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Purification of Protease Inhibitor Protein from Pigeonpea Seeds and its Insecticidal Potential against *Helicoverpa armigera* (Hubner)

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Protease Inhibitors in legumes are one of the most promising weapons that confer resistance against insects by inhibiting proteases present in the gut of larvae. In this study, the Protease Inhibitor (PI) protein was isolated from defatted meal of four varieties of pigeonpea and the crude extract was precipitated using ammonium sulphate precipitation. It was found that the ammonium sulphate fraction 40-80% of the variety Pusa-33 has higher PI activity and was further purified using ion-exchange chromatography and gel-filtration. The purified Pigeonpea Protease Inhibitor (PPI) proteins form Pusa-33 showed a single band on SDS-PAGE corresponding to molecular mass of 26,000 D and molecular weight of PPI was also confirmed as 26 kD by gel filtration chromatography. The toxicity of PPI protein was evaluated by incorporation in to semi synthetic diet at three levels of treatments and the larvae of pod borer *Helicoverpa armigera* were fed on these diets. It was found that the PPI protein reduced the mean larval weight at molting due to partial starvation. Feeding trial revealed the larvae mortality up to 46%and extension of larval period by 12 days. The PPI protein also affected the molting and pupal weight significantly. Mean fecal output by the larval fed on PPI protein diet was significantly low as compared to control. It may be concluded from the study that pigeonpea protease inhibitor protein has insecticidal potential against the *Helicoverpa armigera* and could be used for insect control.

Key words: Pigeonpea, Protease Inhibitor Protein, Ion exchange chromatography, pod borer and *Helicoverpa armigera*.

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 major constraint of pigeonpea production includes feeding by insects on the developing pods in the field as well as during the grain storage and infections caused by viral and fungal pathogens in the field. Among the insect pests causing economic losses pod borer, *Helicoverpa armigera* is the most damaging pest of developing pods of pigeonpea, which causes heavy losses every year (Reed and Lateef, 1990). *Helicoverpa armigera*, Hubner (Lepidoptera: Noctuidae), a highly devastating, polyphagous crop pest has a broad host spectrum and geographical distribution and causes a significant yield losses in many agriculturally important crops,

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like cotton, chickpea, pigeon pea, corn, maize, tomato, okra, sorghum, pearl millet, sunflower, and groundnut Volpicella *et al*., 2003. The losses caused only by this pest reported up to US$ 17 million in crops like, cotton, pigeonpea, chickpea, groundnut, sorghum, pearl millet, tomato and others of economic importance (Chaturvedi, 2007).

Crop protection plays a major role in enhancing crop productivity through minimizing the crop loss by the insect pests. Conventional methods of mainly rely on the use of chemical insecticides and pesticides which not only have the higher cost but also has major concern of food safety and environmental pollution. Hence, there is an urgent need to develop substitution technologies, which would allow a much more limited use of chemicals.

Plants naturally synthesize certain biologically active substances like, proteinase inhibitors, alpha-amylase inhibitors, lectins and chitin binding proteins to resist herbivorous insects, pathogens and wounding (De Leo *et al*., 2001). The defense related proteins like proteinase inhibitors (PIs), amylase inhibitors, lectins and class of pathogenesis-related proteins play a major role in plant defense against insect pests and microbial attacks (Garcia-Olmedo *et al*., 1987; Ryan, 1990; Chrispeels and Raikhel, 1991; Tatyana *et al*., 1998; Connors *et al*., 2002). Proteinase inhibitor becomes a defense alternative by creating an insect-resistant plant (Ryan, 1990). PI proteins mainly found in leguminous plants and are specific to each of the four classes of proteolytic enzyme *viz.* serine, aspartic and cysteine and metallo- protease. PIs are the most exploited class of plant defense proteins for their use in developing insect resistance in plants (Jouanin *et al*., 1998).PI play important role in plant defense mechanism by preventing proteolysis in the midgut of insect larvae leading to their starvation and subsequent death (Johnston *et al*., 1991; Gatehouse *et al*., 1999).The protease inhibitor leads to decline in feeding behavior of the insect, resulting in a decrease in growth causing death in several days (Pulliam *et al*., 2001).

