Purification and Characterization of Protease Inhibitors from A. correntina and A. cardenasii and their Inhibitory Effect on Midgut Proteases of Spodoptera litura and Achaea janata

Thesis submitted for the degree of
DOCTOR OF PHILOSOPHY
by

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Dedicated to
My Teachers

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DECLARATION

I hereby declare that the work embodied in this thesis entitled ***"Purification and Characterization of Protease Inhibitors from A. correntina and A. cardenasii and their Inhibitory Effect on Midgut Proteases of Spodoptera litura and Achaea janata"*** has been carried out by me under the supervision of Prof. Aparna Dutta Gupta and this has not been submitted for degree or diploma of any other university earlier.

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CERTIFICATE

This is to certify that Mr. K. Vijayaprasadarao has carried out research work embodied in this thesis under my supervision and guidance for a full period prescribed under the Ph.D. ordinance of this University. We recommend his thesis **"***Purification and Characterization of Protease Inhibitors from A. correntina and A. cardenasii and their Inhibitory Effect on Midgut Proteases of Spodoptera litura and Achaea janata***"** for submission for degree of Doctor of Philosophy of this University.

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Abbreviations

AChE: Acetylcholinesterase
BApNA: N-α-benzoyl-DL-arginine-p-nitroanilide
BBI: Bowman-Birk inhibitor
BCIP : 5-Bromo-4-chloro-3-indolyl phosphate
BCTI: *Brassica campestris* trypsin inhibitor
BjTI: Brassica juncia trypsin inhibitor
BSA : Bovine serum albumin
°C : degree centigrade / Celsius
CpTI: Cowpea trypsin inhibitor
cDNA : Complementary DNA
DMSO : Dimethylsulfoxide
DNA : Deoxyribonucleic acid
EDTA : Ethylenediaminetetraacetic acid
FAO: Food and Agriculture Organisation
IPM: Integrated Pest Managemant
mg : Milligram
mM : Millimolar
MTI: Mustard trypsin inhibitor
NaPI: *Nicotiana alata* proteinase inhibitor
NBT : Nitrotetrazolium blue
ng : Nanogram
nM : Nanomolar
PAGE : Polyacrylamide gel electrophoresis
PIN2: Proteinase inhibitor2
PI-II: Protease inhibitor2
PIs: Protease inhibitors
PMSF : Phenylmethylsulfonyl fluoride
SaPIN2: *Solanum americanum* Proteinase inhibitor2
SBBI: Soybean Bowman-Birk inhibitor
SBTI: Soybean trypsin inhibitor
SDS : Sodium dodecyl sulfate
SDS-PAGE : Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SPTIs: Sweet potato trypsin inhibitors
TBS : Tris buffered saline
TCA : Trichloroacetic acid
TEMED : N, N, N`, N`, tetramethylethylenediamine
TIs: Trypsin inhibitors
TLCK: Nα-Tosyl-Lys-chloromethylketone
TPCK: N-tosyl-L-phenylalanine chloromethyl ketone
Tris : Tris (hydroxymethyl) aminomethane
v/v : Volume/volume
w/v : Weight/volume
WHO: World Health Organisation
µg : microgram

Introduction and review of literature

Need for improving crop productivity

The global population already exceeded 6 billion in 2000 and is expected to reach approximately 9.2 billion by the year 2050 with an approximate increase of over 40% (U.S. Census Bureau, 2006). Particularly in developing countries the annual increase in human population (2.5-3%) is more than that of annual increase in food production which is a meager 1% (Lawrence and Koundal, 2002). Thus, to meet the needs of the growing human population, it will be necessary to produce more food in the next 50 years than it has since the onset of agricultural production approximately 10,000 years ago. During the last few decades, major progress has been made in increasing crop productivity worldwide. The best example that could be cited is world's rice production which has increased from 25 million tonns in 1966 to 92 million tonns in 1999 (Khush, 1999, 2001).

The increase in food production can be achieved by increasing the crop area, crop yield and/or reducing the crop losses due to pests and pathogens. However, there is no increase in the area of the agricultural land and infact, due to various human activities, it is getting reduced. In 1961, the average of cultivated land per capita was 0.44 ha. Today, this has dropped to 0.26 ha and based on the population projections, until the year 2050 the average will further drop to 0.15 ha of cultivated land per capita (Ce´lia and Maria, 2002). Hence, the increase in food production could be achieved only in the available agricultural land by generating crop varieties with high yield and stability. World wide crop losses due to insect pest attacks account for 15%, despite the

use of insecticides, which represents over US $ 100 billion (Krattiger, 1997; Oerke and Dehne, 2004;). This problem is more acute in tropics and sub-tropics, where the climate provides an absolutely conducive environment, not only for the survival but also for the reproduction and breeding of a wide range of insects. Thus, in order to feed the ever expanding population, crop protection plays a vital and integral role in the present day agricultural practice to minimize losses and thereby increasing production. Hence, the crop varieties with high yield and stability are required.

Crop damage due to insect pests

Arthropods are undoubtedly the most widespread and diverse group of animals with an estimated 4–6 million species worldwide (Novotny *et al.,* 2002). While only a small percentage of arthropods are classified as pest species, they nevertheless cause major losses of crops by destroying around 18% of the world annual crop production (Oerke and Dehne, 2004). The losses are far more significant in food crops, e.g., 52% in wheat, 83% in rice, 59% in maize, 74% in potato, 58% in soybean, and 84% in cotton and in addition to direct losses caused by insects, there are additional costs in the form of pesticides applied for pest control (Sharma *et al.,* 2000). Apart from the damage caused by feeding, insects and mites cause additional yield losses by carrying and infecting crops with disease causing pathogens. Over 200 plant diseases are known to be transmitted by insects, mites and nematodes (Haq, *et al.,* 2004). Phytophagous (plant-eating) insect and mite pests are a major threat to food production for human consumption and the larval forms of

lepidopteran insects are considered the most destructive organisms (Nicholson, 2007).

Classical insect control methods

To date, the major method of pest control has been the widespread use of classical organic agrochemical pesticides. These chemical pesticides were first introduced in the 1940s with the remarkable success of *p,p'*-dichloro-diphenyl-trichloroethane (DDT). DDT was not only a successful agricultural pesticide, but also was used initially in successful malaria eradication programs (Attaran *et al.,* 2000) and is still in use in certain third world countries. The subsequent introduction of organophosphate and carbamate pesticides in the 1960s (Casida and Quistad, 1998) encouraged the customary view that chemical pesticides could produce widespread control of insect pests. However, the extensive and indiscriminate use of the above agrochemical pesticides, with nervous system as target, has inevitably resulted in widespread resistance among arthropod population (Ngowi *et al.,* 2007). Further, massive application of pesticides also leaves harmful residues not only in the food but also in nature and thereby causes adverse effects on (i) non-target organisms like birds, human etc., and (ii) environment (Main *et al.,* 2009).

Drawbacks in using classical insect control methods

Despite extensive plant breeding and pest management efforts, over $10 billion is spent worldwide each year on the chemical control of insect (Khush, 1999). Heavy reliance on chemical pesticides may not be viable as

they provide ephemeral benefits, often with serious adverse side effects, and in some instances actually worsen the farmer's overall pest problems (Way and Heong, 1994; Manichon, 1996; Estruch *et al.,* 1997; Kogen, 1998; Cook, 2000; Matteson, 2000; Sharma and Ortiz, 2002). Thus, the major challenge is how to increase and sustain crop productivity with less use of chemicals.

