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Toxicity of Trypsin Inhibitor from *Phaseolus vulgaris* L. Cultivar against *Pieris brassicae*

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ABSTRACT

Trypsin inhibitor (TI), present in the seeds of a local yellow cultivar of French bean (*Phaseolus vulgaris* L.), which is native to the Himalayan region, was tested using the gut enzyme of *Spodoptera littoralis.* The midguts of cold-anesthetized larvae were dissected and homogenized in ice-cold 0.1 M sodium phosphate buffer (pH 7.5). The seed extract was tested for TI activity using gut extract. The Trypsin Units Inhibited (TUI) per gram of seed weight thus obtained were 892.67. TI was purified to near homogeneity by ammonium sulfate precipitation, ion exchange chromatography and gel permeation chromatography. The level of TI significantly increased in the leaves of *P. vulgaris* following mechanical wounding, indicating a strong systemic response of plants to injury. Purified TI was liquefied in distilled water, coated on cabbage leaf discs and fed to *Pieris brassicae* larvae along with distilled water-coated control leaf discs. It showed a marked effect on larval survival and development. Feeding inhibition and lesser fecal matter was observed in larvae reared on cabbage leaf discs coated with purified inhibitor as compared to control. The feeding bioassays showed that percent mortality of larvae fed on leaf discs coated with 150 µg and 300 µg of purified TI increased from 53.33% (after one day) to 78.67% (after four days) and 46.67% (after one day) to 90.00% (after five days), respectively. Further, excreta and size of the larvae were much smaller than controls. Therefore, the gene expressing this protease inhibitor could be used in vegetable crops to confer resistance against insect pests.

Keywords: insect bioassay, protease inhibitor, *Pieris brassicae*, *Spodoptera littoralis*

INTRODUCTION

Proteins which act as strong and specific inhibitors of proteolytic enzymes are widely distributed in plant tissues and termed protease or proteinase inhibitors, or PIs (Liener and Kakade 1980). They are diverse in number and in specificity towards various proteolytic enzymes (Yehulith 2003). On the basis of their specificity they have been classified into four categories viz., serine, cysteine, metallo and aspartyl protease inhibitors. The serine PIs possess two active sites which inhibit trypsin and chymotrypsin. Serine and cysteine PIs are abundant in seeds of legumes (Guillamon *et al.* 2008) and storage tissues of plants (Reeck *et al.* 1997). The presence of proteins with the ability to inhibit trypsin, chymotrypsin and other proteolytic enzymes has been demonstrated in *Phaseolus vulgaris* seeds. Trypsin inhibitors (TIs) were studied in *Phaseolus vulgaris* cv. 'Canadian Wonder', *P. vulgaris* (Red Kidney, Pinto, Navy), *Phaseolus lunatus* (Hove and King, 1979) and *P. vulgaris* var. Rosinha G_2 (Garbieri and Whitaker 1981). The TI content found in seeds of *P. vulgaris* cv. 'Canadian Wonder', *P. vulgaris* (Red Kidney), *P. vulgaris* (Pinto), *P. vulgaris* (Navy) and *P. lunatus* was 10.5, 11.6, 3.4, 10.8 and 20.2 mg/g of seed weight, respectively.

PIs have been proposed to function in regulating endogenous proteases during the metabolism of storage proteins (Richardson 1991) and factors that protect from insect and pathogen attack (Ryan 1990). The activity of PI on gut proteases attenuates amino acid assimilation and slows the growth of feeding insects (Jongsma and Boulter 1997). The proteolytic enzymes in the insect guts are primarily responsible for the breakdown of plant proteins. The proteins are digested in the insect midgut by digestive enzymes, particularly trypsin and chymotrypsin (Srinivasan *et al.* 2006) that are active in slightly alkaline (Lepidoptera) (Terra *et al.* 1996) to slightly acidic pH (Coleoptera) (Applebaum 1985). Serine proteases (trypsin, chymotrypsin and elastase endoprotease) are most active in alkaline (pH 10-12) guts of Lepidopteran insects (Christeller *et al.* 1992). So, the serine protease inhibitors can be used against these insects. Other species of insects use thiol proteases (cysteine proteases) as their primary protein digestive enzymes and they can be targeted with thiol protease inhibitors. Several proteases has been purified and well characterized from many insect species viz., *Manduca sexta* (Peterson *et al.* 1995), *Locusta migratoria* (Lam *et al.* 1999), *Rhyzopertha dominica* (Zhu and Baker 1999), *Lasioderma serricorne* (Oppert *et al.* 2002), *Spilosoma oblique* (Anwar and Saleemuddin 2002), *Helicoverpa zea* (Volipicella *et al.* 2003) and *Conogethes punctiferalis* (Josephrajkumar *et al.* 2007). These studies reveal that trypsin and chymotrypsin are the prominent digestive proteases of Lepidopterans and Coleopterans. PIs have been found to be effective against many Coleopteran (Elden 2000) and Lepidopteran (Liao *et al.* 2007) pests. Therefore, a thorough understanding of insect gut proteases is required for the development of pest resistant transgenics using PIs. Thus, the natural protective role of PIs against phytophagous insects, and the availability of PI-encoding sequences, encouraged the development of pest resistance programs based on PI expression in transgenic plants. Several plant species have been transformed with the PI genes for controlling insect pest (Michaud 2000). Pest-resistant tobacco (Johnson *et al.* 1989; Marchetti *et al*. 2000), alfalfa (Thomas *et al.* 1994), rice (Duan *et al.* 1996) and potato (Gatehouse *et al*. 1997; Marchetti *et al*. 2000), among other plants, have been developed. TIs could be used in conjunction with other insecticidal genes such as *Bt* genes (Zhang *et al*. 2002) and other protease inhibitors (Amirhusin *et al.* 2007) to increase the level and diversify the basis of resistance in transgenic plants. Despite several reports on successful protection of plants against insect pests through PIs, defense strategies based on PI expression in plants has not

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resulted in any commercial application so far.

