Full Length Research Paper

Effect of plant seeds protein extract on the Sunn pest, *Eurygaste integriceps* Puton, growth and development and its gut serine protease activity

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The sunn pest *Eurygaster integriceps* Puton (Hemiptera: Scutelleridae) is a major pest of wheat and barley in wide areas of the world that cause 100% crop loss when no control measures are taken. The aim of this study was to investigate the effect of the seed proteinous extract of different plant species including Chickpea *Cicer arietinum* (Fabaceae), bean *Phaseolus vulgaris var. naz* (Fabaceae), triticale *Triticosecale wittmack* (Poaceae), *Celosia argentea* (Amaranthaceae) in artificial diet against the Sunn pest growth and development as well as gut serine proteinase such as trypsin and chymotrypsin activities. The obtained results showed that protease inhibitors present in the seed extract affected nymphal development, adult weight and survivability to some extent. Mean developmental time of third and fourth instar nymphs were not significantly affected by the presence of seed extracts. However, developmental time of the fifth instar nymph was affected by seeds proteinous extracts. Bean proteinous extract increased developmental time significantly by almost two days followed by Chickpea that increased developmental time by one day (P < 0.01). Amaranthus seed proteinous extract caused slight increase in development time. Triticale extract did not affect nymph growth and development and there were no significant differences between developmental time of triticale extract and control. Azocaseinolytic activity of gut extract of *E. integriceps* was affected greatly by seed proteinous extracts. There were significant differences in general protease activity between control and all treatments. General protease activity dropped significantly low when treated with bean and cowpea extracts (P< 0.01). Almost the same trend was observed when trypsin and chymotrypsin activities were measured using BApNA and SAAPFpNA as substrates, respectively. It is concluded that seed extracts from non host plants of the Sunn pest caused significant reduction of general and specific protease activity *in vitro*.

Key words: Sunn pest, growth, development, seed extracts, protease activity.

INTRODUCTION

The sunn pest *Eurygaster integriceps* Puton (Hemiptera: Scutelleridae) is a major pest of wheat and barley in wide areas of the near and middle east, west and central Asia, north Africa, and eastern and south Europe (Brown, 1965; Critchley, 1998; Parker et al., 2002). Sunn pest infestations in some areas are devastating and cause 100% crop loss when no control measures are taken.

Pesticide spraying is the main method of Sunn pest control in areas where infestation is high. In addition to the high cost of chemical control, insecticides pose a risk to the balance of nature, human health, water quality, wildlife and the environment as a whole (Javahery, 1995). Thus, to meet the demand for food of the world population, there is need of new ways for protecting plant crops against predators and pathogen while avoiding the use of pesticide chemicals. A milestone in the new control methods was introduction of gene expressing *Bacillus turingiensis* (BT) entomotoxic proteins (Carlini and Grossi-de-sa, 2002; Christou et al., 2006; Ferry et al., 2006). So, alternative to pesticide use could be exploring and exploiting the plant's own defense

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mechanisms by expression of their endogenous defense proteins or introducing an insect control gene derived from another plant. So, nowadays attention is being focused on the idea of using digestive enzyme inhibitors affecting the growth and development of pest species (Mehrabadi et al., 2010). Two main roles of digestive proteases in the insect digestive system are: (a) breaking down proteins into amino acids which are essential for growth and development and (b) inactivating protein toxins ingested during feeding (Terra et al., 1996). Disruption of both or either of these processes has the potential to suppress populations of phytophagous insect pests by limiting nutrient availability or by increasing the pests' vulnerability to toxic proteins.

