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Differential inhibition of *Helicoverpa armigera* gut proteinases by proteinase inhibitors of pigeonpea (*Cajanus cajan*) and its wild relatives

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Abstract

The seeds of 36 pigeonpea [*Cajanus cajan* (L) Millsp.] cultivars, resistant and susceptible to pests and pathogens and 17 of its wild relatives were analysed for inhibitors of trypsin, chymotrypsin, and insect gut proteinases to identify potential inhibitors of insect (*Helicoverpa armigera*) gut enzymes. Proteinase inhibitors (PIs) of pigeonpea cultivars showed total inhibition of trypsin and chymotrypsin, and moderate inhibition potential towards *H. armigera* proteinases (HGP). PIs of wild relatives exhibited stronger inhibition of HGP, which was up to 87% by *Rhynchosia* PIs. Electrophoretic detection of HGPI proteins and inhibition of HGP isoforms by few pigeonpea wild relative PIs supported our enzyme inhibitor assay results. Present results indicate that PIs exhibit wide range of genetic diversity in the wild relatives of pigeonpea whereas pigeonpea cultivars (resistant as well as susceptible to pests and pathogens) are homogeneous. The potent HGPIs identified in this study need further exploration for their use in strengthening pigeonpea defence against *H. armigera*.

Keywords: *Cajanus*; *Helicoverpa armigera*; *Rhynchosia*; Pigeonpea; Insect gut proteinase inhibitors

1. Introduction

Pigeonpea [*Cajanus cajan* (L.) Millsp.] is a multipurpose, hardy grain legume crop grown by resource poor farmers of many developing countries in semi-arid tropics and subtropics. It occupies an important position in human diet as a protein source especially in the vegetarian population (Singh et al., 1984). The most important constraints of pigeonpea production are (i) insect feeding on the developing pods in the field as well as during the grain storage and (ii) infections caused by fungal and viral pathogens in the field. Among insect pests, *Helicoverpa armigera* is the most damaging pest of pigeonpea pods, which causes heavy losses every year (Reed and Lateef, 1990). The current pest control measures are not high enough to protect pigeonpea from such a voracious feeder (Armes et al., 1996). Therefore, the study of host plant resistance can play an important role in identifying the antidigestive or antifeedant compounds and their further use in the pest management strategies (Lewis et al., 1997).

Plants have capacity to synthesize certain biologically active substances, which play a major role in plant defence against insect pests and microbial attacks. Some of these include defence proteins like proteinase inhibitors (PIs), amylase inhibitors, lectins and class of pathogenesis-related proteins (Garcia-Olmedo et al., 1987; Ryan, 1990; Chrispeels and Raikhel, 1991; Tatyana et al., 1998). Several studies have demonstrated that these proteins are specifically produced in the plant upon biotic stress and protect the plant tissue from the damage (Ryan, 1990; Schaller and Ryan, 1995; Conconi et al., 1996; Tatyana et al., 1998). PIs are the most exploited class of plant defence proteins for their use in developing insect resistance in plants (Jouanin et al., 1998).

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Pigeonpea seeds contain proteinaceous inhibitors of trypsin, chymotrypsin and amylases (Singh and Eggum, 1984; Singh et al., 1984) as well as phytolectins, and secondary metabolites (Bressani and Elias, 1979; Grant et al., 1983), which constitute the defence machinery. Biochemical characterization of pigeonpea PIs has revealed that these are Kunitz type PIs having inhibitory activity against trypsin and chymotrypsin (Godbole et al., 1994). Previously, seven isoforms of trypsin/chymotrypsin inhibitors (TCIs) and two isoforms of trypsin inhibitors (TIs) have been reported from pigeonpea seeds (Pichare and Kachole, 1994). In spite of such a broad spectrum of defence compounds in pigeonpea seeds, pre-harvest damage due to insect pests on developing seeds and post harvest losses due to storage pests are severe. Several conventional strategies are being employed to improve the genetic makeup of pigeonpea for strengthening plant defence, which include classical hybridization and mutation breeding methods. Some success has been achieved in obtaining resistance to microbes but achieving insect resistance is still a challenge to the pigeonpea breeders. Although, it is a known fact that wild relatives of pigeonpea possess considerable insect resistance, the biochemical mechanism involved in the resistance has not been investigated. In the present paper, we have screened pigeonpea cultivars and its wild relatives for proteinase inhibitors. We have identified PIs in several wild relatives of pigeonpea showing higher potential against insect proteinases, which is substantiated by several biochemical evidences. This information can be exploited for planning the strategies for developing insect resistance in pigeonpea.