Studies have indicated the relevance of proteinase inhibitor for plant defense and have been shown to act as a defensive compound against phytophagous insects by the direct assay or expression in transgenic crop plants (Koiwa *et al*., 1998, Vain *et al*., 1998) and blocking of major part of *H. armigera* gut proteinase activity by soybean kunitz trypsin inhibitor (Johnston *et al*., 1991, Harsulkar *et al*., 1999). Numerous insect-feeding bioassays and experiments with transgenic plants have also shown the delayed growth and development of the insect (Koiwa *et al*., 1998; Parde *et al*., 2010). Biochemical characterization of pigeonpea PIs has revealed that these are Kunitz type PIs having inhibitory activity against trypsin and chymotrypsin (Godbole *et al*., 1994). Studies demonstrated the retardation in the growth and development of insect pests fed on diets incorporating PIs, or on transgenic plants expressing PIs (DeLeo and Gallerani, 2002; Murdock and Shade, 2002; Telang *et al*., 2003). Trypsin inhibitor was isolated from *Cicer aeretienum* and proved effective against *H. armigera* (Kansal *et al*., 2008). In addition, certain parameters like. insect gut pH, larval developmental stage, concentration of PI and the better understanding of how insects respond and adapt to PIs influence its effectiveness (Dunse and Anderson, 2011). Keeping the above facts and importance in view, the present investigation was conducted to isolate and purify the high potential protease inhibitor (PI) protein from mature seeds of pigeonpea. Bioassays were performed to ascertain the effectiveness of the purified PI protein in inhibiting the growth and development of *H. armigera* larvae. The information generated in the study could be exploited for planning the strategies for developing insect resistance transgenic plants in the future.

MATERIALS AND METHOD

Seed Material

The seeds of four cultivars of pigeonpea (*Cajanus cajan* L.) *viz.* Pusa-855, Pusa-33, Pusa-987 and Pusa-84 were obtained from the Division of Genetics, Indian Agricultural Research Institute, New Delhi, India.

Extraction of Protease Inhibitor Protein

The seeds of pigeonpea were grounded in a Wiley laboratory mill and defatted flour of four varieties *viz.* Pusa-855, Pusa-33, Pusa-987 and Pusa-84 were used for the isolation of Protease Inhibitor proteins following the protocol developed

by Maggo *et al*., (1999) with minor modification of addition of 2% PVP in buffer. The defatted flour (10 g) was shaken with 0.1 M sodium phosphate buffer (pH 7.6) for four hours at 200 rpm in a rotary shaker and supernatant was heat denatured at 80°C for 20 min in shaking water bath and snap cooled in ice. The extract was again centrifuged at 10,000 rpm for 15 min at 20°C (Sorvall RC 5 Plus centrifuge). The crude extract was subjected to ammonium sulfate fractionation (0-20%, 20-40%, 40-60%, 60-80% and 80-100%) and the precipitates obtained on centrifugation were dissolved in 1-2 ml of 0.1 M sodium phosphate buffer (pH 7.6) and were dialyzed extensively against the same buffer. The protein content of the dialyzed extract was determined by Lowry's method (Lowry *et al*., 1951).A standard spectrophotometeric assay was used to measured protease inhibitor activity.

Purification of Protease Inhibitor Protein

The fraction 40-80% has highest Protease Inhibitor (PI) activity used for purification of PI protein through ion-exchange chromatography (DEAE-cellulose column of 50x2cm). The dialyzed protein was loaded on the DEAE-cellulose column. The unbound proteins were washed with 0.1M sodium phosphate buffer (pH 7.6). Bound proteins were eluted using 0.01M-0.1M NaCl gradients in 0.1M sodium phosphate buffer (pH 7.6) in 5 ml fractions with the flow rate of 36 ml/hr. Eluted fractions were monitored at 280nm. Fractions having protein were lyophilized and used for protein estimation. These fractions were used for PI assay using Bovine trypsin enzyme and BApNA as a substrate. Fraction showing PI activity were pooled and passed through Biogel-P100 column (60x2 cm) for gel filtration and 5 ml fraction were collected. Each fraction was again monitored at 280nm and the inhibitory protein was then lyophilized and PI assay was performed with known quantity of protein and PI activity. Fraction showing PI activity was resolved on SDS-PAGE 15% using the following protocol Laemmli (1970) along with suitable molecular weight marker for estimation of molecular weight of PI protein.

