP of larvae fed on two different hosts pointed to different specificities in the trypsin-like activities in the *H. armigera* gut. Bown *et al*. (1997) [26] reported that although the serine PIs antipain, leupeptin, and benzamidine inhibited 98% of HaGP activity, a general serine proteinase inhibitor PMSF inhibited only 28% of HaGP activity in larvae reared on artificial diet. Further, presence of at least 28 genes of trypsin- and chymotrypsin-like proteinases in *H. armigera* with 90% homology and minor differences near the active sites of these proteinases was shown by Bown *et al*. (1997) [26]. Mazumdar-Leighton *et al*. (2000) [36] reported the presence of two transcripts for trypsin-like proteinases in *H. armigera*. Moderate to high level of chymotrypsin-like activity was detected in the larvae fed on pigeonpea, whereas very low activity was detected in larvae fed on chickpea. The gut composition of larvae fed on pigeonpea contrasted with that of *H. armigera* larvae fed on chickpea. *Helicoverpa armigera* fed on pigeonpea revealed the presence of metalloproteinase,

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Table 2. Protein content in the mature seeds, maximum HGP inhibition, *in vitro* inhibition, and percentage inhibition at different pH levels in wild relatives of pigeonpea against the pod borer, *Helicoverpa armigera*.

Species	Accession	Protein (mg/g)	Maximum inhibition of HGP (%)[a]	*In vitro* inhibition (%) HGP at 30 min[b]	HGP inhibition (%)[c]	
					pH 7.8	pH 10.0
C. acutifolius	ICPW 1	16.000 ± 3.151	78 ± 1.84, (240)	73 ± 1.4, (240)	77 ± 5	68 ± 3
	ICPW 2	14.587 ± 1.058	81 ± 1.12, (255)	75 ± 1.5, (255)	75 ± 2	71 ± 4
C. albicans	ICPW 13	10.243 ± 2.025	83 ± 1.71, (278)	83 ± 1.3, (278)	85 ± 3	81 ± 5
	ICPW 14	12.052 ± 4.687	85 ± 1.68, (285)	80 ± 1.7, (285)	82 ± 7	75 ± 2
C. cajanifolius	ICPW 28	10.825 ± 3.235	63 ± 1.28, (325)	55 ± 1.5, (325)	62 ± 4	54 ± 1
	ICPW 29	13.360 ± 2.254	68 ± 1.45, (340)	64 ± 1.92, (340)	63 ± 3	58 ± 3
C. lineatus	ICPW 40	10.705 ± 1.369	59 ± 1.40, (218)	51 ± 1.45, (218)	60 ± 5	54 ± 3
C. sericeus	ICPW 159	13.333 ± 2.314	71 ± 1.11, (270)	75 ± 1.61, (270)	70 ± 4	62 ± 5
	ICPW 160	9.979 ± 2.058	68 ± 1.45, (255)	70 ± 1.61, (255)	71 ± 6	64 ± 4
C. platycarpus	ICPW 68	9.254 ± 1.952	81 ± 1.47, (245)	80 ± 1.25, (245)	82 ± 4	72 ± 3
C. scarabaeoides	ICPW 83	9.648 ± 2.149	70 ± 1.81, (428)	68 ± 1.34, (428)	64 ± 2	54 ± 3
	ICPW 90	14.458 ± 2.104	69 ± 1.78, (410)	70 ± 1.24, (410)	62 ± 8	57 ± 2
	ICPW 94	7.639 ± 1.205	74 ± 1.17, (439)	72 ± 2.14, (439)	65 ± 3	59 ± 1
	ICPW 116	6.132 ± 0.824	69 ± 1.18, (395)	70 ± 1.45, (395)	62 ± 2	52 ± 5
	ICPW 125	9.591 ± 2.138	68 ± 1.24, (402)	71 ± 1.25, (402)	68 ± 4	59 ± 1
	ICPW 130	9.274 ± 1.854	70 ± 1.71, (411)	68 ± 1.45, (411)	63 ± 2	57 ± 3
	ICPW 137	8.701 ± 2.196	67 ± 1.45, (395)	64 ± 1.35, (395)	69 ± 5	61 ± 5
	ICPW 141	10.594 ± 1.496	69 ± 1.71, (430)	68 ± 2.11, (430)	70 ± 1	64 ± 2
	ICPW 152	12.318 ± 2.110	71 ± 1.24, (412)	68 ± 1.45, (412)	68 ± 2	64 ± 1
	ICPW 278	13.927 ± 3.578	65 ± 1.92, (450)	64 ± 1.77, (450)	67 ± 4	65 ± 1
	ICPW 280	12.350 ± 2.314	68 ± 1.45, (465)	65 ± 1.45, (465)	70 ± 2	62 ± 3
	ICPW 281	16.631 ± 2.143	71 ± 1.03, (410)	70 ± 1.28, (410)	72 ± 3	64 ± 4
F. bracteata	ICPW 192	10.810 ± 2.021	67 ± 1.58, (214)	65 ± 1.82, (214)	62 ± 4	55 ± 4
F. stricta	ICPW 202	13.379 ± 1.356	72 ± 1.35, (198)	73 ± 1.95, (198)	73 ± 5	60 ± 7
P. scariosa	ICPW 207	14.050 ± 1.048	69 ± 1.72, (210)	69 ± 1.45, (210)	70 ± 2	62 ± 4
R. bracteata	ICPW 214	11.876 ± 2.486	85 ± 1.45, (187)	80 ± 1.25, (187)	87 ± 7	81 ± 4
R. aurea	ICPW 210	12.572 ± 3.194	75 ± 1.85, (145)	72 ± 1.24, (145)	70 ± 3	62 ± 4
C. cajan	ICPL 87	15.979 ± 2.487	38 ± 1.29, (380)	40 ± 1.45, (380)	42 ± 1	33 ± 3
	ICPL 332	8.817 ± 2.488	65 ± 1.92, (395)	61 ± 1.22, (395)	68 ± 3	57 ± 5