2. Results and discussion

2.1. Differential inhibition of H. armigera gut proteinases (HGPs) by PIs of pigeonpea and its wild relatives

Table 1 presents maximum HGP inhibition by PIs of pigeonpea and wild relatives when different concentrations of protein (10–550 μg) were independently tested against HGP. The concentration of protein used for highest possible HGP inhibition was more than that required for total inhibition of bovine trypsin activity. Among the 36 pigeonpea cultivars analysed, the highest inhibition of HGP was 56% whereas lowest was 9% (Table 1). Only four pigeonpea cultivars showed around 50% inhibition of HGP whereas out of seventeen, fifteen wild relatives showed more than 70% inhibition of HGP activity. The highest HGP inhibition was detected in *Rhynchosia rothii* ICP 15859 (87%) and the lowest inhibition was detected in *Dunbaria ferruginea* ICP 15777 (45%). More importantly, protein concentration required for maximum inhibition of HGP is relatively low in some of the wilds (*R. rothii* 58 μg protein, *R. sublobata* 124 μg, *R. minima* 145 μg, *R. bracteata* 147 μg). Low inhibition potential of HGPs explains susceptibility of pigeonpea and *in toto* successful feeding of *H. armigera* larvae on the pigeonpea despite the presence of broad spectrum of defence compounds. This might be due to adaptation of insects to their host defence; it is possible that insect might synthesize novel compounds to nullify the effect of host plant defence. For example, ineffectiveness of pigeonpea PIs to insect proteinases might be the result of such process during host-pest interaction. Another important observation of earlier studies is late accumulation of pigeonpea PIs and amylase inhibitors during the seed development. In other words, insect chooses a developmental stage of plant tissue where host defence is inadequate (Ambekar et al, 1996; Giri and Kachole, 1998). It has also been demonstrated that the insect inactivates host defence by expressing inhibitor resistant or inhibitor degrading proteinases (Bown et al., 1997; Giri et al., 1998; Giri and Kachole 1998; Harsulkar et al., 1998; 1999; Patankar et al., 1999; 2001). Therefore, screening of non-host plants and/or wild relatives for identification of strong insect gut PIs is a prerequisite for the application of PI-based strategy for developing insect resistant transgenic plants. Screening of several wild relatives of chickpea failed to identify strong inhibitors of HGP (Patankar et al., 1999). *H. armigera* is a polyphagous pest feeding on about 200 plant species belonging to 45 different plant families (Manjunath et al., 1989). It is possible that the insect is exposed and acclimatized to different PIs present in the wide range of host group. This certainly makes it difficult to find potential PIs for HGP, even from the non-host plants. In this scenario, present investigation demonstrates that wild relatives of pigeonpea are good sources of powerful HGP inhibitors and add to the list of HGPIs identified earlier (Giri et al., 2003; Harsulkar et al., 1999; Telang et al., 2003).