The Sunn pest is a major pest of cereals with lacerate and flush feeding strategy (Miles, 1972). This mode of feeding suggests that introduction of plant defensive proteins that is lectins, α- amylase inhibitors, polyga-lacturonase-inhibiting proteins, or protease inhibitors would interfere with its normal digestive physiology and normal growth and development thus, suppress the pest population in the field. Proteinase inhibitors occur in the reproductive organs (seeds), storage organs and vegetative tissues of most plant families (Ryan, 1990; Richardson, 1991; Shewry and Lucas, 1997). It is proposed that these inhibitors as storage proteins or as regulators of endogenous enzymes and in defense against pest or pathogen attack. They are known to interfere with the pests digestive process through specifi-cally binding to the proteolytic enzymes present in the gut (Ryan, 1990), thus they function as plant defense molecules and were considered for use in preventing insect predation (Mosolov et al., 2001; Srinivasan et al., 2005). Many studies using plant derived proteinase inhibitors against class of proteinase/s in the gut of the insect showed that these proteins are able to retard growth and development of a wide range of insect pests. For example, soybean Kunitz trypsin inhibitor (the serine proteinase inhibitors SKTI) and cowpea trypsin inhibitor (CpTI) have been shown to have deleterious effect on many lepidopteran insects (Broadway and Duffey, 1985; Johnston et al., 1993; McManus and Burgess, 1995) but were ineffective against coleopterans. When soybean inhibitor was added to the diet of *Manduca sexta*, *Ostrinia nubilalis*, *Heliothis zea*, *Spodoptera exigua* resulted in retarded larval growth and development (Shukle and Murdock, 1983; Steffens et al., 1978; Broadway and Duffey, 1986). Coleopteran insects have been shown to be affected by cysteine proteinase inhibitors such as multicystatin from potato and oryzacystatins from rice seed (Kuroda et al., 1996; Edmonds et al., 1996). Addition of the soybean cysteine protease inhibitor into the *Challosobrachus maculates* diet caused retarded larval growth and development (Koiwa et al., 1998). Also, when *Leptinotarsa decimlineata* larvae feed on E-64 treated leaves, larval growth and development are inhibited (Wolfson and Murdock, 1987).

Enhanced resistance against Lepidoptera and Coleo-ptera has been achieved by expressing of serine and cysteine protease inhibitors into plants of different families. The insecticidal effects of protease inhibitors against sap-sucking phytophagous insects have been also established (Annadana et al., 2002; Rahbe et al., 2003). Thus, plants have evolved defense mechanisms most of which are concentrated in the seeds since these are the vehicles for propagation and survival of the species. Seed tissues accumulate a wide array of defense compounds that confer resistance against phyto-phagous insects (Carlini and Grossi-de-sa, 2002). So, the aim of this study was to investigate the effect of the seed proteinous extract of different plant species including Chickpea *Cicer arietinum* (Fabaceae), Bean *Phaseolus vulgaris var. naz* (Fabaceae), Triticale *Triticosecale wittmack* (Poaceae), *Celosia argentea* (Amaranthaceae) in artificial diet against the Sunn pest growth and development as well as gut serine proteinase such as trypsin and chymotrypsin activities.

MATERIALS AND METHODS

Chemicals

The enzyme substrates BApNA (Na-benzoyl-L-arginine p-nitroanilide), SAAPFpNA (N-succinyl-alanine-alanine-proline-phenylalanin p-nitroanilidine), Azocasein, were obtained from Sigma Company.

Insect culture

The insects were collected from the wheat farm during spring when feeding started. They fed and maintained on wheat grains in the laboratory conditions at 25 ± 2°C and a photoperiod of 14: 10 (L: D) cycles as described by Allahyari et al. (2010).

Preparation of luminal enzyme extract

Enzyme samples from midguts of adults were prepared by the method of Lam et al. (2000) with slight modifications. Briefly, adults were randomly selected and the midgut from these individuals were removed by dissection under a light microscope in ice- cold 50 mM Tris-HCl buffer (pH 8.0) containing 0.01 M $CaCl_2$. The midgut contents exuded into the buffer while stirring on ice, and the exudates was centrifuged at 12000 g for 10 min at 4°C.The supernatant were pooled (as an enzyme source) and stored at -20°C for subsequent analysis.

Extraction of proteinous inhibitors from plant seeds

Proteinous extract from seeds of "triticale, bean, chickpea and amaranthus" was extracted according to Baker (1987) and Melo et al. (1999). Briefly, ground seeds (30 g each) were mixed with a solution of 0.1 M NaCl and stirred for 2 h, followed by centrifugation at 10,000 g for 30 min. The pellet was discarded, and the supernatant was incubated at 70°C for 20 min to inactivate major endogenous enzymes. Fractionation of the supernatant was done using different concentrations of ammonium sulfate (20, 40, 60 and 80%) followed by centrifugation at 10,000 g for 20 min at 4°C. The

60% pellet containing the highest fraction of protease inhibitors was dissolved in ice cold sodium phosphate buffer (0.02 M and pH 7.0) and dialyzed overnight against the same buffer. The dialysed solution was frieze dried, weighed and kept at -20°C as a source of protease inhibitor.