Development of resistance to the pesticides

Insect resistance is defined by the WHO as the 'development of an ability in a strain of an organism to tolerate doses of toxicant, which would prove lethal to the majority of individuals in a normal (susceptible) population of the species'. In 1992 the WHO reported that more than 500 species of insects and mites had developed resistance to one or more classes of insecticides (Georghiou, 1990; World Health Organisation Bulletin, 1992). Further, the development of resistance has been shown against every class of insecticide, including microbial drugs and insect growth regulators (Krogstad, 1996). However, literature survey shows that maximum reports of resistance development pertain to organophosphates (250) followed by synthetic pyrethroids (156), carbamates (154) and others (including chlorinated hydrocarbons) (85) (Sharma *et al.,* 2001). Many species (about 85) of insects have developed resistance to more than two groups of insecticides. About two decades ago, maximum number of insects and mites showing resistances to pesticides have been recorded in vegetables (48), followed by those infesting fruit crops (25), cotton (21), cereals (15) and ornamentals (13) (Rajmohan, 1998). The resistance of insects to insecticides is reported in all parts of the

world (Picollo *et al.,* 2005; Ahmad *et al.,* 2007; Huang and Han, 2007; Margaritopoulos *et al.,* 2007; Pietrantonio *et al.,* 2007; Stara and Kocourek, 2007; Endersby *et al.,* 2008).

The underlying causes of insecticide resistance are many-fold. Due to the wide usage and narrow target range of agrochemicals, arthropods including insects, have been put under a high degree of selection pressure. The resistance marks a genetic change in response to selection. Individuals carrying genetic traits for coping with the chemically hostile environment survive and reproduce, thereby passing on these traits to their progeny. Continued selection pressure exerted by the insecticide rapidly increases the frequency of the genetic trait (resistance) in the population (Feyereisen, 1995). Insecticide resistance can be characterised by: (i) increased metabolic detoxification resulting from elevated esterase, glutathione S-transferase or monooxygenase levels, (ii) decreased target sensitivity, and/or (iii) sequestration or lowered insecticide availability (Brogdon and McAllister, 1998). The molecular mechanisms responsible for the increase in resistance have been identified as: (a) point mutations in the ion channel of the GABA receptor, sodium channel and the acetylcholinesterase (AChE) active site, (b) amplification of the esterase genes and (c) mutations causing up-regulation of detoxification enzymes (Feyereisen, 1995; Brogdon and McAllister, 1998; Hemingway and Ranson, 2000).

Insecticide resistance is now well documented in various lepidopteran pest populations of India (Sharma *et al.,* 2001). *Helicoverpa armigera*

(Hubner) was recorded to exhibit widespread resistance to cypermethrin (23–8022-fold in field strains), while the resistance to endosulfan and chlorpyriphos was moderate to high in different parts of India. The overall resistance of the pink bollworm *Pectinophora gossypiella* (Saunders) to pyrethroids was low; however, high resistance levels of 23–57 fold to endosulfan were recorded in some areas of Central India. The resistance of *P. gossypiella* to chlorpyriphos was high in the Medak (A. P.), Bhatinda and Sirsa strains from North India. The majority of the *Spodoptera litura* (Fab.) strains collected in South India exhibited high resistance levels of 61–148 fold to cypermethrin, while resistance to endosulfan was high only in two strains collected from Bhatinda and Karimnagar (Kranthi, 2002). Further, the *S. litura* strains from South India also exhibited high levels of resistance (45–129-fold) to chlorpyriphos (Kranthi, 2002).

Harmful effects of pesticides on non-target organisms

Selectivity of a chemical is achieved if both pest and non-pest species do not share the same target (Feyereisen, 1995). Unfortunately, because the target sites for various pest control agents/agrochemicals are conserved among insect pests, beneficial insects and vertebrates, the chemical pesticides have a relatively broad-spectrum of toxicity against non-target species. Extensive studies also suggest a number of potentially adverse outcomes of pesticide exposure (Ngowi *et al.,* 2007). More commonly suggested include pancreatic cancer, altered/lowered reproductive function, and neuropsychological dysfunction (Garabrant *et al.,* 1992; Longnecker *et al.,* 2001; van Wendel de *et*

al., 2001; Beard, 2006). Although the evidence is weak and the reported risk is low, these problems have potential to cause a significant increase in disease burden at a population level, given the large number of people exposed to pesticides in developing countries.

Although the application of pesticides to minimize the losses due to insect pests, diseases and weeds is inevitable, the chemical control of insect pests is under increasing pressure because of the adverse effects of the pesticides. This has necessitated the use of target-specific compounds with low persistence in the environment. Thus, there is an increased emphasis on the search for (i) alternative insect control strategies, which are eco-friendly and (ii) more effective technologies that would allow a rational use of biopesticides/biodegradable control agents for sustainable crop production.

Development of transgenic plants

An alternative strategy could be to take advantage of the plant's own defense mechanisms, for example, by manipulating the expression of their endogenous defense proteins, or by introduction of an insect control gene derived from another plant.

Until recently, pest resistant varieties have been developed mainly through the application of principles of classical Mendelian genetics and conventional plant breeding methods. This conventional crop breeding programmes are limited by the need for a source of resistance within the inter-breeding gene pool. The other problem in the conventional programme is the

loss of resistant characters in the breeding process. For example, the susceptibility of the cowpea *Vigna unguiculata*, an important component of human diet in many poor countries, to predation by the bruchid beetle *Callosobruchus maculatus* was shown to be related to an increased digestibility of vicilins, a major class of storage proteins, by the insect gut enzymes, as compared to the protein found in resistant seeds from Nigerian cowpea (Sales *et al.,* 1992, 2000: Macedo *et al.,* 1993).

Using modern plant genetic engineering technology one could overcome such constraints and transfer resistant genes from any source (plant, animal, microbial, synthetic or recombinant) to the crop plant of choice to be introduced into breeding. In this context, different genes encoding toxic proteins have been introduced into genomes of various crops in order to confer the insect and pathogen resistance (Outchkourov *et al.,* 2004; Abdeen *et al.,* 2005; Kondrak *et al.,* 2005; Srinivasan *et al.,* 2005). Many of these plants are now being tested in field condition or awaiting commercialization (Rahbe *et al.,* 2003; Prasad *et al.,* 2008; He´ma *et al.,* 2009; www.aphis.usda.gov). Such crops, referred to as genetically modified (GM) crops; represent a promising opportunity to make an important contribution to integrated pest management (IPM) programme (Romeis *et al.,* 2006). Since the first transgenic tobacco plant expressing foreign protein obtained in 1984 (Horsch *et al.,* 1985), transgenics have been produced for more than 100 species (Brar *et al.,* 1995; Khush and Brar, 1998; Clark *et al.,* 2005; Kumar *et al.,* 2008). The area under

transgenic crops has increased from 1.7 million hectares in 1996 to 44.2 million hectares in 2000 (James, 2000).

Plant defense in nature

In plant-insect interactions, insects play both beneficial and detrimental roles. Beneficial roles include pollination of flowers and predation of insect pests of plants, while the detrimental roles include phytophagy and as carriers of viruses which cause various plant diseases. However, in a wild community of plants subject to phytophagy under natural conditions, a partial protection against a variety of insect pests is achieved by both physical and chemical factors produced by the plants (Boulter, 1993).

In natural communities of plants the resistance against pests and pathogens is of two types: (a) horizontal resistance and (b) vertical resistance. Horizontal resistance consists of many minor genes with no gene-to-gene matching between host and pathogen (Nelson, 1978). It is probably ubiquitous and gives durable resistance. This kind of resistance may be supplemented in some cases by vertical resistance (Plank, 1966). Vertical resistance consists of major genes with gene-to-gene matching between host and pathogen (Colton *et al.,* 2006).

Leaf toughness is a major physical deterrent to numerous herbivores (Feeny, 1976), and surface hairs (trichomes) on leaves are also effective. Ehleringer and Clark (1988) suggested that trichomes are essential for the regulation of leaf spectral properties, temperature and water loss, but defence against herbivores may also be important (Gregory *et al.,* 1986; Agrawal,

1999; Kostina *et al.,* 2001; Traw and Dawson, 2002). High molecular weight compounds such as phenolics, that are immobile, cannot be recycled and are localized in the leaves until they fall (Mooney *et al.,* 1983). Physical constructs are also unlikely to be reusable for other functions. It is therefore expected that old leaves should have stronger defence than young leaves. It has often been reported that the new leaves are of higher quality (more nitrogen, fewer phenolics, reduced toughness and other characteristics) than old leaves, so that they are preferred by herbivores (Feeny, 1970; Hunter and Lechowicz, 1992).