PIs continue to attract the attention of researchers because of their increasing use in medicine and crop biotechnology programmes (Boulter 1993; Koundal and Rajendran 2003). It has been reported that consumption of *P. vulgaris* (black and navy beans) significantly lowered colon cancer incidence and multiplicity in rats (Bennink 2002). PIs are also considered as the most potent medications for HIV (Human immunodeficiency virus). The drugs developed so far are indinavir (Crixivan®), ritonavir (Norvir®), nelfinavir (Viracept®) and saquinavir (Invirase® or Fortovase®). HIV uses protease in the final stages of its reproduction (replication) process. PIs block protease and thus interfere with HIV reproduction. These drugs may improve symptoms and suppress infection but do not cure it (Wilson 1997; Nijhuis *et al.* 2007). They are frequently investigated with regard to the bioavailability of nutrients (Piergiovanni *et al.* 1994), pest resistance (Todd *et al.* 2002), and inter/intra-specific variability (Marconi *et al*. 1993).

The caterpillars of cabbage white butterfly (*Pieris brassicae* and *Pieris rapae*) cause serious damage to commercial crops of Brassicae. They feed on wild or cultivated crucifers (members of the cabbage family) and prefer cultivated cabbages and Brussel-sprouts (varieties of *Brassica oleracea*), as well as oil-seed rape (*Brassica napus*). The TI activity of purified TI from *Cassia obtusifolia* has already been demonstrated against trypsin enzyme present in the larval midgut of *P. rapae* (Liao *et al*. 2007). Similar inhibition of cysteine and aspartyl proteases in alfalfa weevil (*Hypera postica* Gyllenahl) midgut by biochemical and plant-derived PIs has been reported by Wilhite *et al*. (2000). The serine PI, aprotinin and cysteine protease inhibitor, E64 (*trans*-epoxysuccinyl-L-leucylamido-4-guanidino-butane) was administered continuously in artificial diets to neonate and one-week-old larvae of *Listronotus bonariensis* and *Sitona lepidus* by Todd *et al.* (2002). Aprotinin significantly inhibited the growth of *L. bonariensis*. E-64 caused significant reductions in growth of *L. bonariensis* and mortality in *S. lepidus.*

However, there is no report to date on the effectiveness of *P. vulgaris* TI towards the insect *P. brassicae*. The experiments conducted in the present investigation shed light on the role of plant PIs in providing resistance against insect attack. In the future, the *P. vulgaris* TI gene may be used to develop transgenic crucifers resistant to *P. brassicae*.

MATERIALS AND METHODS

Seeds of bean cultivar and collection of insects

The seeds of *P. vulgaris* cv. 'Local Yellow' were procured from the Vegetable Research Station, Kalpa, India. The larvae of *S. littoralis* and eggs of *P. brassicae* were obtained from the Department of Entomology and Apiculture, Dr. Y S Parmar University of Horticulture and Forestry, Solan, India.

Extraction of larval midgut protease of *Spodoptera littoralis*

One day-old actively growing lab-cultured 4th instar *S. littoralis* larvae were selected for gut extraction. Four midguts from cold-anesthetized larvae were dissected out on ice, cleaned with tissue paper to remove the foodstuff and homogenized with 2 ml of 0.1 M phosphate buffer (pH 7.5) in a chilled test tube, using a glass rod. The homogenate obtained was filtered through filter paper (Whatman Grade 1, Diameter 30 mm manufactured by Star Micronic Devices, New Delhi) to obtain a filtrate of *S. littoralis* gut enzymes.

Extraction of inhibitor protein

A crude extract of *P. vulgaris* was prepared according to the method described by Hajela *et al.* (1999). Briefly, seeds of *P. vulgaris* were ground to a fine powder. The flour obtained was defatted with acetone (1:10 w/v) and air-dried 3-4 times. One gram of the defatted flour was shaken with 10 ml (1:10 w/v) of distilled water in a shaking water bath for 4 hours at room temperature. The suspension obtained was centrifuged at 10,000 × *g* for 30 min at 4°C. The supernatant thereby obtained was used for the TI assay and further studies.