Insects Culture

A test colony of the insects (*Helicoverpa armigera*) was maintained in insectary at 24°C±2°C, 50-60% relative humidity (RH) and 14 hrs light. The photo phase started at 5:30 AM. The rearing procedure followed from NRI Bulletin 57 (Armes *et al*., 1992). The eggs of *H. armigera* were incubated in 30 ml transparent cup with unwaxed cardboard lid at 20°C temperature until they hatched. The newly hatched larvae were removed from the cup and transferred to chickpea based semi-synthetic diet (Singh and Rembold, 1992). On fourth day, the larvae were weighed and transferred to semi-synthetic diet containing three levels of PPI treatments.

Bioassay on *Helicoverpa armigera*

The neonate larvae of *Helicovarpa armigera* were reared on semi artificial diet supplemented with PPI. The diet protein casein was reduced to enhance the toxicity of PPI. The purified PPI was added to the basic diet in three different concentrations *i.e.* 5,000 TUI; 10,000 TUI and 20,000 TUI. These treatments were referred to as T1, T2 and T3, respectively (Nandeesha and Prasad, 2001).The dietary protein (casein) was reduced from 0.80% - 0.55 % to increase the PPI concentration from 0.048% - 0.144% and taken the observations at alternate days on developing larvae.

RESULTS AND DISCUSSION

Screening of pigeonpea varieties for protease inhibitor

The seed flour extracts of four selected varieties of pigeonpea *viz.* Pusa-855, Pusa-33, Pusa-987 and Pusa-84 were screened for the presence of protease Inhibitor (PI) activity. The protease inhibitor activity was expressed as trypsin unit inhibited/mg of protein as shown in (Figure - 1 and Table -1). The PI activity was present in all the varieties but showed the variation at inter-varietal level. The highest PI activity was found in the variety P-33 which has 19.7% of total protein out of which 0.62% PI protein. Whereas, the lowest protein content (10.24%) of which 0.69% PI activity was found in the variety P-88.The protein content of commonly grown pigeonpea cultivars ranges between 17.9% and 24.3% for whole grain sample (Salunkhe *et al.* 1986). The variety P-33 was further used for purification of PI protein for the insect bioassay. The molecular weight of the purified PI protein was estimated by

running on SDS- PAGE along with molecular weight marker and found to be approximately 26 kD (Figure-2).

Insect bioassay

The purified PPI protein incorporated in the larval diet showed dose dependent influence on the growth and development of *H. armigera* while all the untreated larvae stopped feeding on the fourteenth days and entered in to the pre-pupal stage. The larvae in the three treatments, *viz.;* T_1, T_2 and T_3 have taken 18, 24 and 26 days, respectively to complete the feeding stages and the larval period extended was 4, 10 and 12 days in the treatment T_1, T_2 and T_3, respectively (Table -2 and Figure-3).

The feeding of pigeonpea protease inhibitor reduced the larval body weight significantly as compared to control diet (without inhibitor) indicating the possible response of ecdysis to semi starvation. The reduction of body weight varied in different treatments (Figure-3A).The final weight of the larvae in control was 380.4 mg whereas; body weight was reduced to 190.4mg, 287.0 and 220.2 mg in T1, T2 and T3, respectively. The increasing concentration of the inhibitor in the diet resulted in progressive reduction of larval growth. The results are in congruence with Johnston *et al*. (1993) reported a reduction of total biomass by 50% in larvae fed on diet supplemented with soybean protease inhibitor. However, significant reduction in larval growth of *H. armigera* was also reported by Sudheendra and Mulimani, (2002) when fed with mungbean and chickpea protein inhibitors.