Diet preparation and insect bioassay

To examine the effects of seed protease inhibitors on the growth and development of the Sunn pest's nymphs, an artificial diet was established. Artificial diets were prepared as described by Saadati and Bandani (2010). The levels of inhibitor used in the diet expressed were as percentage protein of inhibitor per protein of diet (W/W). The inhibitors (lyophilized powder) added to the diet were Triticale (1%), Bean (1%), Chickpea (1%) and Amarantus (1%). The control diet was without inhibitors. Because first instars of *E. integriceps* do not feed and second instar nymphs are so small, third nymphal instar was transferred to the diet with fine brush and its growth and development was monitored until adult (24 h post-molt) was emerged. In each treatment (each dose) 12 newly molted third instar nymphs were used and each treatment had 5 replicates. Diets were replaced twice a week. The insects were kept at 25±2°C and 14:10 (Dark: Light) photoperiod. Nymphal development times, survival rate and adult weights 24 h post molt were measured.

Effects of seeds proteinous inhibitors on the gut proteolytic activity

To analyze the effect of seeds proteinous inhibitors on protease activity, two assays including general protease assay and specific protease assays were conducted. Gut extract from control insects (insects grown on wheat grains) were prepared and then the effect of plant seeds proteinous extract on the gut proteases were tested using general protease substrate (2% azocasein) and specific serine protease substrate namely: 1 mM BApNA and 1 mM SAAPFpNA (Elpedina et al., 2001). To do the assays, the enzymes (gut extract) were preincubated with the appropriate seed proteinous extract at 30°C for 30 min, then substrate was added and the assays were proceeded. General protease assay was done using 2% azocasein (Sigma) as substrate. The buffer used was universal buffer system pH 6.0 (40 mM sodium acetate–phosphate–borate). Reaction mixture was consisted of pre-incubated enzyme (midgut extract) with seed extract, substrate and universal buffer. Reaction started with the addition of substrate and incubation was done at 30°C. 500 µm of reaction mixture were removed at 0, 5, 10, 15, 20 and 30 min time intervals and equal amount of 30% (w/v) trichloroacetic acid (TCA) was added to each in order to terminate reaction. The samples were mixed, left to stand for 30 min at 4°C and centrifuged at 15000 g for 15 min. Supernatant was removed and an equal volume of 1 M NaOH was added to it and absorbance was recorded at 410 nm. Appropriate blanks (no substrate and no enzyme) were run for all assays. Also, the effect of seed proteinous inhibitors on specific protease activity (trypsin and chymotrypsin activity) was carried out according to the method of Gatehouse et al. (1999) with slight modifications. To do this, gut extracts was prepared and enzyme activity in the presence of appropriate seed proteinous extract was measured using 1 mM BApNA (Na-benzoyl-L-arginine p-nitroanilide) and 1 mM SAAPFpNA (N-succinyl-alanine-alanine-proline-phenylalanin p-nitroanilidine) for calculation of trypsin and chymotrypsin activity, respectably. Substrate was dissolved in dimethyl-sulfoxide (DMSO) and then diluted in buffer and final concentration of DMSO in solution was less than 10%.

The hydrolysis of substrates was monitored continuously at 410 nm at 30°C and initial rates were measured from the slopes of absorbance against time.

Proteinase assay using gelatin/PAGE

E. integriceps luminal proteolysis was qualitatively assayed using gelatin/PAGE based on Laemmli (1970) and Walker et al. (1998). Gut extract (3 mg/ml of total soluble protein) was run on a 12.5% resolving gel co-polymerised with 0.1% gelatin. The sample-loading buffer did not contain mercaptoethanol and samples were not boiled prior to loading. To test the effect of inhibitors on protease activity, 20 µl of the gut extracts were mixed with 20 µl of seed extracts and incubated for 1 h at 30°C. Then they were mixed in a 1:1 ratio with sample buffer ,and 25 µl of the final solution loaded on the gel. Gut extracts incubated without inhibitor were used as controls. Electrophoresis was conducted at 4°C and following electrophoresis SDS was eluted from the gel by incubation in 2% (v/v) Triton X-100 for 30 min at 37°C. Then, the gel was incubated in Ttris-buffer solution (pH 8) at room temperature overnight. Then, gel was stained in 40% methanol, 7% glacial acetic acid and 0.05% Coomassie Brilliant Blue R and destained until proteolytic activity was seen as clear bands in a dark background.