Trypsin inhibition assay

The TI assay was performed according to the method described by Hajela *et al*. (1999). One ml of the filtered extract of *S. littoralis* larval midgut was mixed with 0.1 ml of the crude *P. vulgaris* extract (herein referred to as TI extract) and preincubated in a metabolic shaking water bath at 37°C for 10 min, followed by the addition of buffer I (prepared fresh by mixing 10 ml of 0.1 M phosphate buffer (pH 7.5), 4 ml of 0.1 M CaCl2 and 6 ml of distilled water) and 0.3 ml of α-benzoyl-DL-arginine-*p*-nitro anilide (BApNA, Sigma Aldrich, USA). The total volume of the reaction mixture was 2 ml. The reaction mixture was incubated at 37°C in a shaking water bath. After 10 minutes of incubation the reaction was stopped by adding 0.5 ml of 30% acetic acid. In the blank, the same procedure was adopted except for the fact that BApNA was not added to the reaction mixture. In the control, the same procedure was adopted except for the fact that in place of TI extract 0.10 ml of buffer I was added to the reaction mixture. The optical density of the test and control mixtures was measured at 410 nm against the blank using a UV/VIS spectrophotometer to measure trypsin inhibitor activity (TIA). BApNA is an artificial substrate that reveals the non-inhibited trypsin as it becomes yellow when it reacts with trypsin (Kakade *et al.* 1974). So, the trypsin unit inhibited (TUI) were calculated by comparing the absorbance measured for test mixture and control mixture. The decline in optical density (OD) monitored at 410 nm as compared to control of 0.01 OD per minute was taken as TUI.

Mechanical wounding of leaves

P. vulgaris seeds were germinated in a pot containing soil in a greenhouse under natural light. To test the hypothesis that TIs are wound-inducible, ten leaves of approximately the same stage (~ 48.3 cm^2 surface area) were mechanically wounded by punching holes (~ 0.25 cm radius) with a paper punch that had been wiped clean with alcohol. The leaves were detached from the plant by a single cut with a razor blade 48 hours after wounding. Ten leaves of a similar stage that had not been wounded were also collected for use as a control. The leaves were crushed in 0.1 M phosphate buffer (pH 7.5) (1:1, w/v) and the suspension was centrifuged at 10,000 × *g* for 30 min at 4°C. The supernatant obtained was used in a TI assay using trypsin enzyme instead of gut extract as described previously for the crude extract obtained from the *P. vulgaris* seeds.

Feeding bioassays

Insect bioassays were performed with *P. brassicae* larvae using a purified TI extract obtained from the *P. vulgaris* seeds. TI was purified to apparent homogeneity with 55.79% recovery using ammonium sulfate precipitation, ion exchange chromatography and gel permeation chromatography from the *P. vulgaris* seeds. The crude extract was precipitated 70% with ammonium sulphate and the precipitate dialyzed against distilled water and then subjected to gel filtration chromatography on Sephadex G-100. Fractions were collected and monitored for protein content at 280 nm and also analysed for TI activity. The trypsin inhibition assay was performed using trypsin enzyme (Sigma Aldrich, USA) instead of gut extract according to the method described under the heading "trypsin inhibition assay". The fractions showing trypsin inhibition were pooled and subjected to ion exchange chromatography on a DEAE Sephadex column. Fractions of 2 ml each were collected and monitored for protein ($A_{280\ nm}$) as well as TI activity. The peak fractions showing inhibitory activity were pooled together and concentrated by freeze drying at -86°C with an Allied Frost Freeze Dryer-2. The freeze-dried purified TI samples were used for conducting insect bioassays. The purified inhibitor was liquefied in distilled water. A stock of purified inhibitor was prepared fresh by dissolving 10 mg purified inhibitor in 10 ml of distilled water (1

Table 1 Effect of wounding on leaf TI activity (TIA). Experiment replicated three times.

Leaf damage	TIU per ml
Wounded leaf	41.833 ± 0.635
Normal leaf	31.933 ± 0.375
t-value (P<0.05)	23.245

TIU = trypsin inhibitor units

mg = 1000 µg so 10,000 µg of purified TI was present in 10 ml of distilled water and 1000 µg in 1 ml of distilled water). Three cabbage leaf discs (ϕ 6 cm, weight ~0.75 g) were coated with 0.15 ml of liquefied TI (containing 150 µg of purified TI) while another three cabbage leaf discs (ϕ 6 cm, weight ~0.75 g) were coated with 0.15 ml of distilled water alone (control). One hundred and fifty larvae (immediately after hatching) were collected and the feeding assay was conducted by feeding larvae of *P. brassicae* on treated as well as control leaf discs. Twenty five larvae were kept on each of the six leaf discs. All larvae were given fresh leaves after 24 h for 4 days. The % mortality, % leaf area eaten and weight of faecal matter was recorded after 24 h.

A similar feeding bioassay was conducted using one-week-old *P. brassicae* larvae. The experiment was again carried out in three replicates. Ten larvae were exposed per replication and 0.30 ml of liquefied TI (containing 300 µg of purified TI) was coated on leaf discs (ϕ 4 cm, weight ~0.3 g). The control leaf discs were coated with 0.30 ml of distilled water and ten larvae were placed on them. The larvae were given fresh leaves after 24 h for 5 days. The same parameters were recorded as above after an interval of 24 h for 5 days.

Statistics

The student's *t*-test was applied to the data from the mechanical wounding experiment because in case of a small sample size (less than 30) student's *t*-distribution is applicable. In the above-mentioned experiment a paired *t*-test (Agarwal 2003) was used for testing the mean difference of the samples. The experiments were carried out to find the effect of wounding on TI level. Appropriate controls (unwounded leaves) were set for comparing with the treatment. The experiment was conducted in three replicates. The use of paired samples enabled us to perform a more precise and accurate analysis. To test the significance the calculated *t* value was compared with tabulated *t* values at 5% level of significance.