Endogenous chemical protectants have been separated into two groups: (a) micromolecular, e.g., alkaloids and (b) macromolecular, e.g., proteins. The latter are likely to afford vertical resistance, since they are major and monogenic. Further, protein protectants differ significantly from chemical insecticides in their mode of action, where the protein protectants' mode of application is ingestion and they primarily affect digestion..

During the co-evolution of plants and insects, plants are able to synthesise a wide range of molecules including proteins to defend themselves against insect attack (Bate and Rothstein, 1998; Weiler *et al.,* 1998; Chi *et al.,* 2009). The best known plant proteins supposedly involved in defense mechanisms are lectins, ribosome-inactivating proteins (RIPs) of type 1 and 2, inhibitors of proteolytic enzymes and glycohydrolases (Bowles, 1990; Ryan, 1990; Chrispeels and Raikhel, 1991; Barbieri *et al.,* 1993; Peumans and Van Damme, 1995; Koiwa *et al.,* 1997; Zavala *et al.,* 2004; Wang *et al.,* 2005; Macedo *et al.,* 2007; Noghabi *et al.,* 2008). Other plant proteins involved in the

complex mechanisms of defense are the arcelins (Osborn *et al.,* 1988), chitinases (Herget *et al.,* 1990; Cohen, 1993), canatoxin (Carlini *et al.,* 1997) and modified forms of storage proteins (Macedo *et al.,* 1993; Sales *et al.,* 2000). Some of these proteins are relatively thermostable and are only partially inactivated by heat during cooking (Peumans and Van Damme, 1996; Carlini and Udedibie, 1997). Proteinaceous proteinase inhibitors (PIs) active against insect proteolytic enzymes are considered among these molecules (Ryan, 1990; Koiwa *et al.,* 1997; Birk, 2003).

Protease inhibitors

Proteolytic enzymes catalyze the cleavage of peptide bonds in proteins. They are classified according to their mechanism of catalysis and the amino acid present in the active center: (1) serine proteinases, with a serine and histidine; (2) cysteine proteinases, with a cysteine; (3) aspartic proteinases, with an aspartate group and (4) metalloproteinases, with a metallic ion (Zn^{+2}, Ca^{+2} or Mn^{+2}) (Neurath, 1984). The term protease includes both endopeptidases and exopeptidases, where as the term proteinase is used to describe only endopeptidases (Ryan, 1990).

Proteolysis as a key process in all living organisms must be extremely controlled, otherwise it could be very deleterious to their natural environment. It is therefore, not surprising that a large number of naturally occurring PIs have been described in animals, plants as well as in microorganisms and have been extensively studied in order to elucidate their structural and functional properties (Bode and Huber, 1992; Hibbetts *et al.,* 1999; Khatib, 2005; Chye

et al., 2006; Mosolov and Valueva, 2008). Broadly the PIs are classified as serine protease inhibitors, cysteine protease inhibitors, metallo-protease inhibitors and aspartic acid protease inhibitors depending on the type of protease they inhibit (Ryan, 1990; Jouanin, 1998; Mosolov, 1998; Bode and Huber, 2000).

Plant protease inhibitors

Plant protease inhibitors (PIs) are extremely wide spread throughout the plant kingdom. They appear to have been most extensively studied in the Leguminosae, Gramineae and Solanaceae, probably because of the large number of species in these families which form important source of food (Richardson, 1991), while the economically unimportant species were comparatively neglected (Konarev *et al.,* 2004). They are known to be involved in several physiological processes, such as reserve control, regulation of protein turnover, apoptosis, stress tolerance, cell proliferation, defense against pathogens and pests etc. (Koiwa *et al.,* 1997; Belenghi *et al.,* 2003; Megdiche *et al.,* 2008; Zhang *et al.,* 2008; Srinivasan, *et al.,* 2009; Tian *et al.,* 2009). Further, PIs have been shown to be developmentally expressed in seeds and reserve organs (Birk, 1996; Koiwa *et al.,* 1997) or induced by wounding in leaves (Schaller and Ryan, 1995).

The possible role of PIs in plant protection was investigated as early as 1947 when, Mickel and Standish (1947) observed that the larvae of certain insects were unable to develop normally on soybean products. Subsequently the trypsin inhibitors (TIs) present in soybean were shown to be toxic to flour

beetle, *Tribolium confusum* (Lipke *et al.,* 1954). In 1972, Green and Ryan reported the increased levels of PIs detected in plants that have been damaged by insect feeding. Following these early studies, there have been many examples of protease inhibitors active against certain insect species, both in *in vitro* assays against insect gut proteases (Pannetier *et al*. 1997; Koiwa *et al.,* 1998; Tamhane *et al.,* 2007; Ramos et al., 2008; Telang *et al.,* 2009) and in *in vivo* artificial diet bioassays (Urwin *et al.,* 1997; Vain *et al.,* 1998; Bhattacharya *et al.,* 2007; Amorim *et al.,* 2008; Ramos *et al.,* 2009). The natural protective role of PIs against phytophageous insects and the availability of PI-encoding sequences encouraged the development of pest-resistance programmes based on PI expression in transgenic plants (Ryan, 1990; Birk, 2003).

First transgenic plant expressing plant PI was produced by Hilder *et al.,* (1987), by transferring trypsin inhibitor gene from *Vigna unguiculata* to tobacco, which conferred resistance to wide range of insect pests including lepidopterans such as *Heliothis* and *Spodoptera*, coleopterans such as *Diabrotica*, *Anthonomnous* and orthoptera such as *Locusts*. Furher there is no concrete evidence for its toxic or deleterious effects on mammals. Many of these protease inhibitors are rich in cysteine and lysine, contributing to better and enhanced nutritional quality (Ryan, 1989). Protease inhibitors also exhibit a very broad spectrum of activity including suppression of parhogenic nematodes like *Globodera tabaccum*, *G. pallida,* and *Meloidogyne incognita* by cowpea trypsin inhibitor (CpTI) (Willimson and Hussey, 1996), inhibition

of spore germination and mycelium growth of *Alternaria alternate* by buckwheat trypsin/chymotrypsin inhibitor (Dunaewskii *et al.,* 1997). Cysteine PIs from pearl millet inhibit growth of many pathogenic fungi including *Trichoderma reesei* (Joshi *et al.,* 1998). These properties make PIs an ideal choice to be used in developing transgenic crops resistant to insect pests. Further, transformation of plants with PI encoding cDNA clones appears attractive not only for the control of plant pests and pathogens, but also as a means to produce recombinant PIs in substantial amounts, useful in alternative systems and the use of plants as factories for the production of heterologous proteins (Sardana *et al.,* 1998; Rival *et al.,* 2008; Vancanneyt *et al.,* 2009).

The advantages of using PIs as insect-control agents include: (i) their activity against a wide range of insects, (ii) their use as a second mechanism to help in preventing development of insects that are resistant to Bt endotoxin, (iii) their inactivation with cooking and (iv) the common nature of such inhibitors in food of human as well as animals. The major disadvantages are (i) the high levels of protein required for insect killing and (ii) the potential need to regulate protein expression to specific plant organs (Brunke and Meeusen, 1991).

Further, as the PIs are primary gene products, their genes are excellent candidates for engineering pest-resistance into plants (Boulter, 1993). The availability of diverse genes from different plant sources is in itself an advantage, as two or more genes can be transferred in combination and their products could target different physiological aspects (Urwin, 1998).

Classification of plant PIs

Laskowski and Kato (1980) have classified the "Proteinase inhibitors" into several families based on extensive homology among its members, topological relationships between the disulfide bridges and the location of the reactive site. Plant serine PIs that obey the standard mechanism are grouped into soybean (Kunitz), Bowman–Birk, potato I and II, and squash families (Laskowski and Kato, 1980; Birk, 2003; Schirra *et al.,* 2008). Several other inhibitor families, such as barley, ragi 1 and 2, thaumatin and serpin have also been suggested (Ryan, 1990; Dahl *et al.,* 1996; Ascenzi *et al.,* 1999).