Protein determination

Protein concentration was measured according to the method of Bradford (1976), using bovine serum albumin (Bio-Rad) as a standard.

Statistical analysis

One-way analysis of variance (ANOVA) was done to compare nymphal development times and adult weights of the insects reared on different diets.

RESULTS

Effect of inhibitors on the Sunn pest development time

Analysis of variance (ANOVA) indicated that protease inhibitors affected nymphal development to some extent (Table 1). Mean developmental time of third and fourth instar nymphs were not significantly affected by the presence of seed extracts (Table 1). However, developmental time of the fifth instar nymph was affected by seeds proteinous extracts. Bean proteinous extract increased developmental time significantly by almost two days followed by Chickpea that increased developmental time by one day (P < 0.01). For example developmental time in control, bean seeds extract, chickpea extract was 7.1, 9.12 and 8.41 days, respectively. Amaranthus seed proteinous extract caused slight increase in development time. Triticale extract did not affect nymph growth and development and there were not significant differences between developmental time of triticale extract and control (P > 0.01).
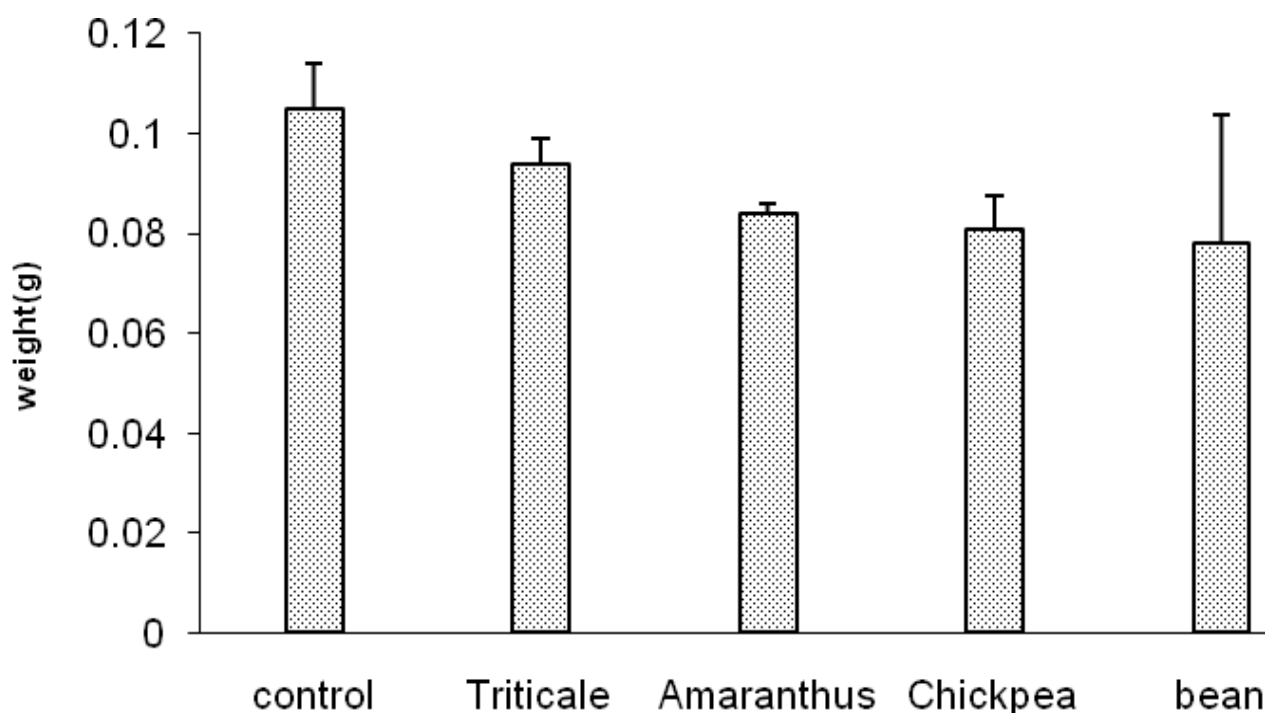
Effect of seed proteinous extract on adult weight and survivability

Adult weight measurement 24 h post-emergence showed

Table 1. The effects of seed proteinase inhibitors incorporated into diet on the Sunn pest nymphal development time.