The feeding bioassays were laid out in a completely randomized Design factorial (CRD factorial) (Cockran and Cox 1992). In factorial experiments the effect of the number of different factors can be investigated simultaneously. The experiments were conducted in three replicates. The F-ratio for the treatments and control is significant at the 5% level.

Angular transformation is applicable for the data expressed in percentage. The precise transformation is defined as arcsin square root of the data (Bhattacharyya 2000). Tables of angular value corresponding to percentages are available. For data spreading over a wide range of percentages angular transformation is used. In the feeding bioassays there was per cent data pertaining to the leaf area eaten, hence transformation was applied.

RESULTS

P. vulgaris cv. 'Local Yellow' seeds were found to contain 892.67 ± 0.333 trypsin units inhibited per gram of seed weight. This clearly shows that the TI present in seeds of this cultivar can efficiently inhibit the trypsin enzymes present in the guts of *Spodoptera littoralis* larvae.

The effect of mechanical wounding on leaf TI activity is presented in **Table 1**. The data from the TI assay of wounded and unwounded leaves showed that both leaf types had TI but that the amount of TI was more in wounded leaves. The trypsin units inhibited per ml were significantly greater in wounded leaves than in unwounded leaves.

The feeding bioassay with newly hatched *P. brassicae* larvae showed that the larvae were sensitive to purified TI. The larvae that were fed TI showed a significant reduction in development and survival immediately after hatching compared to control larvae (**Fig. 1**). Reduction in larval development was judged solely from a reduction in size and excreta. The percent mortality increased from 53.33% (after one day) to 78.67% (after four days) on the leaf discs coated in purified TI. The % leaf area eaten and faecal matter of the larvae that were fed on TI were significantly less than the control (**Tables 2, 3**).

The feeding bioassay with one-week-old P. brassicae larvae showed that they were also sensitive to the purified TI (**Fig. 2**). The percent mortality increased from 46.67% (after one day) to 90.00% (after five days). The percent leaf area eaten and faecal matter of the larvae fed on leaves coated with purified TI were significantly less than the control (**Tables 4, 5**).

Table 2 Percent leaf area eaten by *P. brassicae* larvae placed on leaf discs immediately after hatching. The experiment was conducted three times using a completely randomized design. Values expressed in the parenthesis are arcsine transformation of percentage data.

Concentration of Trypsin inhibitor (µg) (C)	Percent leaf area eaten Number of days (D)				Mean
	1st day	2nd day	3rd day	4th day	
Control (0)	3.20 ± 0.15 (1.79)	4.13 ± 0.13 (2.03)	5.58 ± 0.04 (2.36)	8.27 ± 0.12 (2.88)	5.29 (2.26)
150	0.77 ± 0.03 (0.58)	0.58 ± 0.02 (0.76)	0.50 ± 0.03 (0.73)	0.47 ± 0.03 (0.68)	0.59 (0.67)
Mean	1.99 (1.33)	2.36 (1.38)	3.04 (1.54)	4.37 (1.78)	

Effect	SE(m)	$CD_{(0.05)}$
C	0.03	0.03
D	0.22	0.05
C×D	0.03	0.04

Table 3 Faecal matter (mg) produced by *P. brassicae* larvae placed on leaf discs immediately after hatching. The experiment was conducted three times using a completely randomized design.

Concentration of Trypsin inhibitor (µg) (C)	Faecal matter (mg) Number of days (D)				Mean
	1st day	2nd day	3rd day	4th day	
Control (0)	2.77 ± 0.03	4.37 ± 0.17	4.57 ± 0.12	5.23 ± 0.15	4.23
150	0.90 ± 0.06	1.73 ± 0.07	1.17 ± 0.03	1.00 ± 0.00	1.18
Mean	1.84	3.05	2.87	3.12	

Effect	SE(m)	$CD_{(0.05)}$
C	0.07	0.15
D	0.10	0.21
C×D	0.14	0.30

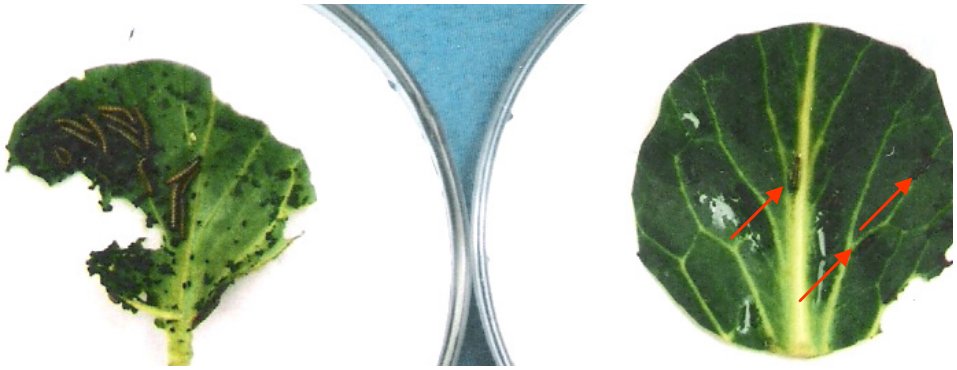

Fig. 1 Feeding inhibition of *Pieris brassicae* larvae fed on cabbage leaf disc coated with 150 µg purified trypsin inhibitor from immediately after hatching until 3 days of age. Left: Larvae grown on leaf without inhibitor (control). Right: Larvae grown on leaf coated with inhibitor (treatment).