Table 1: Families of proteinacious inhibitors of protease in plants

Family	Protease inhibited
A. Serine protease inhibitors Soyabean trypsin inhibitor (Kunitz) family Bowman-Birk family Barley trypsin inhibitor family Potato inhibitor I family Potato inhibitor II family Squash inhibitor family Ragi I-2/maize trypsin inhibitor family Serpin family	Trypsin and Chymotrypsin
B. Cysteine protease inhibitors (Phytocystatins)	Papain, Cathepsin B, H, L

C. Metallo-protease inhibitors	Carboxypeptidase A, B
D. Aspartic protease inhibitor	Cathepsin D

A. Serine protease inhibitors

Serine PIs are universal throughout the plant kingdom. They have been reported from a variety of plant sources and are the most-studied class of PIs. Their physiological roles include the regulation of endogenous proteinases during seed dormancy, the reserve protein mobilization, and the protection against the proteolytic enzymes of parasites and insects (Birk, 2003). Moreover, they may also act as storage or reserve proteins. The two best-characterized families of plant serine PIs are the Kunitz-type and Bowman–Birk type inhibitors. Kunitz-type inhibitors have a molecular mass of 18–22 kDa, one or two polypeptide chains, a low cystine content (usually with four Cys residues in two disulfide bridges), and one reactive site. In contrast, Bowman–Birk type inhibitors have a lower molecular mass (8–10 kDa), high cystine content, two reactive sites and typically found in legume seeds (Birk 1996, 2003). They bind simultaneously and independently to two separate enzyme molecules, such as trypsin and chymotrypsin (Birk, 1985, 2003; Bode and Huber, 1992; Mc Bride *et al.,* 2002; Qi *et al.,* 2005).

In general, serine PIs behave as pseudo-substrates, with the amino acid at position P1 of the inhibitor determining the specificity for the enzyme, either trypsin or chymotrypsin (Bode and Huber, 1992). In spite of differences in primary structure and topology, the reaction center structure and mechanism of

action are well preserved among serine PIs (Qi *et al.,* 2005). Some of the plant serine PIs are bifunctional molecules, being able to inhibit trypsins as well as α-amylase (Strobl *et al.,* 1995; Haq *et al.,* 2005).

B. Cysteine protease inhibitors

Plant cystatins or phytocystatins are the second most studied class of inhibitors. Phytocystatins have been identified in a variety of monocot and dicot species, such as maize, rice, potato, soybean and apple (Kondo *et al.,* 1990; Abe *et al.,* 1991, 1996; Botella *et al.,* 1996; Gruden *et al.,* 1997; Ryan *et al.,* 1998; Tian *et al.,* 2009). One group of phytocystatins contain a single domain and comprise the majority of phytocystatins (Pernas *et al.,* 1998), whereas a second group has multiple domains, such as the multicystatins found in potato tubers, tomato leaves and sunflower seeds (Walsh and Strickland, 1993; Wu and Haard, 2000; Kouzuma *et al.,* 2000). The phytocystatins differ largely from animal cystatins (Kondo *et al.,* 1991; Brown *et al.,* 1997; Arai *et al.,* 2002), by displaying high inhibitory activity towards insect gut proteinases (Bode and Huber, 1992; Koiwa *et al.,* 1997; Martinez *et al.,* 2007) making them attractive as biological control agents of insect pests (Gatehouse and Gatehouse, 1998; Ussuf *et al.,* 2001; Benchabane *et al.,* 2008). The cysteine PIs (present in tomato and potato) confer resistanse and protect the plants from cowpea weevils (Gatehouse *et al.,* 1986; Amirhusin *et al.,* 2004) and Colorado potato beetles (Wolfson and Murdock, 1987) which employ cysteine proteinases as important digestive enzymes. The incorporation of cysteine PIs

into artificial diet and transgenic plants have been shown to have toxic effects on the larvae (Annadana *et al.,* 2002; Outchkourov *et al.,* 2004).

C. Metallo-protease inhibitors

The metallo-protease inhibitors in plants are represented by the metallo-carboxypeptidase inhibitor family in tomato (Rancour and Ryan, 1968) and potato plants (Hass *et al.,* 1975; Graham and Ryan, 1981). However, Shahverdi *et al.,* (2006) purified two matrix metalloproteinase protease inhibitors from *Ferula persica* which exhibited a selective inhibitory effect on tumor cell invasion.

D. Aspartic protease inhibitors

Aspartic PIs are a relatively less studied class, mainly due to their rarity of occurrence. Potato tubers possess an aspartic proteinase (cathepsin D) inhibitor (Mares *et al.,* 1989) that shares considerable amino acid sequence identity with the soybean trypsin inhibitor (SBTI). However, Christeller *et al.,* (1998, 2006) have purified and characterized an aspartic protease inhibitor from the squash (*Cucurbita maxima*) phloem exudates.

Distribution and localization

Soybean trypsin inhibitor was the first PI isolated and characterized. Since then many PIs have been found widely distributed throughout the plant kingdom. Most of the plant PIs that have been characterized are from the Gramineae (Poaceae), Leguminosae (Fabaceae), and Solanaceae families (Brzin and Kidric, 1995). PIs are usually found in storage organs, such as seeds

and tubers, but their occurrence in the aerial part of plants, as a consequence of several stimuli has also been widely documented (De Leo *et al.,* 2002). PIs are also found in non-storage tissues, such as leaves, flowers and roots (Brzin and Kidric, 1995; Xu *et al.,* 2001; Sin and Chye, 2004). PIs may accumulate to about 1 to 10% of the total protein in these storage tissues, while upto 1% in leaves of tomato and potato (Ryan, 1968). Reports also show the presence of PIs in yeast (Matern *et al.,* 1979; Winterburn *et al.,* 2007) and other fungi (Richardson, 1977; Steenbakkers *et al.,* 2008).

Localization studies reveal the presence of a trypsin inhibitor in the cytosol of mung bean cotyledonary cells (Chrispeels and Baumgartner, 1978). Soybean trypsin inhibitor (SBTI) was reported to be mainly present in the cell wall, with lesser amount in protein bodies, cytoplasm, and the nuclei of cotyledonary and embryonic cells. Soybean Bowman- Brik inhibitor (SBBI) was mainly found in protein bodies, the nuclei, and to a lesser extent in the cytoplasm. In contrast to SBTI, small quantity of SBBI was also located in the intercellular space but not in the cell wall (Horisberger and Tacchini-Vonlanthen, 1983). The wound-induced inhibitors accumulate in vacuoles of tomato, wild tomato, and potato leaves. Xu *et al.,* (2004) described the expression of a PIN2 protein from *S. americanum* (Mill.) in phloem of stem, in addition to roots and leaves, suggesting a novel endogenous role for PIN2 in phloem. Further research showed that both SaPIN2a and SaPIN2b are expressed in floral tissues (Sin and Chye, 2004). The exact cellular distribution

of many of the PIs is still unknown, therefore, further research is needed in this area to elucidate their exact subcellular location.

Properties and functions

Plant PIs usually have a high content of cysteine residues (Richardson, 1991) that form disulfide bridges (Greenblatt *et al.,* 1989; Hung *et al.,* 2003) and confer resistance to heat, wide pH ranges, and proteolysis (Richardson, 1991). For example, a trypsin inhibitor purified from seeds of *Brassica campestris* (BCTI) with molecular weight of 8 kDa was found to be a thermostable Bowman-Birk type trypsin inhibitor that inhibits trypsin at the molar ratio 1:1. The stability of BCTI is apparently related to the presence of the disulfide bridges (Hung *et al.,* 2003). The plant PIs normally have little or no carbohydrate moiety (Richardson, 1977).