2.2. Loss of PI diversity during domestication of pigeonpea

Pigeonpea cultivars exhibited monomorphism in terms of TI and CI isoforms (results not shown) contrary to the diverse inhibitory profiles of pigeonpea wild relatives (Fig. 1A and B). *C. cajanifolius* ICP 15632, *R. bracteata* ICP 15815, *C. albicans* ICP 15625, *C. lineatus* ICP 15642, *C. sericeus* ICP 15760, *R. bracteata* ICP 15815 showed higher number of TI and CI bands (Fig. 1A and B, lanes 4, 9 to 12 and 17, respectively), on the other hand lowest number of TI and CI isoforms were detected in *F. stricta* ICP 15803 and *C. platycarpus* ICP 15665 (Fig. 1A and B, lanes 7 and 13). Close examination of TI and CI profiles of wild relatives revealed that *Cajanus lanceolatus* ICP 15659, *C. acutifolius* ICP 15603, *C. platycarpus* ICP 15665 exhibit PI

Table 1
Inhibition potential of pigeonpea accessions and wild relatives towards *H. armigera* gut proteinases. All the samples showed 100% inhibition of trypsin and chymotrypsin. Hence are not indicated in the table. The %HGP inhibition indicated in the table is the highest possible inhibition, which is causing at least 100% inhibition of trypsin with respective seed extract. The values are averages of three different independent assays with replicates. The values in the parentheses are protein content (μg) of the seed extract used to obtain inhibition of HGP indicated. The results are shown as Mean±S. E. ($n=3$)

Accession	Maximum inhibition of HGP (%)	Wild genotype	Maximum inhibition of HGP (%)
PBR ICP 11967	40±1.26 (326)	*C. crassus* ICP 15774	84±1.80 (126)
PBR ICP 13198	51±1.09 (406)	*C. lanceolatus* ICP 15639	79±1.37 (233)
PBR ICP 13199	34±1.72 (340)	*C. cajanifolius* ICP 15632	71±1.22 (481)
PBR ICP 11966	27±0.98 (298)	*C. acutifolius* ICP 15603	81±1.29 (270)
PBR ICP 11964	47±1.25 (547)	*C. sericeus* ICP 15760	69±1.64 (550)
PBR ICP11962	56±1.89 (593)	*C. platycarpus* ICP 15665	80±1.39 (257)
PBR ICP 13200	27±1.28 (354)	*C. scarabaeoides* ICP 15712	69±1.20 (470)
PBR ICP 11953	23±1.57 (266)	*C. lineatus* ICP 15642	58±1.40 (207)
PBS ICP 7203	25±1.43 (272)	*C. albicans* ICP 15625	80±1.78 (303)
PFR ICP 13210	52±1.21 (287)	*R. rothii* ICP 15859	87±1.90 (58)
PFR ICP 13204	30±1.59 (342)	*R. sublobata* ICP 15868	86±1.20 (124)
PFR ICP 11965	44±1.17 (534)	*R. minima* ICP 15838	75±1.83 (145)
PFR ICP 13203	29±1.52 (354)	*R. densiflora* ICP 15828	75±1.62 (160)
PFR ICP 11968	50±1.61 (470)	*R. bracteata* ICP 15815	84±1.34 (147)
PFR ICP 11957	56±1.92 (486)	*F. semialata* ICP 15802	69±1.76 (114)
PFR ICP 11951	47±1.36 (221)	*F. stricta* ICP 15803	71±1.35 (102)
PFR ICP 11950	56±1.21 (432)	*D. ferruginea* ICP 15777	45±1.48 (444)
PBR.PFR ICP 11961	43±1.06 (486)		
PBR.PFR ICP 13197	24±1.98 (532)		
PBR.PFR ICP 13201	17±1.24 (510)		
PBR.PFR ICP 13207	34±0.87 (366)		
SM.PB.R. ICP 11301	37±1.79 (461)		
SM.PB.R. ICP 11302	25±1.60 (285)		
SM.PB.R. ICP 11303	33±1.63 (526)		
SM.PB.R. ICP 11300	56±1.72 (535)		
SM.PB.R. ICP 8466	36±1.69 (384)		
W.PB.R. ICP 11287	18±1.52 (283)		
W.PB.R. ICP 8868	38±1.89 (230)		
W.PB.R. ICP 10958	38±1.32 (434)		
W.SM.R. ICP 11297	40±1.81 (545)		
W.SM.R. ICP 11298	50±1.93 (316)		
W.SM.R. ICP 11290	9±1.65 (454)		
W.SM.R. ICP 11289	35±1.09 (238)		
W.SM.PB.R. ICP 5097	43±1.92 (547)		
W.SM.PB.R. ICP 11294	12±1.77 (314)		
W.SM.PB.R. ICP 8094	40±1.29 (257)		