Treatment	Developmental time (day)		
	3rd instar nymph (mean ± se)	4th instar nymph (mean ± se)	5th instar nymph (mean ± se)
Control without seed extract	7.68 ± 1.4[a]	8.03 ± 0.93[a]	7.1 ± 0.1[b]
Triticale seed extract	8.28 ± 0.81[a]	8.66 ± 0.63[a]	7.19 ± 0.22[b]
Amarantus seed extract	8.73 ± 1.52[a]	8.45 ± 0.41[a]	8.06 ± 0.09[ab]
Bean seed extract	7.2 ± 0.5[a]	9.37 ± 1.65[a]	9.12 ± 0.88[a]
Chick pea seed extract	7.2 ± 0.77[a]	9.37 ± 1.09[a]	8.41 ± 0.95[a]

Means (± se) followed by the same letter indicate no significant differences ($P < 0.05$) between data based on Turkey test.

Figure 1. The effect of protein extracts incorporated into diet on the Sunn pest weight. Seed protein extracts incorporated into the diet and offered to the third instar nymphs and adult weight were measured at 24 h post emergence.

that there are no significant differences between adult weights in different treatments ($P> 0.01$) (Figure 1).

However, data obtained showed that slight change in body weight occurs in different treatment. For example the lowest body weight obtained when nymphs fed on bean seed extracts followed by chickpea, amaranthus, and triticale (Figure 1). Figure 2 shows the survivability of the Sunn pest when feeds on the seed extracts of different plant species. As shown, amaranthus seed extract had the great effect and caused more than 50% mortality whilst the other three plant extracts (triticale, chickpea and bean) had less activity against the insect survivability.

Effect of seed proteinous extracts on midgut protease activity

Azocaseinolytic activity of gut extract of *E. integriceps* was affected greatly by seed proteinous extracts (Figure 3). There were significant differences in general protease activity between control and all treatments. General protease activity dropped significantly low when treated with bean and cowpea extracts ($P< 0.01$). Order of protease activity reduction when treated by extracts was as bean > cowpea > triticale > control. Almost the same trend was observed when protease activity was measured using BApNA as a substrate ($P < 0.01$) (Figure
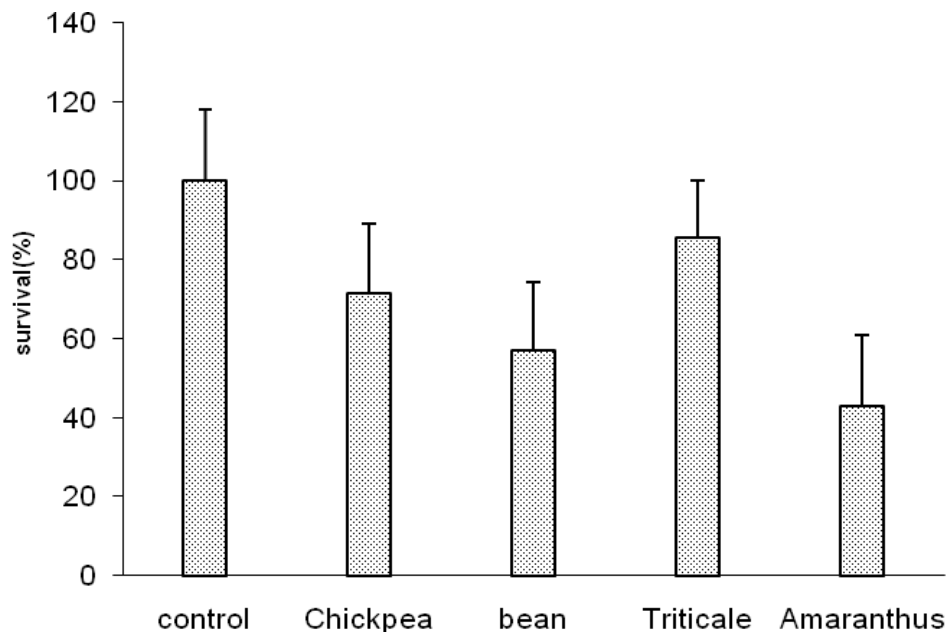
Figure 2. The effect of protein extracts incorporated into diet on the Sunn pest survival. Seed protein extracts incorporated into the diet and offered to the third instar nymphs and their survival were measured at 24 h post emergence of adult.
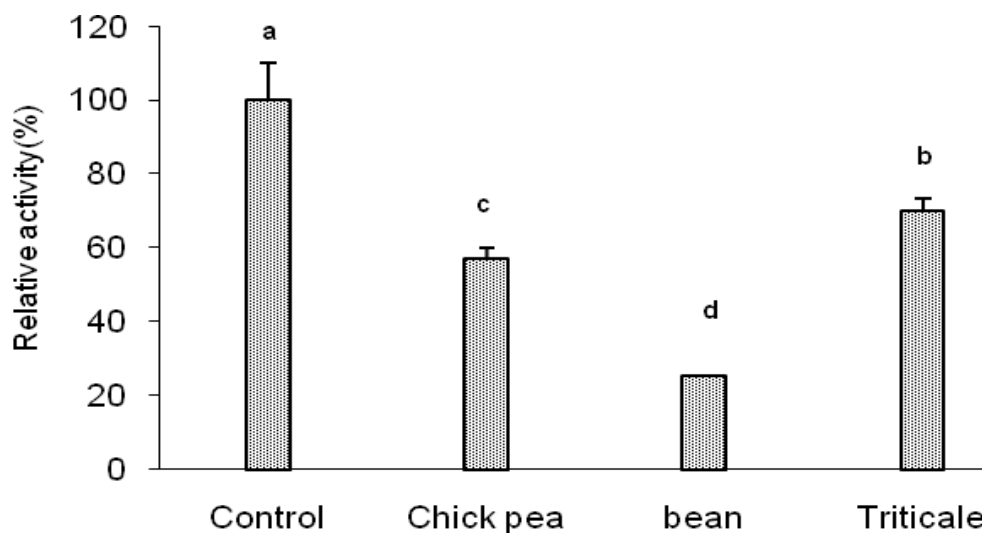