Fig. 2 Feeding inhibition of one week-old *Pieris brassicae* larvae fed on cabbage leaf disc coated with 300 µg purified trypsin inhibitor for 3 days. Left: Larvae grown on leaf without inhibitor (control). Right: Larvae grown on leaf coated with inhibitor (treatment).

Table 4 Percent leaf area eaten by one week old *P. brassicae* larvae. Each treatment was replicated three times using a Completely Randomised Design. Values expressed in the parenthesis are arcsine transformation of percentage data.

Concentration of trypsin inhibitor (µg) (C)	**Percent leaf area eaten Number of days (D)**					**Mean**
	1st day	**2nd day**	**3rd day**	**4th day**	**5th day**	
Control (0)	76.33 ± 0.12 (60.89)	85.10 ± 0.06 (67.30)	94.87 ± 0.19 (76.94)	100.00 ± 0.00 (88.35)	100.00 ± 0.00 (88.35)	91.26 (76.37)
300	27.10 ± 0.06 (31.37)	18.03 ± 0.03 (25.13)	14.17 ± 0.17 (22.11)	5.33 ± 0.17 (13.35)	3.47 ± 0.15 (10.73)	13.62 (20.54)
Mean	51.72 (46.13)	51.57 (46.22)	54.52 (49.53)	52.67 (50.85)	51.74 (49.54)	

Effect	SE(m)	$CD_{(0.05)}$
C	0.07	0.15
D	0.12	0.24
C×D	0.16	0.34

Table 5 Faecal matter (mg) produced by one week-old *P. brassicae* larvae feeding on leaf discs. Each experiment was replicated three times using a Completely Randomised Design.

Concentration of trypsin inhibitor (µg) (C)	**Faecal matter (mg) Number of days (D)**					**Mean**
	1st day	**2nd day**	**3rd day**	**4th day**	**5th day**	
Control (0)	60.67 ± 0.67	64.33 ± 0.67	500.33 ± 0.33	999.70 ± 0.33	999.50 ± 0.29	524.90
300	1.07 ± 0.67	11.40 ± 0.70	73.33 ± 6.67	30.67 ± 0.67	20.00 ± 0.00	27.29
Mean	30.87	37.87	286.83	515.19	509.76	

Effect	SE(m)	$CD_{(0.05)}$
C	1.36	2.85
D	2.16	4.50
C×D	3.05	6.37

DISCUSSION

The protein was extracted in distilled water following the protocols of Hajela *et al.* (1999) and Maggo *et al.* (1999). Albumin-like (water soluble) inhibitors have been extracted from wheat, rye and chickpea in water (Kashlan and Richardson 1981; Poerio *et al.* 1989). However, globulin-type inhibitors have been extracted in 0.1 M NaCl (Richardson *et al.* 1987; Duarte *et al.* 1992), which may be buffered with sodium phosphate buffer (0.02 to 0.1 M, pH 6.0 to 7.6) (Richardson *et al.* 1986; Sastry and Murray 1987).

The gut extract was prepared in 0.1 M sodium phosphate buffer (pH 7.5). Mid gut proteases have been extracted from many lepidopteran species (Mohan and Gujar 2003). An elastase-like chymotrypsin was extracted from the guts of *Conogethes punctiferalis* larvae using 20 mM Tris HCl buffer (pH 8.0) by Josephrajkumar *et al.* (2007). The TI activity of seed extract was measured using BApNA as substrate and larval gut extract as source of enzyme. BApNA is an artificial substrate that reveals the non-inhibited trypsin as it becomes yellow when it reacts with trypsin (Kakade *et al.* 1974). The trypsin enzyme present in the gut extract was inhibited by the addition of PI and the residual trypsin activity was measured by adding BApNA. The reaction was terminated by 30% acetic acid. The inhibition of TIs has been determined in a similar manner by Hajela *et al.* (1999), Maggo *et al.* (1999) and Mulimani *et al.* 2002).

The trypsin activity of the gut extract from *S. littoralis*

larva was efficiently inhibited by the TI present in the crude extract of *P. vulgaris* cv. 'Local Yellow' seeds. This indicates the efficacy of TI against this insect pest.

The activity of TI in *P. vulgaris* leaves in this study significantly increased on wounding thereby indicating a strong systemic response of these bean plants to injury. A similar type of induction of TIs has been reported in the leaves of *Vigna umbellate* T. (rice bean) plants (Maggo *et al*. 1999). The leaves of rice bean plants were wounded on the periphery by making pin holes and sampled at different intervals viz, 4, 8, 16, 24 and 48 hours after wounding. The leaves of unwounded plants exhibited a very low level of TIA which increased sharply up to 8 hours after wounding. Thereafter, the activity remained almost constant. Similarly, PI was induced to accumulate in the aerial tissues of *Lycopersicon esculentum* (tomato) and *Solanum tuberosum* (potato) plants as a direct consequence of insect damage or mechanical wounding (Green and Ryan 1972). The findings that PI accumulates in large quantities in leaves as a result of insect damage demonstrate that insect behavior can rapidly and effectively influence the protein composition of plant leaves. The potential threat of the wound-induced PIs to the metabolism of the insect suggests that its function may be that of protection against insect attack.