bands that appeared as just TI or CI (compare Fig. 1A and B, lanes 3, 5, and 13). Other wild relatives included in our study were also rich in presence of specific TI or CI proteins than possessing both TI and CI activities to single protein.

PI isoforms of wild germplasm have revealed significant variation in number, band pattern and protein specificities towards trypsin, chymotrypsin and HGP as compared to those in pigeonpea cultivars. A similar observation has been reported in chickpea where higher level of PI variation was exhibited in mature seeds of wild relatives than the cultivated ones (Patankar et al., 1999). However, this screening failed to find out potential PI against HGP. The maximum inhibition of gut proteinase activity by chickpea wild relatives was below 55%. This also supports a general consideration that during domestication, crops have lost genetic diversity of their defence tools whereas the pest counterpart has achieved remarkable success by diversifying their biochemical weaponry to combat the plant defence.

2.3. *In vitro* stability of pigeonpea wild relative PIs to HGP

PIs of four *Rhynchosia* species were studied for their stability against HGPs (Fig. 2). Prior to assessment for the inhibition of HGP, PIs of *R. rothii, R. sublobata, R. bracteata* and *R. densiflora* were incubated with HGP for 0 min, 30 min and 3 h as food retention time in the larval gut of *H. armigera* is 3 h. After 3 h incubation, there was a slight increase in the HGP inhibition by *Rhynchosia* PIs on the other hand pigeonpea PIs lost their inhibitory potential (Fig. 2). These results clearly indicate that *Rhynchosia* PIs are highly stable to HGPs