Figure 3. Effect of plant seed protein extract on general protease activity *in vitro* using azocasein as a substrate. Means (± se) followed by the same letter above bar indicate no significant differences (P < 0.05) between data based on Tukey test.

4). Trypsin activity significantly reduced compared with the control and reduction of protease activity was in the order of bean > triticale > amaranthus > chickpea> control (Figure 4). When protease activity was measured using chymotripsin substrate (SAAPFpNA), activity was suppressed more by triticale seed extract followed by bean, chickpea, amaranthus and control, respectively

(Figure 5). So, as can be seen the amaranthus seed extract had the least effect on chymotrypsin activity.

Proteinase assay using gelatin/PAGE

Effect of seed proteinous extract on protease activity was
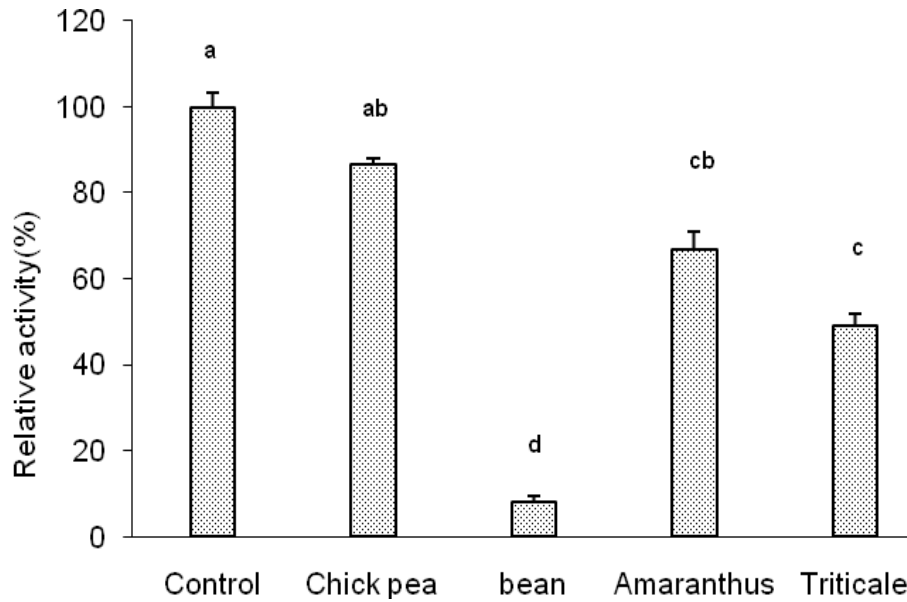
Figure 4. Effect of plant protein on trypsin activity *in vitro* using BApNA as a substrate. Means (± se) followed by the same letter above bar indicate no significant differences (P < 0.05) between data based on Tukey test.