In the present study, the feeding inhibition and reduction in *P. brassicae* larval growth was indicated by a significantly lower per cent leaf area eaten and lesser amount of fecal matter produced by the larvae when compared to the controls. This may be due to direct inhibition of the larval digestive enzymes and consequential depletion of essential amino acids by the TI. The reduction of larval survival and feeding found here is similar to that found in other studies. For example, transgenic tobacco plants expressing Cowpea Trypsin Inhibitor (CPTI) were found to be resistant against *Heliothis virescens* (Hilder *et al.* 1987). Young transgenic plants were infested with newly emerged larvae of *H. virescens* (tobacco budworm) in a growth cabinet. After seven days the larvae were removed, their size recorded and the extent of leaf damage was measured. Insect survival on and damage to ~20% of the transformants was clearly decreased compared to controls in repeated trials. Schuler *et al*. (1998) found that the serine PI, KTi 3 from soybean, resulted in up to 100% mortality of 1st instar cotton leaf worms (*S. littoralis*) when expressed in transgenic tobacco. In another study, the survival of the larvae of the noctuid, *Helicoverpa armigera,* was lower when reared on artificial diet impregnated with soybean TI (49% survival) compared to untreated control diet (90% survival) (Shukla *et al*. 2005).

On the contrary, the findings of Nandi *et al.* (1999) indicated that growth and development of young (3 day-old) *H. armigera* larvae were unaffected when fed the leaves of transgenic tobacco plants expressing very high levels of Soybean Trypsin Inhibitor (SBTI). However, when the purified SBTI extracted from transgenic plants was mixed with an artificial diet, significant reduction in growth of *H. armigera* larvae was observed. This was explained by the authors as free amino acids and amides present in the growing plants that could perhaps help the insect overcome the stress imposed by the presence of the antimetabolite, implying that the free amino acids and amides present in young tobacco leaves may be sufficient to sustain growth and development of these insects even in the presence of SBTI. The artificial diet, however, contained much less free amino acids and amides that are not sufficient for sustenance of the insects. Insects belonging to the order Hemiptera are known to nourish on free amino acids and amides of plants. This was not the case in our studies as the bioassays were conducted using cabbage leaf discs. Moreover, the test insect used in the present studies was *P. brassicae*. Thus, the present findings point to the possibility of using this TI in crop protection against insect damage.

We conclude that *P. brassicae* and *S. littoralis* larvae could be controlled by the TI present in *P. vulgaris* L cv. 'Local Yellow'. The effects of plants transformed to express the TI on the survival and development of these two insect pest larvae would need to be tested to confirm this. The effect of consumption of TI-transformed crops on human health is likely to be minimal as local yellow cultivar has been part of the human diet for a long time.

REFERENCES

Agarwal BL (2003) *Basic Statistics*, New Age International (P) Ltd. Publishers, New Delhi, pp 197-201

Amirhusin B, Shade RE, Koiwa H, Hasegawa PM, Bressan RA, Murdock LL, Zhu-Salzman K (2007) Protease inhibitors from several classes work synergistically against *Callosobruchus maculates*. *Journal of Insect Physiology* **53 (7)**, 734-740

Anwar A, Saleemuddin M (2002) Purification and Characterization of digestive enzymes from the larvae of *Spilosoma oblique*. *Archive of Insect Biochemistry and Physiology* **51**, 1

Applebaum SW (1985) Biochemistry of digestion. In: Kerkut GA, Gilbert LI (Eds) *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (Vol IV), Pergamon Press, Toronto, p 279-311

Bennink MR (2002) Consumption of black beans and navy beans (*Phaseolus vulgaris*) reduced Azoxymethane-induced colon cancer in rats. *Nutrition and Cancer* **44 (1)**, 60-65

Bhattacharyya BK (2000) *Statistics in Applied Sciences*, Kalyani Publishers, Noida, UP, India, pp 304-306

Boulter D (1993) Insect pest control by copying nature using genetically engineered crops. *Biochemistry* **34**, 1453-1466

Christeller JT, Liang WA, Markwick NP, Burgress EPJ (1992) Midgut protease activity in 12 phytophagous lepidopteran larvae: dietary and protease inhibitory interactions. *Insect Biochemistry and Molecular Biology* **22**, 248

Cockran WG, Cox WM (1992) *Experimental Design*, John Wiley & Sons (Asia), Inc., Singapore, pp 148-182

Duan X-L, Xue Q, Abo-El-Saad M, Xu D, Wu R (1996) Transgenic rice plants harboring an introduced potato proteinase inhibitor II gene are insect resistant. *Nature Biotechnology* **14**, 494-498

Duarte PR, Bergeron D,Nielsen SS (1992) Screening of heat-stable trypsin inhibitors in dry beans and their partial purification from Great Northern beans (*Phaseolus vulgaris*) using Anhydrotrypsin-Sepharose Affinity Chromatography. *Journal of Agricultural and Food Chemistry* **40**, 32-42

Elden TC (2000) Effects of proteinase inhibitors and plant lectins on the adult alfalfa beetle (Coleoptera: Curulionidae). *Journal of Entomology Science* **35**, 62-69