Studies on the biosynthesis of several plant PIs demonstrated that they are synthesized either as prepro-proteins (Graham *et al.,* 1985a) or as pre-proteins (Graham *et al.,* 1985b) that are processed *in vivo,* either during or after synthesis to produce the native PIs (Nelson and Ryan, 1980). Some small PIs are derived *in vivo* from the post-translational processing of multidomain precursors (Sanchez- Serrano *et al.,* 1986; McManus *et al.,* 1994a; Miller *et al.,* 2000). Variety of PIs are known to be produced in response to various stress conditions like pathogens, insect attack, wounding, and environmental stresses such as high salt *etc*. (Koiwa *et al.,* 1997). They are also synthesized during normal course of development including programmed cell death (Solomon *et al.,* 1999)

Sweet potato PIs (SPTIs), which inhibit endogenous serine protease activity, also proved to have both dehydroascorbate reductase and monodehydroascorbate reductase activities and respond to environmental stresse (Hou and Lin, 1997). Further, trypsin inhibitors in sweet potato account for about 60% of total water soluble proteins and could be recognized as storage proteins (Lin and Chen, 1980; Yeh *et al.,* 1997a).

Protease inhibitors are now well established as a class of cancer chemopreventive agents (Kennedy, 1998; Lippmann and Matrisian, 2000; Birk, 2003). Several PIs are undergoing further evaluation in human clinical trials (Fear *et al.,* 2007). The use of protease inhibitors as therapeutic agents, in particular, their use in cellular transformation, blood clotting disorders, osteoporosis, obesity, cardiovascular, neurodegenerative and retroviral diseases is under thorough investigation (Hocman, 1992; Birk,, 2003; Fear *et al.,* 2007).

Specificity

In some cases the PIs exhibit a narrow range of specificity, being capable of inhibiting either only one or two closely related proteinases, whilst others of broad specificity are active against a wide range of different enzymes. Trypsin inhibitors are generally inhibitory towards related enzyme chymotrypsin (Kassel, 1970; Birk, 2003). In some cases the reactive site of the inhibitor is same for both enzymes. However, there are large number of inhibitors which are either double 'headed' or 'polyvalent', *i.e.,* containing different reactive sites for the independent inhibition of the two proteolytic enzymes (McBride *et al.,* 2002; Birk *et al.,* 2003; Qi *et al.,* 2005). It should be

noted that some potent inhibitors of trypsin are inactive or only weakly active against chymotrypsin (Wilson and Laskowski, 1973) and vice versa (Iwasaki *et al.,* 1971; Kiohara *et al.,* 1973). Sometimes the trypsin or chymotrypsin inhibitors are strictly specific for these two enzymes (Belew *et al.,* 1975). However, there are many of them which inhibit a range of other serine proteases such as elastase (Wilson and Laskowski, 1975), thrombin, plasmin and kallikrein (Sakato *et al.,* 1975). They were also shown to inhibit enzymes of other groups, for example, chymotrypsin inhibitors from broad bean also inhibited the sulfhydryl enzyme papain (Birk, 2003). Similarly the PIs isolated from cultivated cells of *Scopolia japonica* included the acidic proteinase pepsin in their broad spectrum of specificity (Sakato *et al.,* 1975). The soyabean trypsin inhibitor has been shown to inhibit clostripain, a microbial enzyme similar to trypsin, but having the catalytic site of an –SH protease (Siffert *et al.,* 1976).

Mechanism of action

The mechanism of binding of the plant PIs to the insect proteases appears to be similar with all the four classes of inhibitors. The inhibitor binds to the active site on the enzyme to form a complex with a very low dissociation constant (10^{-7} to 10^{-14} M at neutral pH values), thus effectively blocking the active site. A binding loop on the inhibitor, usually "locked" into conformation by a disulphide bond, projects from the surface of the molecule and contains a peptide bond (reactive site) cleavable by the enzyme (Terra *et al.,* 1996; Walker *et al.,* 1998; Birk, 2003). This peptide bond may be cleaved in the

enzyme inhibitor complex, but cleavage does not affect the interaction, so that a hydrolyzed inhibitor molecule is bound similar to an unhydrolyzed one. The inhibitor thus directly mimics a normal substrate for the enzyme, but does not allow the normal enzyme mechanism of peptide bond cleavage to proceed to completion *i.e.,* dissociation of the product (Walker *et al.,* 1998).

PIs inhibit the protease activity of insect gut proteolytic enzymes and reduce the quantity of proteins that can be digested (Ramos *et al.,* 2009), and also cause hyperproduction of the digestive enzymes which enhances the loss of sulfur amino acids (Shulke and Murdock, 1983; Konrad *et al.,* 2009)) as a result of which, the insects become weak with stunted growth and ultimately die. Retardation of insect development, slower rate of growth and reduced fitness for survival would allow a much wider window within which the other pesticides including biopesticides could be successfully employed for the management of insects. This would help to generate greater confidence in integrated pest management (IPM) by farmers, who normally prefer complete insect control based on chemicals (Sharma *et al.,* 2000).

Transgenic plants expressing PI gene

Transformation of plant genome with PI-encoding cDNA clones as well as PI genes appears attractive, not only for the control of plant pests and pathogens, but also because it can produce PIs useful for pharmaceutical and therapeutic applications. The potential of these inhibitors has already been demonstrated by diet incorporation assays or by *in vitro* inhibition studies of digestive proteinases. In the last few years, cDNA sequences encoding

different PIs have been incorporated in the genome of variety of plants such as cereals, rapeseed, tobacco, potato *etc.,* and protective effects have been obtained in some cases, mainly against lepidopteran pests (Table 2)

Table 2: Protease inhibitors conferring resistance against insect pests.

Inhibitor specificity and plant source	**Target insect**	**Test condition**	**Reference**
Serino proteinase inhibitors *V. unguiculata* (cowpea) (trypsin inhibitor)	Lepidoptera *Heliothis virescens* *Manduca sexta* *Helicoverpa zea* *Chilo suppressalis* *Spodoptera infestans* *Lacanobia oleracea* *Pieris rapaee* *Heliothis armigera* Coleoptera *Otiorhynchus sulcatus* *Euscepes postfaciatus*	Transgenic tobacco Transgenic tobacco Transgenic tobacco Transgenic rice Transgenic rice Transgenic potato , artificial diet Transgenic cabbage Transgenic cabbage Transgenic strawberry Transgenic sweetpotato	Hilder *et al.,* 1987 Hilder *et al.,* 1987 Hoffmann *et al.,* 1992 Xu *et al.,* 1996 Xu *et al.,* 1996 Gatehouse *et al.,* 1997, 1999a; Bell *et al.,* 2001 Hao and Ao, 1997 Hao and Ao, 1997 Graham *et al.,* 1996 Golmirizaie *et al.,* 1997
G. max (soybean Kunitz trypsin inhibitor)	Lepidoptera *Heliothis virescens* *Lacanobia oleracea* *Diatrea saccharalis* *Spodoptera littoralis*	Transgenic tobacco Transgenic potato, Artificial diet Artificial diet Transgenic tobacco, Artificial diet	Gatehouse *et al.,* 1993 Gatehouse *et al.,* 1999a,b Pompermayer *et al.,* 2001 Marchetti *et al.,* 2000
Leucaena leucocephala (trypsin inhibitor)	Lepidoptera *Heliothis armigera*	Artificial diet	Nandeesha and Prasad, 2001