The larval mortality was also found in dose dependent manner with highest mortality of 46 % was reported in the diet mixed with 20,000 PI units/ml followed by 33% and 20% in the diet containing 10,000 and 5,000 PI units/ml, respectively, However, no mortality of larvae was found in the control (diet without PPI protein) conditions. The pupal weight was drastically reduced in the treatments, T2 and T3, the difference in the pupal weight between the control and T1 was 64.6 mg (Figure- 3B). The adults were observed to be inactive and failed to mate and lay

Table 1. Protease inhibitor activity in 40-80% fraction of pigeonpea varieties

Pigeonpea varieties	P-855	P-33	P-971	P-84
Protein Conc. (mg/g seeds)	14.27	19.79	13.24	10.24
PI Activity (U/mg)	809	860	788	696

Table 2. Effect of protease inhibitor (PI) protein on growth parameters of *Helicoverpa armigera*

Age of larvae (days)	Control (wt.mg.)	T-1 (wt.mg.) 5,000U/ml	T-2 (wt.mg.) 10,000U/ml	T-3 (wt.mg.) 20,000U/ml
6	40.51(15)	40.49(15)	40.20(15)	40.20(15)
8	182.10(15)	120.10(15)	111.00(15)	98.10(15)
10	250.15(15)	244.10(13)	151.10(13)	130.20(12)
12	290.50(15)	251.10(13)	199.20(12)	157.10(10)
14	380.40(15)	311.00(13)	272.30(10)	212.50(9)
16	-	328.00(12)	278.00(10)	211.20(8)
18	-	351.05(12)	280.00(10)	218.20(8)
22	-	-	287.00(10)	219.50(8)
26	-	-	-	220.20(8)
Pupal wt.	314.40(15)	289.80(12)	264.40(10)	204.00(8)
Mortality (%)	0%	20%	33%	46%
Extended Larvae growth	Normal	6days	8days	10days

eggs and did not survive more than 2-3 days. Some adult emergence from the larvae feeding on diet containing protease inhibitor were abnormal deformed and have crippled wings (Figure -4).

The result of the study showed that PPI retarded the growth and development of *H. armigera* larvae. The presence of PPI in diet significantly reduced the average body weight of larvae and the sizes of larvae were small in all the treatments as compared to control. Increase the level of PPI in the diet increased the mortality of larvae and reduced their growth rate. PPI also disrupt the normal development of larvae and adult emergence. The fecal output was also reduced up to 30-45% at the two lower concentrations and up to 60% at the highest concentration. The results of feeding trail showed that PPI has a good potential for protection of crop plants against *H. armigera*. The results are in agreement with Johnston *et al*. (1993)reported the protease inhibitor, interfering within the normal proteolysis and cause starvation of the larvae. The protease inhibitor leads to decline in feeding behavior of the insect, resulting in a decrease in growth causing death in several days (Pulliam *et al*., 2001).

PPI is the good candidate for incorporating resistance trait to develop insect pest resistance in crop plants. The expression of the PPI gene should be up to 0.144- 0.15%, which will give the significant effect on targeted insect pest. The study indicated that 0.144% of PPI in diet level have the 46% mortality and which also delayed the period of larvae to pupation by 12 days. The *in-vivo* studies showed effectiveness of PPI on the targeted insect pest. The PI gene strategy is effective against the insect pest with minimal risk of counter adverse effect on human being due to low soluble protein. In the post defense strategy involving the parameter for crop protection is that at the minimal basal level of expression of gene should a high adverse effect on larval mortality or in delaying the larval growth, should be achieve (De-Leo *et al*., 2001).