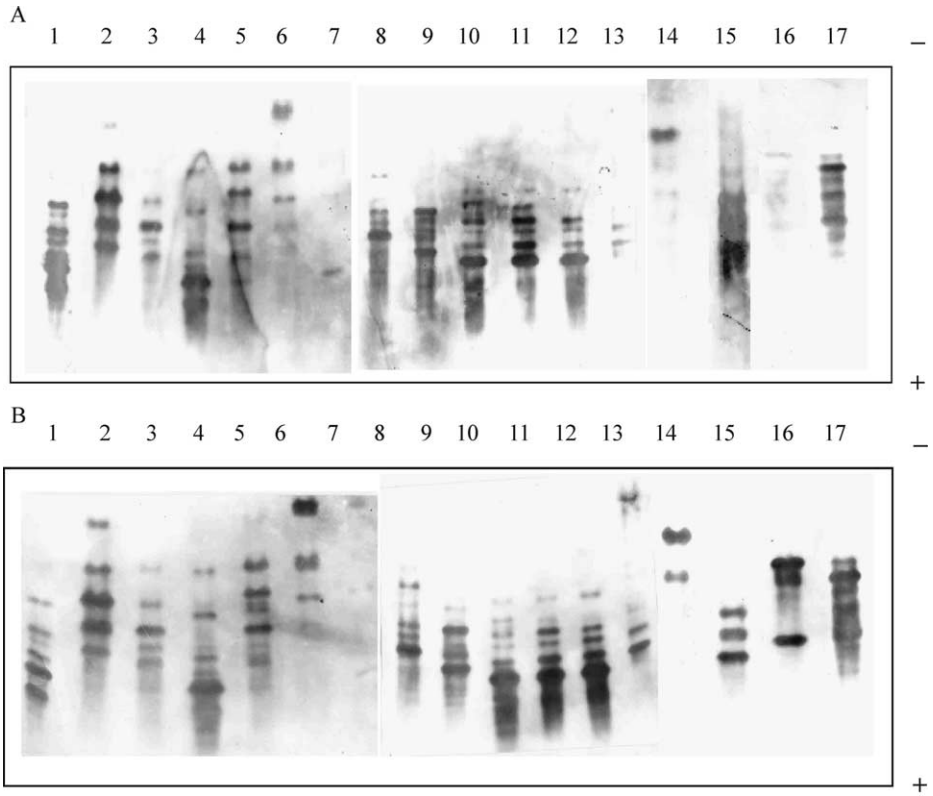

Fig. 1. (A) TI profiles (B) CI profiles of pigeonpea and its wild relatives. Electrophoresis was carried out on 10% non-denaturing polyacrylamide gel. TI and CI activity bands were visualized by the gel X-ray film contact print technique as described in Section 3.4. Equal protein (40 μg) was loaded in each lane except *F. stricta* ICP 15803 and *C. platycarpus* ICP 15665(70 μg), *R. sublobata* ICP 15868 and *R. bracteata* ICP 15815 (20 μg). Lane 1 to 17, *C. cajan*, *C. crassus* ICP 15774, *C. lanceolatus* ICP 15639, *C. cajanifolius* ICP 15632, *C. acutifolius* ICP 15603, *F. semialata* ICP 15802, *F. stricta* ICP 15803, *R. minima* ICP 15838, *R. sublobata* ICP 15868, *C. albicans* ICP 15625, *C. sericeus* ICP 15760, *C. lineatus* ICP 15642, *C. platycarpus* ICP 15665, *C. scarabaeoides* ICP 15712, *D. ferruginea* ICP 15777, *R. densiflora* ICP 15828, and *R. bracteata* ICP 15815, respectively.

and they may also remain stable in the insect gut. Similar study has been carried out to assess stability of non-host PIs (winged bean, peanut, potato, bitter gourd etc.) to HGP by Harsulkar et al., (1999) and Telang et al., 2003, and it has been showed that incorporation of these PIs in the artificial diet inhibits insect growth and development significantly over their control counterparts. While describing the ideal PI for developing insect resistant plants several researchers (including us) have proposed that the identified PIs should have strong inhibitory potential and stability against insect gut proteinases (Giri and Kachole, 1998; Harsulakar et al., 1999; Jongsma and Bolter, 1997; Jouanin et al., 1998; Telang et al., 2003).

2.4. Identification of HGPI proteins and their specificities towards HGP isoforms

Fig. 3 represents the electrophoretic profiles of HGPIs in seed extracts of pigeonpea and its wild species. *Rhynchosia* group showed presence of high activity HGPI bands as compared to pigeonpea and other wild *Cajanus* species. Two HGPI bands were detected in *R. sublobata* ICP 15868 (lane 5) while *R. bracteata* ICP 15815 contains five HGPI bands (lane 6). On the other hand *Cajanus cajan*, *C. crassus* ICP 15774, *C. lineatus* ICP 15642, *F. semialata* ICP 15802 (lanes 1–4) showed presence of very low HGPI activity. This indicates that