Solanum tuberosum (potato) (trypsin inhibitor I) (trypsin/chymotrypsin inhibitor II)	Lepidoptera *Chrysodeixis eriosoma* *Manduca sexta* *Sesamia inferens* Coleptera *Plagiodera versicolor*	Transgenic tobacco Transgenic tobacco Transgenic rice Transgenic poplar	McManus *et al.*, 1994 Johnson *et al.*, 1989 Duan *et al.*, 1996 Klopfenstein *et al.*, 1997
Lycospersicum esculentum (tomato) (trypsin/chymotrypsin inhibitor II)	Lepidoptera *Manduca sexta*	Transgenic tobacco	Johnson *et al.*, 1989
Ipomea batatus (sweetpotato) (trypsin inhibitor)	Lepidoptera *Spodoptera litura*	Transgenic tobacco	Yeh *et al.*, 1997b
Sinapis alba (white mustard) multidomain inhibitor	Lepidoptera *Plutella xylostella* *Mamesrra brassicae* *Spodoptera littoralis*	Transgenic Arabidopsis/oilseed rape Transgenic Arabidopsis/oilseed rape Transgenic Arabidopsis/tobacco	De Leo *et al.*, 2001 De Leo *et al.*, 2001 De Leo *et al.*, 1998
Schistocerca gregaria proteinase inhibitor	Lepidoptera *Leptinotarsa decemlineata*	Transgenic potato	Kutas *et al.*, 2004
Nicotiana alata proteinase inhibitor	Lepidoptera *Epiphyas postvittana*	Transgenic apple	Maheswaran *et al.*, 2007
Potato PI-II & Carboxypeptidase inhibitor	Lepidoptera *Heliothis obsolete* *Liriomyza trifolii*	Transgenic tomato	Abdeen *et al.*, 2005
Mustard trypsin inhibitor MTI-2	Lepidoptera *Spodoptera littoralis*	Transgenic tobacco	DeLeo & Gallerani, 2002
Brassica juncea trypsin inhibitor (BjTI)	Lepidoptera *Spodoptera litura*	Transgenic tobacco Transgenic tomato	Mandal *et al.*, 2002

Cystein proteinase inhibitors *Oryza sativa* (rice) (oryzacystatins I and II)	Coleoptera *Chrysomela tremulae* *Callosobruchus chinensis* Hemiptera *Riptortus clavatus* Lepidoptera *Cnaphalocrocis medinalis* Thysanoptera *Frankliniella occidentalis*	Transgenic poplar Artificial diet Artificial diet Transgenic rice Transgenic potato	Leple *et al.*, 1995 Kuroda *et al.*, 1996 Kuroda *et al.*, 1996 Han *et al.*, 2007 Outchkourov *et al.*, 2004
G. max (soybean) (soyacystatins N and L)	Coleoptera *C. maculatus*	Artificial diet	Koiwa *et al.*, 1998
Arabdopsis thaliana	Coleoptera *Chrysomela populi*	Transgenic poplar	Delledonne *et al.*, 2001

Groundnut and its protease inhibitors

Cultivated groundnut, also known as peanut (*Arachis hypogaea* L.), is grown on nearly 24 million hectares between latitudes 408 ^{0}N and 408 ^{0}S with a total global production of 34.5 million tones (FAO, 2000). Although originated in South America, the vast majority of groundnut is produced in Asia and Africa [Asia 68% (23 Mt), Africa 24% (8 Mt)].

The largest groundnut acreage in Asia occurs in India. However, China is frontrunner in total production. The average yield of groundnut in China is 3.1 t/ha while in India it is 1.0 t/ha. The key factors contributing to higher yields in China are (i) deployment of improved varieties which cover 90% of

the groundnut plantation area, (ii) adoption of improved cultural practices including crop rotation and polythene film mulching, (iii) recognition of groundnut growers which produce high yield, and (iv) national policies for price support systems and marketing opportunities (Shuren *et al.,* 1996).

Groundnut is one of the world's most important oilseeds crop (FAO, 1999). It is one of the major sources of vegetable protein and edible oil in both developed and developing countries (Dwivedi *et al.,* 2003). Groundnut is also a rich source of minerals (phosphorus, calcium, magnesium, and potassium) and vitamins (E, K, and B group) (Savage and Keenan, 1994). Oleic, linoleic, and palmitic fatty acids, together, account for over 80% of the total fat in groundnut seeds (Dwivedi *et al.,* 1993a). The cake remaining after oil extraction is extremely used as animal feed (Savage and Keenan, 1994). Groundnut haulms which is rich in proteins, constitute approximately 45% of the total plant biomass, and provide excellent forage for cattle (Cook and Crosthwaite, 1994).

In India groundnut is one of the major oilseed crops, it accounts approximately 32% of the oilseed area and 39% of oilseed production. It is grown in three seasons, *i.e.* kharif (monsoon or rainy season), rabi (post rainy winter season) and summer. The rainy season groundnut, which is grown during the south west monsoon period (June–November) is spread over the entire country and is generally rainfed. The post rainy season groundnut is confined to South India and Orissa and is raised mostly in rice fallows during

October–March. The summer groundnut is mostly grown in the Central Indian States of Gujarat, Maharashtra and Madhya Pradesh from January–May.

Despite its high production potential, the actual yields on farmer's fields are quite low largely because of insect pests and diseases. More than 350 species of insects damage this crop in different parts of the world (Stalker and Campbell, 1983), among which lepidopteran insects like *Spodoptera litura*, *Spodoptera littoralis, Spodoptera frugiperda, Helicoverpa zea* and *Helicoverpa armigera* are very important. Two decades ago, *Spodoptera litura* was a minor pest of groundnut. However, it has now become a major pest particularly of irrigated groundnut in Andhra Pradesh and Karnataka states of India (Amin, 1988; Ghewande and Nandagopal, 1997).

Host plant resistance is one of the most economical and environment-friendly method of keeping pest population below economic injury levels (EILs). Opportunities for more sustainable use are offered by an integrated approach based on IPM. Given the costs of production and pest losses, it could be most economic and feasible to develop an IPM programme for groundnut.

The levels of resistance to insect pests in cultivated groundnut (*Arachis hypogaea*) germplasm was found to be fairly low (Sharma *et al.,* 2003). On the other hand screening studies with wild species of *Arachis* clearly revealed their multiple resistances not only against various insect pests but also against fungal pathogens (Stevenson *et al.,* 1993; Sharma *et al.,* 2003). Hence wild *Arachis* species are likely to harbor genes capable of imparting high levels of resistance to diseases and insect pests.

Although few attempts have been made to purify and characterize the Bowman-Birk protease inhibitors (Norioka *et al.,* 1983; Tsunog *et al.,* 1986; Suzuki *et al.,* 1987, 1993) as well as clone their cDNAs from *A. hypogaea* (Dodo *et al.,* 2004; Boateng *et al.,* 2004), but to the best of our knowledge it has not been attempted for wild species of groundnut which show multiple insect resistance.

One of the major concerns in this area is that many of the wild *Arachis* species are not cross compatible with cultivated groundnut. However, efforts to overcome incompatibility in wide crosses have started to liberate resistance genes in interspecific progenies. But these progenies carry a lot of linkage drag. Hence, identification of PIs from the wild species and their transfer to the cultivable species seems to be a good alternative to be tested for developing insect resistant transgenic groundnut plants.

With this background in view the following objectives were formulated for the present study:

- ✓ Survey of wild species of groundnut for their antifeedant/insecticidal activity
- ✓ Correlation of leaf surface structure with larval feeding
- ✓ Purification and partial characterization of protease inhibitors from leaf ectracts of the wild ground nut plants

Materials and methods

Chemicals

Brilliant blue G-250, BSA (bovine serum albumin, fraction V), 2-mercaptoethanol, Brilliant blue- R-250, Freund's complete adjuvant, Freund's incomplete adjuvant, sodium azide, NBT/BCIP (nitro blue tetrazolium /5-bromo-4-chloro-3-indolyl phosphate), polyvinyl polypyrrolidone, polyethyleneimine, Sepharose 4B, BApNA (N-benzpoyl-DL-arginine-*p*-nitro anilide), PMSF (phenyl methyl sulfonyl fluoride) were obtained from Sigma Chem. Co. (St. Louis, USA). Tricine, sodium dodecyl sulfate, acrylamide, N,N'- methylene bis-acrylamide, glycerol, bromophenol blue *etc.,* were obtained from Sisco Research Laboratories Limited, Mumbai. Tween-20, sodium ascorbate and gelatin were procured from HiMedia Laboratories Pvt. Limited, Mumbai. Alkaline phosphatase conjugated anti-rabbit IgG was obtained from Bangalore Genei, Bangalore. All other chemicals used were of analytical grade and were obtained from local sources in India. Whatman No. 1 filter paper and nitrocellulose membrane were obtained from Pall Life Sciences, USA.