The PI and other defensive proteins is the direct gene product. These defensive effects have been tested by genetic transformation in the number of plant species. The multidomain structure of PI may allow plant to produce inhibitor against a broad spectrum that retains their defensive function in the different chemical environment. These multidomain structures probably allow plant to target a large number of different protease within a relatively short period of time (Miller *et al*., 2000). The first PI gene from cowpea (CpTI) was successfully transferred into tobacco, rice, oilseed, resulting in enhancing resistance against *Lepidopteron*, *Manduca sexta* and *Spodoptera litura* insect (Hilder *et al*., 1987; Gatehouse *et al*., 1999). These studies provided direct evidence for the effectiveness of CpTI against specific insects. CpTI was tested against *Spodoptera litura* in the feeding tail under laboratory condition in rice, reduced 50% biomass at 3-5 mg of CpTI in fresh leaves of transgenic plant. The Soybean Kunitz trypsin inhibitor level

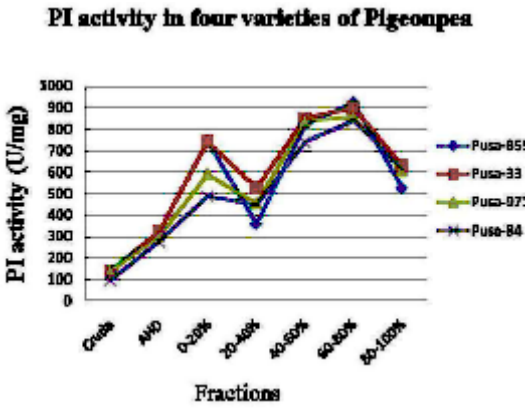

Fig. 1. Estimation of protease inhibitor (PI) activity in different varieties of pigeonpea

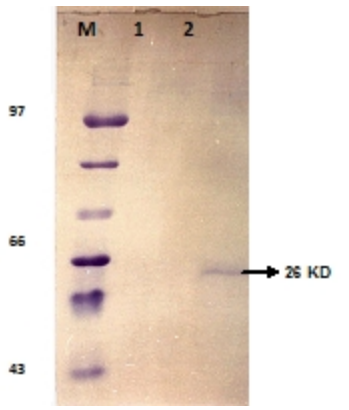

Fig. 2. Determination of molecular mass of PI protein on SDS-PAGE

in transgenic rice was found to be 0.05-0-2.5% of soluble protein (Lee *et al.*, 1995) and transgenic was resistance to brown plant hopper (*Nilaparvata lugens*).

PIs are highly specific for a particular class of digestive enzyme. However insect have shown enough flexibility to switch proteinase composition in their gut to overcome the particular protease inhibitor expressed in the transgenic plants (Johnston *et al.*, 1995). Insect belonging to

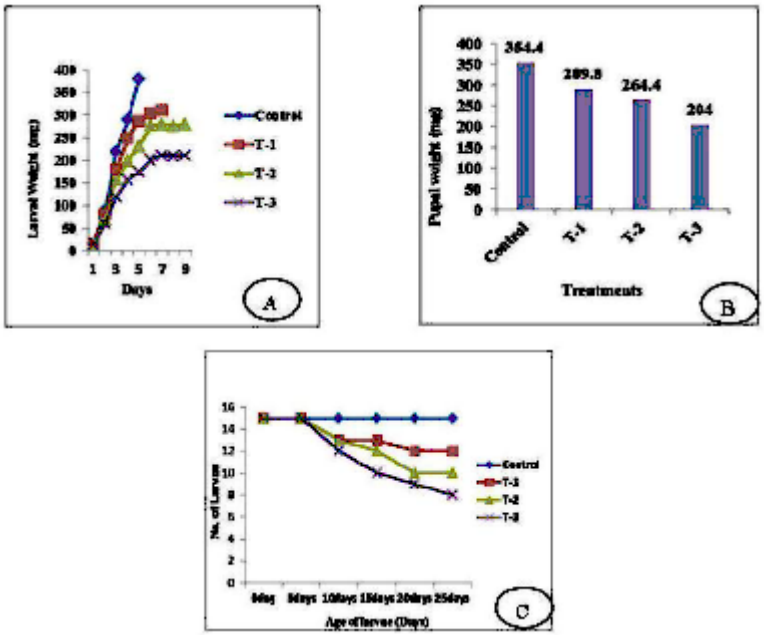

Fig. 3. Effect of pigeonpea protease inhibitor protein (PPI) on (A) larval weight; (B) pupal weight and (C) Survival rate of *Helicoverpa armigera*