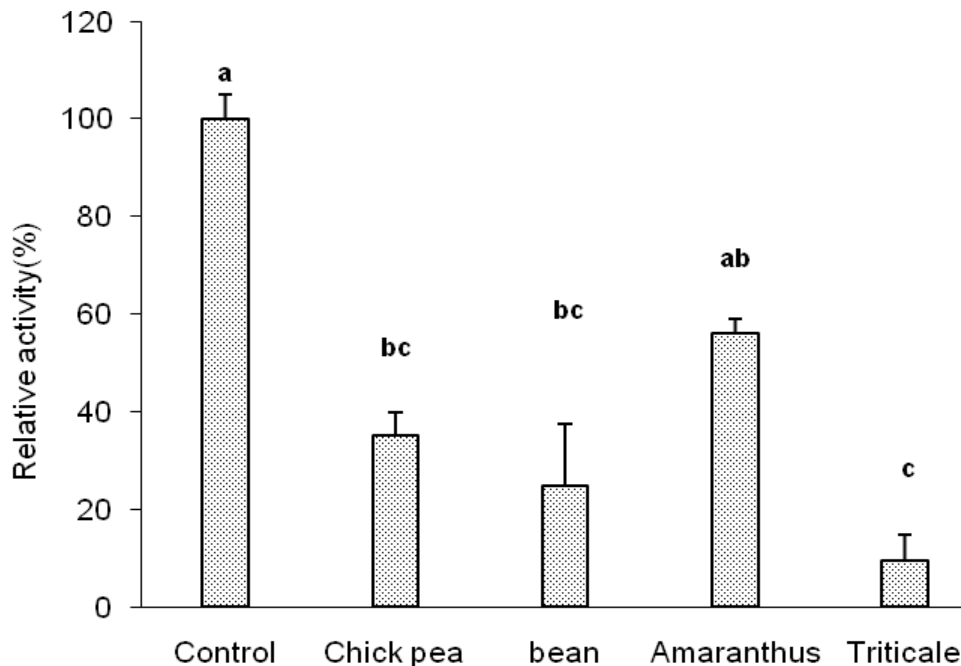

Figure 5. Effect of plant inhibitors on chymotrypsin activity *in vitro* using SAAPFpNA as a substrate. Means (± se) followed by the same letter above bar indicate no significant differences (P < 0.05) between data based on Tukey test.

tested using gelatin gel. Results showed that bean proteinous extract almost completely removed proteinase activity. It showed that bean extract was the most effective inhibitor against Sunn pest proteases. The other most effective inhibitor was cowpea that although, it was not as strong as bean, it had some effect on the Sunn pest proteases. The other seed extract (triticale) had the lowest inhibitory effect among seed proteinous extract
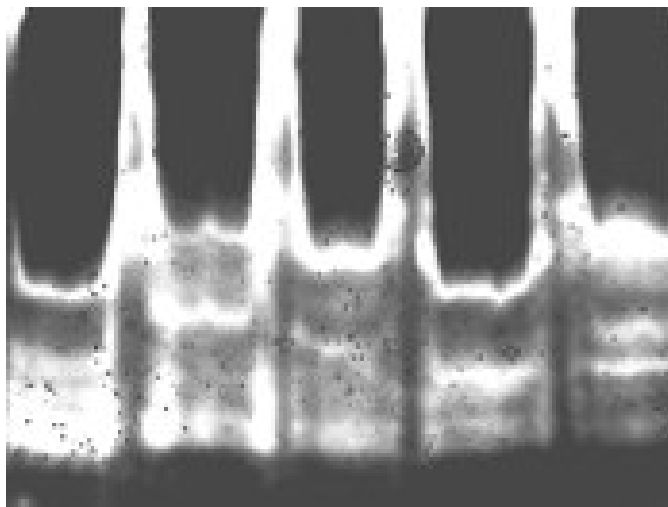
1: Control   2: Chick pea   3: Bean   4: Amarantus 5: Triticale

Figure 6. Effect of plant protein on general protease activity *in vitro* using gelatin as a substrate in the SDS-Page electrophoresis.

tested (Figure 6).