Gatehouse AMR, Davison GM, Newell CA, Merry weather A, Hamilton WDO, Burgess EPJ, Gilbert RJC, Gatehouse JA (1997) Transgenic Potato plants with enhanced resistance to the tomato moth, *Lacanobia oleracea*: Growth room trials. *Molecular Breeding* **3**, 49-63

Green T, Ryan CA (1972) Wound induced proteinase inhibitor in plant leaves: a possible defense mechanism against insects. *Science* **175**, 776-777

Guillamon E, Pedrosa MM, Burbano C, Sancher M de C, Muzguiz M (2008) The trypsin inhibitors present in seed of different grain legume species and cultivar. *Food Chemistry* **107**, 68-74

Hajela N, Pande AH, Sharma S, Rao DN, Hajela K (1999) Studies on a double headed protease inhibitor from *Phaseolus mungo*. *Journal of Plant Biochemistry and Biotechnology* **8**, 57-60

Hilder VA, Gatehouse AMR, Sheerman SE, Barker RF, Boulter D (1987) A novel mechanism of insect resistance engineered into tobacco. *Nature* **330**, 160-163

Hove EL, King S (1979) Trypsin inhibitor contents of lupin seeds and other grain legumes. *N Z Journal of Agricultural Research* **22**, 41-42

Johnson R, Narvaez J, An G, Ryan C (1989) Expression of proteinase inhibitors I and II in transgenic tobacco plant: effects on natural defense against *Manduca sexta* larvae. *Proceedings of the National Academy of Sciences USA* **86**, 9871-9875

Jongsma MA, Boulter C J (1997) The adaptation of insect to plant protease inhibitors. *Journal of Insect Physiology* **43**, 885-895

Josephraj Kumar A, Chakrabarty R, Thomas G (2007) Purification of elastase-like chymotrypsin from cardamom shoot and capsule bore. *Indian Journal of Experimental Biology* **45**, 998-1002

Kakade ML, Rackis JJ, McGhee JE, Puski G (1974) Determination of trypsin inhibitor activity of Soy products: A collaborative analysis of an improved procedure. *Cereal Chemistry* **51**, 375-382

Kashlan N, Richardson M (1981) The complete amino acid sequence of a major wheat protein inhibitor of alpha-amylase. *Phytochemistry* **20**, 1781-1784

Koundal KR, Rajendran P (2003) Pant insecticidal proteins and their potential for developing transgenics resistant to insect pests. *Indian Journal of Biotechnology* **2**, 110-120

Lam W, Coast GM, Rayne RC (1999) Isolation and characterization of two chymotrypsins from the midgut of *Locusta migratoria*. *Insect Biochemistry and Molecular Biology* **29**, 653

Liao H, Ren W, Kang Z, Tiang JH, Zhao XJ, Du LF (2007) A trypsin inhibitor from *Cassia obtusifolia* seeds: isolation, characterization and activity against *Pieries rapae. Biotechnology Letters* **29**, 653-658

Liener IE, Kakade ML (1980) Protease inhibitors. In: Liener IE (Ed) *The*

Toxic Constituents of Plant Food Stuffs (2nd Edn), Academic Press, New York, pp 7-71

Maggo S, Malhotra SP, Dhawan K, Singh R (1999) Purification and characterization of protease inhibitor from rice bean (*Vigna umbellata* T.) seeds. *Journal of Plant Biochemistry and Biotechnology* **8**, 61-64

Marchetti S, Delledonne M, Forgher C, Chiaba C, Chiesa F, Savazzini F, Giordano A (2000) Soybean Kunitz, C-II and P-IV inhibitor genes confer different levels of insect resistance to tobacco and potato transgenic plants. *Theoretical and Applied Genetics* **101**, 519-526

Marconi E, Ng NQ, Carnovale E (1993) Protease inhibitors and lectins in cowpea. *Food Chemistry* **47**, 37-40

Mohan M, Gujar GT (2003) Characterization and comparison of midgut proteases of *Bacillus thuringienesis* susceptible and resistant diamondback moth (Plutellidae: Lepidoptera). *Journal of Invertebrate Pathology* **82**, 1-11

Michaud D (Ed) (2000) *Recombinant Protease Inhibitors in Plants*, Biotechnology Intelligence Unit 3, Texas, USA, 242 pp

Mulimani VH, Sudheendra K, Giri A (2002) Detection of legume protease inhibitors by the Gel-X-ray film contact print technique. *Biochemistry and Molecular Biology Education* **30 (1)**, 40-44

Nandi AK, Basu D, Das S, Sen SK (1999) High level expression of soybean trypsin inhibitor gene in transgenic tobacco plants failed to confer resistance against damage caused by *Helicoverpa armigera*. *Journal of Biosciences* **24**, 445-452

Nijhuis M, Maarseveen Noortje M. van, Lastere S, Schipper P, Coakley E, Glass B, Rovenska M, Jong Dorien de, Chappey C, Goedegebuure Irma W, Heilek-Snyder G, Dulude D, Cammack N, Brakier-Gingras L, Konvalinka J, Parkin N, Krausslich Hans-Georg, Brun-Vezinet F, Boucher CAB (2007) A Novel substrate-based HIV-1 protease inhibitor drug resistance mechanism. *PLoS Med* **4 (1)**, in press