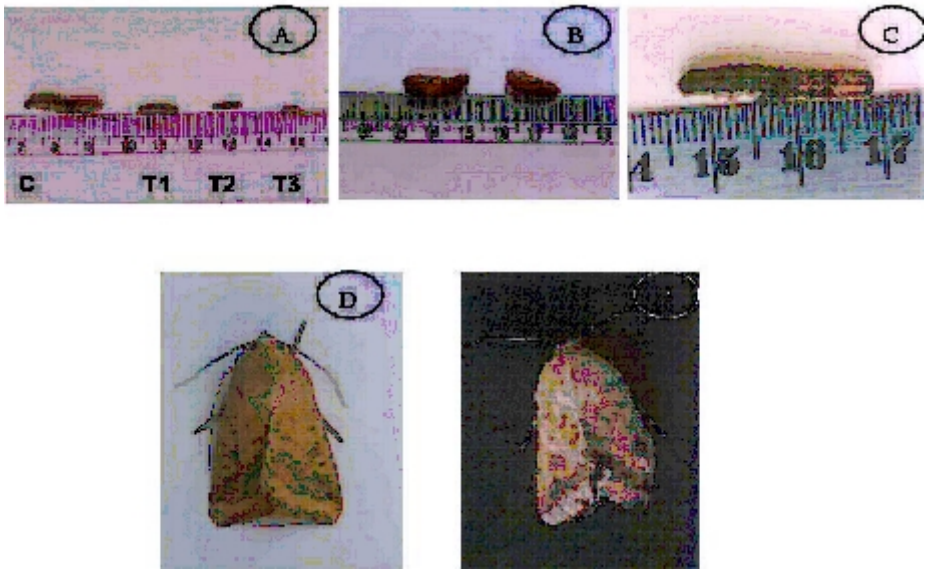

Fig. 4. Insect Bioassay: Effect of purified pigeonpea protease inhibitor protein on pupation of *H. armigera* larva (A-C) Larval stages and pupal stages (D) Normal adult of *H. armigera* (E) Deformed adult after treatment of purified protease inhibitor (PI) protein of pigeonpea

both lepidopteron and coleopteran orders can over express existing gut protease or overcome the production of new types that are insensitive to the protease inhibitor to overcome the deleterious effect of PI ingestion (Bhatia and Mitra, 1998). This may be contributing for the decrease effectiveness of PIs expressed in transgenic plant. The high level of expression of Soybean PI gene in tobacco plant failed to confer resistance against *H. armigera* (Nandi *et al*., 1999). The gut protease not the target affected by PIs but they can also affect the water balance, moulting and enzyme regulation of insect (Boulter, 1993).

Little information on the biosafety evaluation of Genetically Modified (GM) food crops harboring PI genes is available. Since the PIs are the plant-derived genes produced and are easily inactivated by cooking from new host plant cause no problem in human (Bishnoi and Khetarpaul, 1994). Another important factor is that gene transfer to other species will not be creating any environmental hazard (Ussuf *et al*., 2001). The nutrition value of transgenic pea expressing bean amylase inhibitor has been investigated. It has minimal detrimental effect on the nutritional value of the pea fed at 30% of diet. The study indicated that transgenic pea could be used in diet without harmful effect on growth (Pusztai *et al*., 1999).

CONCLUSIONS

The protease inhibitor protein isolated from the different cultivars of pigeonpea showed the variable protease activity and was found to inhibit *Helicoverpa armigera* gut protease. The results of the insect bioassay reported the insecticidal potential of pigeonpea protease inhibitor against the *H. armigera*. PPI protein revealed its anti-metabolite activity as it affected both the growth and digestive physiology of *H. armigera*, leading to starvation and death. The PI protein of pigeonpea showed considerable potential against the *H. armigera* and could be considered as potential candidates for use in genetic transformation of crops for pest management. Furthermore, a combination of different plant protease inhibitors might produce a greater insecticidal effect. It is likely that PIs expressed and produced in higher amounts in the agronomically important crops would lead to development of resistance against a variety of polyphagous insects.

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