Plants

Seeds of *A. duranensis, A. cardenasii, A. correntina, A. stenosperma* (all wild species) and JL-24 (cultivated species) were procured from ICRISAT, Patancheru, Hyderabad, germinated, planted and grown in plant culture facility of the university.

Experimental insects

(A) *Spodoptera litura* (Fabricius)

It is commonly known as tobacco cutworm or cluster caterpillar and belongs to the order Lepidoptera and family Noctuidae. It is a polyphagous pest affecting many crop plants which include tobacco, cotton, tomato, rice, castor, groundnut and many vegetables. The damage may reach upto 100% if the the insect infests at the early stages of crop.

Its life cycle consists of distinct larval, pupal and adult stages. The larvae or caterpillars are nocturnal in habit and it is the active feeding stage of the insect which feeds on the crop plant.

Rearing of *Spodoptera litura*

Healthy second instar larvae were procured from Directorate of Oil Seeds Research (DOR), Hyderabad, India and were reared in clean plastic troughs at insect culture facility of the department. Fresh castor leaves were provided to the larvae everyday. Pupae were collected and maintained in troughs containing moist sand and allowed to reach adult stage. The adults were transferred to egg laying cage and fed with 20% commercial honey (Honeyrex) containing vitamin E (Evinal-200) using cotton swab. The egg

masses were collected alternate day and sterilized. They were maintained on a moist filter paper and allowed to hatch. The cultures were maintained in insect culture room at 26 ± 1 ^{0}C, 60 ± 5% relative humidity (RH) and 14:10 h light:dark (LD) photoperiod.

(B) *Achaea janata*

It is commonly known as castor semilooper, belongs to the order Lepidoptera and family Noctuidae. The caterpillar primarily feeds on castor species Occasional hosts include banana, cabbage, Chinese cabbage, crown of thorns, *Ficus,* macadamia, mustard, poinsettia, rose, sugarcane and tomato as well as some legumes, teas, and other *Brassica* species.

Its life cycle consists of distinct larval, pupal and adult stages. The larvae or caterpillars are nocturnal in habit and it is the active feeding stage of the insect which feeds on the crop plant.

Rearing of *Achaea janata*

Healthy and actively feeding larval forms were collected from local castor field and maintained on fresh castor leaves in insect culture room at 26 ± 1°C, 60 ± 5% RH and 14:10 h LD photoperiod. Pupae were allowed to reach adult stage on a cotton bed in a plastic trough. The adults were collected and maintained in breeding cages and fed with 20% commercial honey (Honeyrex) containing vitamin E (Evinal-200) using cotton swab. The eggs were collected and allowed to hatch on a moist filter paper. The freshly emerged larvae were transferred to fresh tender leaves

Stages used for the present study

2^{nd}, 3^{rd}, 4^{th}, 5^{th} and 6^{th} instar larvae of *Spodoptera litura* and last (6^{th}) instar larvae of *Achaea janata* were used for the present study.

Bioassays

Extensive bioassays were carried out using various larval stages of *Spodoptera litura*. For each assay six to eight larvae of respective instars were placed in plastic boxes containing detached leaves of the different *Arachis* species, which include *A. duranensis, A. cardenasii, A. correntina,* and *A. stenosperma.* The boxes were kept under greenhouse conditions. Damp absorbent paper provided humidity in the boxes. Leaves were replaced by fresh ones every day and the weight of larvae was noted every 48 h. For all the bioassays JL-24 fed larvae were used as control.

Electron microscopic studies

For scanning electron microscopic studies, young and old leaves from above mentioned species were analysed using ESEM (XL-30) of FEI (at Central Instrumentation Laboratory of the university). The clean and fresh leaves were detached from plants and taken to CIL in a moist filter paper. They were gold coated using sputter coater for 5 min. A uniform thickness of around 400 ^{0}A was maintained. After gold coating the samples were introduced into vacuum chamber of the scanning electron microscope and the samples were analyzed under different magnifications and resolutions depending upon the requirement.

Protein sample preparation

Midgut homogenates from Spodoptera litura and Achaea janata

The *S. litura* and *A. janata* midgut homogenates were prepared according to the method of Ahmad *et al.,* (1980) with slight modification. The final instar larvae were anaesthetized on ice, midgut was dissected out in an ice cold iso-osmotic saline (0.15 M NaCl) and homogenized in 0.1 M. Tris-HCl buffer pH 8.0 (1 ml for each gut). The slurry was centrifuged at 12,000 x g for 20 min at 4 ^{0}C. The clear supernatant, designated as midgut homogenate, was transferred to a pre-chilled microfuge tube. This midgut homogenate enzyme preparation was divided into aliquots of 200 µl and stored at -20 ^{0}C until further use (upto two weeks).

Midgut luminal enzyme preparation from S. litura and A. janata

The final instar larvae of *S. litura* and *A. janata* were anaesthetized on ice and the digestive tract was dissected using ice cold iso-osmotic saline (0.15 M NaCl). It was then cleaned of unwanted adhering tissues and the gut luminal contents were collected into a pre-chilled microfuge tube, containing 0.1 M Tris-HCl (pH 8.0), by gently squeezing the wall of the midgut. The collected sample was centrifuged at 12000 x g for 20 min at 4 ^{0}C and the clear supernatant was designated as midgut luminal enzyme preparation. It was divided into aliquots of 200 µl. The samples were stored at -20 ^{0}C until further use (upto two weeks).

Macromolecular quantification

Protein

Protein content in various samples was estimated using micro-protein assay method of Bradford (1976).

Preparation of protein reagent

10 mg of Brilliant blue G-250 (Sigma) was dissolved in 5 ml of 95% ethanol. To this solution, 10 ml of 85% (w/v) orthophosphoric acid was added. The resulting solution was diluted to a final volume of 100 ml with distilled water, filtered through Whatman No. 1 filter paper and stored in an amber colored bottle at 4 ^{0}C.

Procedure for protein estimation

An aliquot of the sample was taken into a tube and the volume was adjusted to 0.1 ml with 10 mM Tris-HCl (pH 7.4). To this, 1 ml of protein reagent was added and mixed. After 10 min, absorbance at 595 nm was measured spectrophotometrically against a protein sample blank. The protein content in the sample was calculated using a standard curve prepared using BSA (fraction V).

Polyacrylamide gel electrophoresis

Denaturing gel electrophoresis (SDS-PAGE)

For protease inhibitor studies Tris-tricine sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) with acrylamide:N,N'-bisacrylamide (49.5% T, 3% C; T denotes the total percentage concentration of both monomers i.e. acrylamide and bisacrylamide and C denotes the

percentage concentration of the crosslinker relative to the total concentration T) was used according to the procedure of Schagger and Jagow (1987) with slight modification. The 12% T and 3%C gel was used as a uniform resolving gel which is overlaid by a 4% T, 3% C stacking gel (1 cm). Electrophoresis was carried out at 30 V when the sample is in stacking gel and 150 V when the sample is in resolving gel at 4 ^{0}C.

For studying the larval gut proteases, SDS-PAGE method of Laemmli (1973) was used.

The sample was prepared by mixing an aliquot of the protein sample with sample buffer containing 0.125 M Tris-HCI (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.002% bromophenol blue followed by incubation at 100°C for 1 min.

Non-reducing gel electrophoresis

This was carried out as described in denaturing gel electrophoresis except the change of sample buffer. The sample buffer did not contain any reducing agent. The 1X sample buffer consisted of 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 0.025% bromophenol blue and 1% SDS. The sample, after mixing with the sample buffer, was loaded on to the gel without boiling.