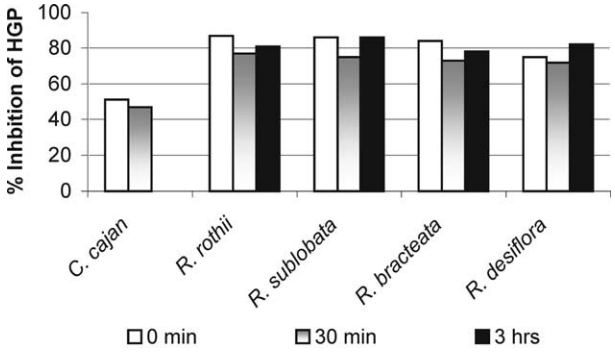

Fig. 2. In vitro stability of *Rhynchosia* group of wild relatives of pigeonpea PIs against *H. armigera* gut proteinases. Stability assay was performed using protein amounts identified to have maximum possible inhibition of HGP (*Cajanus cajan* 280 μg, *Rhynchosia rothii* ICP 15859 58 μg, *Rhynchosia sublobata* ICP 15868 124 μg, *Rhynchosia bracteata* ICP 15815 147μg, *Rhynchosia densiflora* ICP 15828 160 μg). These protein amounts were mixed with HGP extract and reaction mixture was analysed for unhibited proteolytic activity at different time interval of 0 min, 30 min and 3 h.

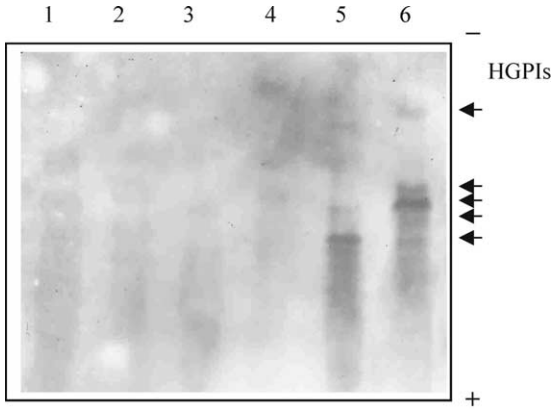

Fig. 3. HGPI profiles of pigeonpea accessions and wild relatives. Selected seed extracts of pigeonpea and its wild relatives were separated on native 10% polyacrylamide gels. After electrophoresis, HGPI bands were visualized as described in the experimental Section 3.4. Equal TI units were loaded in each lane. Lane 1 *Cajanus cajan*, lane 2 *C. crassus* ICP 15774, lane 3 *C. lineatus* ICP 15642, lane 4 *F. semialata* ICP 15802, lane 5 *R. sublobata* ICP 15868, lane 6 *R. bracteata* ICP 15815.

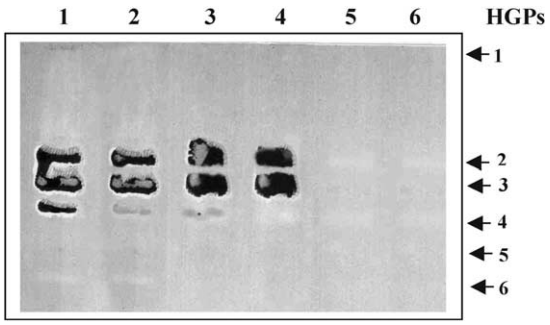

Fig. 4. *H. armigera* proteinase profiles after incubation with seed extracts containing PIs of pigeonpea accessions and its wild relatives. Equal TI units were incubated with HGP extract at 37 °C for 30 min. HGP isoforms were separated on 10% non-denaturing polyacrylamide gel and visualised as described in experimental Section 3.4. Equal amount of HGP extract was loaded in each lane. Lane 1 untreated HGP extract, Lanes 2 to 6, HGP extract treated with PIs of *Cajanus cajan*, PBS ICP 7203, PBR ICP 13198, *Rhynchosia rothii* ICP 15859, *Rhynchosia sublobata* ICP 15868 respectively.

Cajanus cajan and other wilds have low potential against HGPs while *Rhynchosia* group PIs are more potent.

To determine specificities of PIs towards HGP isoforms, HGP extract was incubated with seed extracts (equal TI units). Further, HGPs were resolved on polyacrylamide gel and proteinase activity bands were visualized as described in materials and methods. The wild *Cajanus* PIs exhibited strong specificities and activities towards HGP isoforms (Fig. 4). HGP extract was resolved into six HGP isoforms, three major (HGP-2, 3 and 4) and three minor (HGP-1, 5 and 6) activity bands (Fig. 4, lane 1). *R. rothii* and *R. sublobata* PIs were able to inhibit all HGP isoforms (Fig. 4, lanes 5 and 6). PIs of two pigeonpea cultivars (PBS ICP 7203 and PBR ICP 13198) did not inhibit major HGP isoforms indicating their low potential against insect proteinases (Fig. 4, lanes 3 and 4).