The values are average of three replicates ± SD. [a]The % inhibition indicated in the table is the highest possible inhibition, which causes @ 100% inhibition of trypsin with respective seed extract. The values in the parenthesis are protein content (µg) of the seed extract used to obtain maximum inhibition of HGP. [b]*In vitro* stability of wild pigeonpea protease inhibitors against HGP. Inhibitors were pre-incubated with HGP for 30 min at 37˚C. The values in the parenthesis are protein content (µg) of the seed extract used to obtain maximum inhibition of HGP. [c]Activity assays were performed at pH 7.8 and 10.0. Double concentration of gut extract was required to obtain equivalent units of BApNAase activity at pH 7.8 than at pH 10.0. Five concentrations of inhibitor extract were used to assess the potential of inhibitor for inhibiting HGP activity.

and a lower percentage of aspartic and cysteine proteinases, which were absent in the gut of larvae fed on chickpea.

When, HaGP of second- and fifth-instars of *H. armigera* fed on artificial diet were assessed for their inhibition by chemical inhibitors, HaGP of second-instars showed 55% to 75% inhibition by serine PIs such as leupeptin, pefabloc, aprotinin, and soybean trypsin inhibitor, with the exception of antipain, which inhibited only 45% of HaGP activity (**Table 4**). Interestingly, substantial inhibition was shown by pepstatin (42%), E-64 (38%), EDTA (32%), and chymostatin (30%), indicating the presence of specificity of other than serine proteinases in the second-instars. HaGPs of fifth-instars showed inhibition by all the serine PIs viz., antipain (68%), leupeptin (71%), pefabloc (87%), aprotinin (52%),

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Table 3. Effect of chemical inhibitors on gut proteinase activity of *H. armigera* fed on artificial diet. Inhibition of gut proteinase activity was measured using casein as a substrate.

Chemical inhibitor	Specificity of the inhibitor	Effective concentration	Inhibition of gut proteinase activity of larvae fed on artificial diet (%)*	
			Chickpea	Pigeonpea
Antipain	Serine Proteinases	275 μM	83 ± 4	65 ± 2
Leupeptin	Serine Proteinases	25 μM	84 ± 10	75 ± 8
Pefabloc	Serine Proteinases	10 mM	85 ± 5	92 ± 8
Aprotinin	Serine Proteinases	1.8 μM	65 ± 10	87 ± 8
Soybean trypsin Inhibitor	Trypsin	1 mM	54 ± 8	72 ± 8
Chymostatin	Chymotrypsin	280 μM	50 ± 10	80 ± 8
E-64	Cysteine Proteinases	150 μM	0	28 ± 10
Pepstatin	Aspartic Proteinases	10 μM	0	28 ± 10
EDTA-Na2	Metallo-Proteinases	1 mM	0	58 ± 10

*The values are average of three replicates ± SE.