Oppert B, Hartzer K, Zuercher M (2002) Digestion proteinases in *Lasioderma serricorne* (Coleoptera: Anobiidae). *Bulletin of Entomology Research* **92**, 331-336

Peterson AM, Fernando GJP, Wells MA (1995) Purification, characterization and cDNA sequence of an alkaline chymotrypsin from the midgut of *Menduca sexta*. *Insect Biochemistry and Molecular Biology* **25**, 765-774

Piergiovanni AR, Gatta CD, Sergio L, Perrino P (1994) High antinutrient levels and bruchid resistance of cowpea (*Vigna unguiculata*) seeds. *Euphytica* **80**, 59-62

Poerio E, Carrano L, Garzillo AM, Buonocore V (1989) A trypsin inhibitor from the water soluble protein fraction of wheat kernel. *Phytochemistry* **28**, 1307-1311

Reeck GR, Kramer KJ, Baker JE, Kanost MR, Fabrick JA, Behnke CA (1997) Protease inhibitors and resistance of transgenic plants to insects. In: N Carozzi, M Koziel (Eds) *Advances in Insect Control: The Role of Transgenic Plants*, Taylor and Francis, London, pp 157-183

Richardson M, Campos FAP, Xavier-Filho J, Macedo MLR, Maia GMC, Yarwood A (1986) The amino acid sequence and reactive (inhibitory) site of the major trypsin isoinhibitor (DES) isolated from the seeds of the Brazilian Carolina tree (*Adenanthera pavonia* L.). *Biochimica et Biophysica Acta* **872**, 134-140

Richardson M, Valdes-Rodriguizo S, Blanco-Labra A (1987) A possible function for thaumatin and a TMV-induced protein suggested by homology to a maize inhibitor. *Science* **327**, 432-433

Richardson M (1991) Seed storage proteins: the enzyme inhibitors. In: Rogers LJ (Ed) *Methods in Plant Biochemistry, Amino Acids, Proteins and Nucleic Acid* (Vol 5), Academic Press, New York, pp 259-305

Ryan CA (1990) Genes for improving defenses against insects and pathogens. *Annual Review of Phytopathology* **28**, 425-449

Sastry MCS, Murray DR (1987) The contribution of trypsin inhibitors to the nutritional value of chickpea seed protein. *Journal of the Science of Food and Agriculture* **40**, 253-261

Schuler TH, Poppy GM, Kerry BR, Denholm I (1998) Insect resistant transgenic plants. *Trends in Biotechnology* **16**, 168-175

Sgarbieri VC, Whitaker JR (1981) Partial characterization of trypsin-chymotrypsin inhibitors from bean (*Phaseolus vulgaris* L. var. Rosinha G2): Chemical and physical properties. *Journal of Food Biochemistry* **5 (3)**, 215-232

Shukla S, Arora R, Sharma HC (2005) Biological activity of Soybean trypsin inhibitor and plant lectins against cotton bollworm/legume pod borer, *Helicoverpa armigera*. *Plant Biotechnology* **22**, 1-6

Srinivasan A, Giri AP, Gupta VS (2006) Structural and functional diversities in Lepidopteran serine proteases. *Cellular and Molecular Biology Letters* **11 (1)**, 132-154

Terra WR, Ferreira C, Jardao BP, Dillon RJ (1996) Digestive enzymes. In: Lehane MJ, Billingsley RF (Eds) *Biology of the Insect Midgut*, Chapman and Hall, London, p 153-186

Thomas JC, Wasmann CC, Echtc Dunn RL, Bonnert HJ, McCoy TJ (1994) Introduction and expression of an insect proteinase inhibitor in alfalfa. *Plant Cell Reports* **14**, 31-36

Todd JH, Malone LA, Gatehouse HS, Burgress EPJ, Christeller JT, Philip BA, Tregidata EL (2002) Effects of two protease inhibitors on larvae of Argentina stem weevil and clover root weevil. *New Zealand Plant Protection* **55**, 416-420

Volpicella M, Ceci LR, Codewener J, America T, Gallerani R, Bode W, Jongsma MA, Beekwilder J (2003) Properties of purified gut trypsin from *Helicoverpa zea* adapted to proteinase inhibitors *European Journal of Biochemistry* **270**, 10

Wilhite SE, Elden TC, Brzin J, Smigocki AC (2000) Cysteine and aspartyl proteases in alfalfa weevil midgut with biochemical and plant-derived proteinase inhibitors. *Insect Biochemistry and Molecular Biology* **30**, 1181-1188

Wilson BA (1997) Understanding Strategies for Treating HIV. *Medical Surgical Nursing* **6**, 109-111

Yehudith B (2003) *Plant Protease Inhibitors – Significance in Nutrition, Plant Protection, Cancer Prevention and Genetic Engineering*, Springer, Berlin, 107 pp

Zhang JH, Wang CZ, Qin JD (2002) The interaction between the soybean trypsin inhibitor and δ-endotoxin of *Bacillus thuringinensis* in *Helicoverpa armigera* larva. *Journal of Invertebrate Pathology* **75**, 259-266

Zhu YC, Baker JE (1999) Characterization of midgut trypsin like enzymes and three trypsinogen cDNAs from the lesser grain borer , *Rhyzopertha dominica* (Coleoptera: Bostrichidae). *Insect Biochemistry and Molecular Biology* **29**, 1053