In summary, the present study showed that pigeonpea cultivars irrespective of their classification as tolerant or susceptible to pests revealed monomorphic PI profiles and exhibited weak inhibition of HGPs. In contrast, almost all wild relatives (except *D. ferruginea*) showed relatively strong inhibition of HGP and diverse inhibitor profiles. *Rhynchosia* group is identified to have relatively higher number of PI isoforms, which were stable to HGP and three of PIs detected as HGPIs. Taking into consideration, Jongsma and Bolter's hypothesis (1997), these PIs might be acting synergistically and prevent each other's digestion by insect gut proteinases resulting in higher inhibition potential. However, PIs of pigeonpea are not strong inhibitors of HGP as well as not very stable to HGPs. In the present study, it has been observed that not all PIs of pigeonpea wild relatives appeared as HGPIs indicating that specific PIs of *R. bracteata* and *R. sublobata* possess strong inhibitory activity against HGP. However, further studies are necessary to characterize *Rhynchosia* PIs for developing strategies for introducing pest resistance in pigeonpea by improving PI diversity.

3. Experimental

3.1. Procurement of seeds of pigeonpea and its wild relatives

Seventeen wild relatives of pigeonpea *Cajanus crassus* ICP 15774, *Flemingia semialata* ICP 15802, *Rhynchosia rothii* ICP 15859, *C. lanceolatus* ICP 15639, *C. cajanifolius* ICP 15632, *C. acutifolius* ICP 15603, *F. stricta* ICP 15803, *R. minima* ICP 15838, *R. sublobata* ICP 15868*C. C. sericeus* ICP 15760, *Dunbaria ferruginea* ICP 15777, *R. densiflora* ICP 15828, *C. platycarpus* ICP 15665, *C. scarabaeoides* ICP 15712, *C. lineatus* ICP 15642, *C. albicans* ICP 15625, and *R. bracteata* ICP 15815, and 36 accessions of pigeonpea: pod borer resistant (PBR) ICP 11967, ICP 13198, ICP 13199, ICP 11962, ICP 11964, ICP 11966, ICP13200, ICP 11953, pod borer susceptible (PBS) ICP 7203, Pod fly resistant (PFR) ICP 13210, ICP 13204, ICP 11965, ICP 13203, ICP 11968, ICP 11957, ICP 11951, ICP 11950, pod borer and pod fly resistant (PBR.PFR) ICP 13197, ICP 13201, ICP 13207, ICP 11961, wilt, sterility mosaic virus and phytophthora blight resistant (W.SM.PB.R.)ICP 5097, ICP 11294, ICP 8094, wilt, sterility mosaic virus resistant (W.SM.R.) ICP 11297, ICP 11290, ICP 11289, ICP 11298, sterility mosaic virus and phytophthora blight resistant (SM.PB.R.)ICP 11300, ICP 11301, ICP 11302, ICP 11303, ICP 8466, and wilt and phytophthora blight resistant (W.PB.R.) ICP 11287, ICP 8868, ICP 10958 were provided by International Crop Research Institute for Semi Arid Tropics, Patancheru Hyderabad, India.

3.2. Extraction of PIs and HGPs

Extraction of PIs was carried out as described by Pichare and Kachole (1996). Mature seeds were washed with distilled water and ground to fine powder. The powder was defatted with hexane and acetone washes and suspended in distilled water (1/6 W/V) containing 1% polyvinylpyrrolidone (PVP) and incubated overnight at 15 °C for extraction of proteinase inhibitors. The suspension was centrifuged at 10,000 rpm for 10 min. at 4 °C and supernatant was used for further studies. Fourth instar larvae of *H. armigera* were collected from the fields. Mid gut tissue was isolated from dissected larvae and stored frozen. The mid gut tissue was homogenised with 0.1 M glycine–NaOH buffer (1:3 w/v) pH 10 for 15 min. at 10 °C. The suspension was centrifuged at 10,000 rpm for 10 min. at 4 °C and supernatant was used as a source of *H. armigera* gut proteinases (HGP). Protein in seed extracts was estimated by Lowry's method (Lowry et al., 1951) with BSA as standard.