Table 4. Effect of chemical inhibitors on gut proteinase activities of second and fifth instar of *H. armigera* larvae fed on artificial diet. Inhibition of gut proteinase activity was measured using casein as a substrate.

Chemical inhibitor	Specificity of the inhibitor	Effective concentration	Inhibition of HGP (%)* of	
			Second instar	Fifth instar
Antipain	Serine Proteinases	275 μM	45 ± 5	68 ± 10
Leupeptin	Serine Proteinases	25 μM	75 ± 8	71 ± 3
Pefabloc	Serine Proteinases	10 mM	70 ± 2	87 ± 7
Aprotinin	Serine Proteinases	1.8 μM	59 ± 10	52 ± 7
Soybean trypsin Inhibitor	Trypsin	1 mM	55 ± 8	64 ± 7
Chymostatin	Chymotrypsin	280 μM	30 ± 2	55 ± 3
E-64	Cysteine Proteinases	150 μM	38 ± 7	20 ± 4
Pepstatin	Aspartic Proteinases	10 μM	42 ± 5	38 ± 4
EDTA-Na2	Metallo-Proteinases	1 mM	32 ± 3	23 ± 4

*The values are average of three replicates ± SE.

soybean trypsin inhibitor (64%), and chymostatin (55%), but low inhibition by inhibitors of pepstatin (38%), EDTA (23%), and E-64 (20%). The results suggested increased dominance of serine proteinases in insect development. The differential susceptibilities of the proteinases to chemical inhibitors observed in the two larval instars suggested the dynamic nature of expression of the gut proteinases possessing different specificity during the course of larval development.

The *in vitro* stability of pigeonpea PIs against the HaGPs was evaluated by enzyme assays after incubation with 0.02 BA*p*NAase units of HaGP for 30 min (**Table 2**). *In vitro* stability of the pigeonpea PIs against HaGPs was reflected by the extent of inhibition after HaGP treatment for 30 min. All accessions of pigeonpea showed inhibition between 60% to 90%, except in the susceptible pigeonpea cultivar, ICPL 87 (40%). Inhibition of HaGP was also evaluated at pH 7.8 and pH 10.0 because two groups of proteinases showing activity at specific pH were observed in the HaGP complement. All accessions of wild relatives of pigeonpea showed high to moderate levels of inhibition at pH 7.8, except in ICPL 87 (42%). PIs from wild pigeonpeas showed more stability at pH 7.8 as compared to pH 10.0. Cultivated pigeonpea exhibited monomorphism in terms of TI and CTI isoforms, contrary to the diverse inhibitory profiles of wild pigeonpeas. *Cajanus albicans*, *C. platycarpus*, *C. scara-*

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Table 5. Trypsin and chymotrypsin inhibitors in wild relatives of pigeonpea against the pod borer, *Helicoverpa armigera*.

Genotype	Trypsin inhibitors	Chymotrypsin inhibitors	TIs + CTIs	Total PIs
C. acutifolius	1	2	2	3
C. albicans	7	5	5	12
C. cajanifolius	5	4	3	9
C. lineatus	4	5	3	9
C. sericeus	3	4	3	7
C. platycarpus	6	5	4	11
C. scarabaeoides	8	4	3	12
C. ferruginea	6	2	2	8
F. bracteata	4	0	0	4
F. stricta	2	1	1	3
P. scariosa	3	4	3	7
R. bracteata	5	7	4	12
R. aurea	6	4	3	10
C. cajan	7	5	4	12

baeoides, and *R. bracteata* showed more number of TI and CTI bands. Lowest number of TI and CTI isoforms was observed in *C. acutifolius*, *F. bracteata*, and *F. stricata* (**Table 5**). Some of the wild relatives exhibited both TI and CTI activities.

Protease inhibitor isoforms of wild relatives of pigeonpea showed significant variation in number, band pattern, and protein specificities towards trypsin, chymotrypsin, and HaGPs as compared to that of the cultivated pigeonpea. Similar observations have been reported in chickpea, where high variation in PIs was recorded in mature seeds of wild relatives than in the cultivated ones [29].

In the present study, it has been observed that PIs from the wild relatives of pigeonpea appeared as HGPIs, indicating that specific PIs from *R. bracteata*, *C. albicans* and *C. platycarpus* possessed strong inhibitory activity against HGP. However, further studies are necessary to characterize the PIs from wild relatives of pigeonpea to developing strategies for expressing PIs from the wild relatives in the cultivated pigeonpea for resistance to *H. armigera*.

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