DISCUSSION

In this study, it was found that proteinous extract from seeds had anti-insect activity when incorporated into the Sunn pest diet. Although, there was not significant mortality when nymphs fed on treated diet, seeds proteinous extract can disrupt normal growth and development of the Sunn pest. The same is true for the effect of seeds proteinous extract on the Sunn pest weight. Among the seed extracts, bean was the most effective in disrupting the Sunn pest growth and develop-ment as well as weight and survival. It has been reported that plants produce protease inhibitors to defend themselves against herbivorous insects (Ryan, 1979; Broadway et al., 1986). There are many reports indicating the lack of effect of plant proteinase- and a-amylase inhibitors upon the digestive enzymes of the insects adapted to a particular plant species (Broadway, 1996; Chrispeels, 1996; Jongsma and Bolter, 1997). The same inhibitors are, on the other hand, very efficient in blocking enzymes from mammalian source or from other insects that do not feed on that plant. Thus, to increase plant resistance to particular insect species is achievable through genes found in non-host plants. Protease inhibitors are proteins or polypeptides bind to proteolytic enzymes thus, interfering with normal digestive process of the insect. It was Gatehouse et al. (1979) who first announced that protease inhibitors protect plants in the field against insect herbivores. So, this trait was initially exploited by conventional plant breeder followed by biotechnologist to create transgenic plants expressing protease inhibitors (Redden et al., 1983). The effect of

these metabolites on the larval growth and development of two orders of phytophagous insects including Lepidoptera and Coleoptera have been studied the most. The effect of cowpea trypsin inhibitor against Cowpea weevil, *Callosobruchus maculatus*, was studied and the results showed that incorporation of cowpea trypsin inhibitor into the artificial diet caused significant antimetabolic effects (Gatehouse and Boulter, 1983).

In this study, it was found that bean seed extract inhibited general protease activity almost by 80% followed by chickpea that inhibited protease activity by 40%. However, interestingly, when these seed extracts have been incorporated into diet it did not caused significant mortality or growth disruption. It showed that the insect somehow can cope with the inhibitors when incorporated into their diet or into their host (transgenic plants). Since it has been reported that insect deals with protease inhibitors in two ways either producing high level of inhibitor-resistant protease or induction of novel inhibitor-insensitive protease (Bown et al., 1997). Thus, it is highly likely that the Sunn pest develop the same kind of reaction to the presence of protease inhibitor in their diet. Serine and cysteine protease inhibitors have been shown to have deleterious effects on Lepidoptera and Coleoptera when incorporated into their diet. These effects include reduced fecundity, decrease weight, increased mortality and even in some cases deformation (Kuroda et al., 1996; Gruden et al., 1998; Elden, 2000). In the recent years, genes encoding different proteinase inhibitors have been transferred to the genome of different plants such as cereals, rapeseed, tobacco and potato (Lecardonnel et al., 1999; Ussuf et al., 2001).

Thus, transgenic plants have been protected mainly against phytonematodes and lepidopteran pests. However, there have not been any reports on the successful use of protease inhibitor genes to produce insect-resistant transgenic plants (McManus and Burgess, 1995). For example Johnston et al. (1993) reported a significant reduction in both larval growth and survival of of *Helicoverpa armigera* by SKTI *in vitro*. However, transgenic tobacco plants expressing SKTI failed to show any significant levels (Carlini and Grossi-de-sa., 2002). The same trend has been achieved when tested SKTI against proteolytic activity of *Heliothis virescens in vitro* (Gatehouse et al., 1994). However, transgenic plant expressing SKTI did not show enhanced resistance to lepidopteran pests (Confalonieri et al., 1998). It also had been found that the lepidopterans *H. zea* and *Lymantria dispar* produced increased levels of inhibitor-resistant trypsin-like proteases as a response to chronic ingestion of cabbage protease inhibitors (Broadway, 1995). Also, Wu et al. (1997) showed that larvae of *H. armigera* adapt to transgenic tobacco plants expressing giant taro protease inhibitor by a change in the types of protease.

It is concluded that seed extracts from non host plants of the Sunn pest caused significant reduction of general and specific protease activity *in vitro*. However, when these seed proteinous extracts were incorporated into the insect diet they did not affect significantly growth and development and the insect weight.

ACKNOWLEDGEMENT

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