3.3. Estimation of PIs and HGPI activities

Bovine trypsin and chymotrypsin were obtained from Sisco Research laboratory, Mumbai, India. Trypsin and chymotrypsin activities were determined using synthetic substrates benzoyl-arginyl-*p*-nitro-anilide (BApNA) (Erlanger et al., 1961) and *n*-glutaryl 1-phenylalanine *p*-nitroanilide (GLUPHEPHA) (Muller and Weder, 1989), respectively. HGP activity was also estimated separately using both the substrates to measure trypsin-like and chymotrypsin-like activity in the gut extract. For trypsin assay, 15 μg of trypsin or of HGP extract (equal activity units) was added to 400 μl of 1 mM BApNA (8% DMF in 0.1 M glycine–NaOH, pH 10) and incubated at 37 °C for 10 min. The reaction was terminated by the addition of 200 μl of 30% glacial acetic acid and absorbance was taken at 410 nm. For chymotrypsin assay, 15 μg of chymotrypsin or of HGP extract (equal activity units) was added to 400 μl of 1mM GLUPHEPHA (8% DMF in 0.1 M glycine-NaOH, pH 10) and incubated at 37 °C for 1 h. The reaction was terminated by adding 200 μl of 30% glacial acetic acid and absorbance was taken at 405 nm. PI and HGPI activities were measured using the method described by Giri et al., (1998). An inhibitor assay was performed using linearly increasing amount of proteins of an individual pigeonpea accession or wild genotype and mixing them with HGP extract. Exact protein concentration has been identified, where HGP inhibition gets saturated and this inhibition was defined as maximum possible inhibition of HGP, which is presented in Table 1. One trypsin or chymotrypsin (BApNAase or GLUPHEPAase) activity units were calculated as an increase of one optical density unit of substrate hydrolysis products (*p*-nitroanilide) liberated by proteinase action per min at 37 °C. One proteinase inhibitory unit is defined as inhibition of one proteinase activity unit.

3.4. Visualisation of isoforms of PIs and HGP

Proteinase inhibitors were separated on 10% native polyacrylamide gels and visualised by using X-ray film contact print technique (Pichare and Kachole, 1994). After electrophoresis, gel was equilibrated with respective assay buffer and placed in 0.1 mg/ml trypsin or chymotrypsin in 0.1M Tris-HCl buffer, pH 7.8 for 10 min at 37 °C. The gel was then gently washed with fresh assay buffer and placed on an undeveloped X-ray film for 5–10 min. Hydrolysis of gelatin was monitored visually. At the end of incubation period, gel was removed and X-ray film was washed gently with tap water to detect the trypsin/chymotrypsin inhibitor activity bands.

HGPI isoforms were detected by separating PIs (equal TI units) on 12% non-denaturing polyacrylamide gels. After electrophoresis, gel was incubated in 0.1 M glycine-NaOH buffer, pH 10 or 10 min at 37 °C followed by incubation in HGP extract for 1 h with constant shaking. The gel was then washed gently in fresh buffer assay and overlaid on an undeveloped X-ray film for 5–15 min. The gel was removed and X-ray film was washed gently with tap water to detect HGPI bands.

Mixture of seed extract containing equal TI units and HGP extract was incubated at 37 °C for 30 min, and was used for detection of unbound HGP isoforms. HGPs were separated on 10% native polyacrylamide gel and visualized using gel X-ray film contact print method for proteinase detection (Harsulkar et al., 1998).

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