Visualisation of trypsin inhibitor by substrate gel electrophoresis (Zymography)

Leaf extract proteins or purified PIs were separated by non-reducing SDS-PAGE as previously mentioned with slight modification that the resolving gel was copolymerized with 0.1% (final concentration) gelatin. After running the gel, it was soaked in 2.5% Triton X-100 for 1 h. The gel was then washed

with distilled water for several times and was incubated in the enzyme solution given below for visualization of different inhibitory activity band(s).

For trypsin inhibitory activity: The gel was incubated in 0.1% trypsin in 0.05 M Tris-HCl buffer (pH 8.2) at 4 ^{0}C for 30 min and later for 90 min in the same solution at 37 ^{0}C. Then the gel was stained in comassie Brilliant blue R-250.

For chymotrypsin inhibitory activity: The gel was incubated in 0.05% chymotrypsin in 0.05 M Tris-HCl buffer (pH 7.8) at 4 ^{0}C for 30 min and later for 90 min in the same solution at 37 ^{0}C. Then the gel was stained in comassie Brilliant blue R-250.

For pepsin inhibitory activity: After washing with Triton X-100 and water, the gel was washed with 10 mM HCl for several times. Then incubated in 0.1% pepsin in 10 mM HCl for 30 min at 4 ^{0}C and for 90 min in the same solution at 37 ^{0}C. Then the gel was stained in comassie Brilliant blue R-250.

For papain inhibitory activity: The gel was incubated in 0.1% papain in 50 mM phosphate buffer (pH 6.8) containing 5 mM cysteine and 2 mM EDTA at 4 ^{0}C for 30 min and later for 90 min in the same solution at 37 ^{0}C. Then the gel was stained in comassie Brilliant blue R-250.

Visualisation of protease activity in the SDS-PAGE (Zymography) by casein diffusion method

The protease activity of larval gut proteases was visualized using the method of Garcia-Carreno *et al.,* (1993) with slight modification. Non-reducing gel electrophoresis of larval midgut homogenate and midgut luminal enzyme preparation was carried out as mentioned above. After electrophoresis, the gel was immersed in 2% casein in 50 mM glycine-NaOH buffer (pH 10.5) for 60

min at 4 ^{0}C. Then the casein solution was removed and incubated in 50 mM glycine-NaOH buffer (pH 10.5) at 37 ^{0}C for 90 min. Finally the gel was stained in comassie brilliant blue R-250

Visualization of electrophoretically separated proteins by silver staining

This was carried out according to the procedure of Blum *et al.,* (1987). The gel was incubated in fixative (50% methanol, 12% acetic acid and 50 µl of 37% formaldehyde/100 ml) for 1 h followed with 3 washes in 50% ethanol. Subsequently the gel was pretreated with sodium thiosulphate (20 mg/100 ml) for 1 min and rinsed thrice (20 sec each) with distilled water. The gel was impregnated with silver nitrate (0.2% with 187 µl of 37% formaldehyde) with gentle agitation for 30 min. The impregnated gel was rinsed with distilled water and developed with 6% sodium carbonate (w/v) and 50 µl of 37% formaldehyde (v/v). Finally, the stained gel was thoroughly rinsed with distilled water and stored in 50% methanol.

Coomassie staining of polyacrylamide gels

This was carried out according to the method of Wilson (1983). The gel was incubated for staining in coomassie solution (0.025% Brilliant blue- R250 in 40% methanol and 7% acetic acid) for 30 min. To visualize the reversible binding of stain to peptides, destaining with 5% methanol and 7.5% acetic acid was done to remove background staining.

Generation of polyclonal antibodies against protease inhibitors

The polyclonal antibody was raised against the purified protease inhibitors. For that, three months old male rabbits (New Zealand variety) were

injected with 100 µg of purified protease inhibitor (emulsified with 500 µl of Freund's complete adjuvant) by subcutaneous injections into various sites on the back. Prior to injection, the lateral ear vein was bled to collect pre-immune serum. After a fortnight, first booster injection was given followed by a second booster injection after seven days. For booster injections, 50 µg protein emulsified with Freund's incomplete adjuvant was used. The blood was collected after a week of second booster injection. The collected blood was left overnight at 4 ^{0}C for clotting and the antiserum was collected by centrifugation at 5,000 x g for 20 min. The antiserum was aliquoted and stored at -20 ^{0}C after adding 25% glycerol and 0.001% sodium azide.

Western blotting and immunostaining

The electrophoretically separated polypeptides were transferred (electro-blotted) to nitrocellulose membrane using Trans-Blot apparatus (Bio-Rad) according to the procedure of Towbin *et al.,* (1979). For this, the gel was first equilibrated in Towbin buffer (25 mM Tris, 192 mM glycine and 20% methanol) for 30 min followed by transfer to the membrane for 3 h at 70 V with 250 mA current limit. The transfer of protein to membrane was checked by reversible Ponceau S staining (100 mg Ponceau S in 5% acetic acid). The stain was removed by 3-4 washes with TBST [Tris buffered saline with Tween-20 (10 mM Tris-Cl (pH 7.4), 150 mM NaCl and 0.1% Tween-20 (v/v))]. For immunostaining, the protein blot was processed with 3% BSA (w/v) in TBST for 1 h at room temperature to block the non-specific binding sites followed by washing with TBST (10 min x 5 changes). The blot was then incubated with the primary antibody diluted in TBST containing 3% BSA

(w/v) for 2 h to overnight. This was again followed by a thorough wash in TBST (10 min x 5 changes). Thereafter, the blot was incubated with alkaline phosphatase (ALP) conjugated anti-rabbit IgG for 1 h. Once again the blot was washed in TBST (10 min x 5 changes). The visualization of the specific cross-reactivity was carried out with the substrates of ALP i.e., NBT/BCIP (0.0033% nitroblue tetrazolium and 0.0165% 5-bromo-4-chloro-3-indolyl phosphate in 10 mM Tris-HCl pH 9.5, 5 mM $MgCl_2$ and 10 mM NaCl) for color reaction.

Fractionation and purification of protease inhibitors

Preparation of crude extract

Freshly plucked leaves were thoroughly washed in tap water and then in distilled water. They were blot dried, condensed in liquid nitrogen and ground well. The liquid nitrogen was allowed to evaporate and the powder thus obtained was extracted in extraction buffer by stirring for 1h at room temperature. The extraction buffer contained insoluble polyvinyl polypyrrolidone (equal to the weight of the leaf tissue and presoaked in the buffer for 2 to 4 h), 0.2 M sodium tetraborate, 0.25 M sodium ascorbate, 20 mM sodium metabisulphite, 13 mM β mercaptoethanol, 10 mM EDTA and 2 mM PMSF. The slurry was centrifuged for 30 min at 10000 x g at 4 ^{0}C. In case the supernatant was not clear, it was centrifuged again. The clear and viscous supernatant thus obtained was treated with 0.5% polyethyleneimine to remove the DNA, responsible for the viscosity of the solution (Gegenheimer, 1990; Burgess, 1991). The DNA precipitate was removed by centrifugation at 10000 x g, 4 ^{0}C for 30 min. The clear supernatant was used as crude extract and was immediately subjected to ammonium sulphate fractionation.

Ammonium sulphate fractionation

The supernatant was subjected to 60% ammonium sulfate fractionation by adding slowly and in small amounts the required amount of ammonium sulfate. After completely adding the salt, the supernatant was fractionated for 4-5 h at 4 ^{0}C. Then the solution was centrifuged at 4 ^{0}C for 30 min at 12000 x g. The supernatant was discarded and the precipitate was dissolved in small volume of 50 mM Tris-HCl buffer (pH 7.4) and was dialyzed exhaustively in the same buffer. The dialysate was either used for the trypsin affinity column chromatography immediately or stored at -20 ^{0}C until further use

Trypsin affinity chromatography

The dialysate thus obtained was then loaded onto trypsin-Sepharose column equilibrated with 50 mM Tris-HCl (pH 7.4). The column was washed extensively with the same buffer and the breakthrough as well as the washings were discarded. After washing the column, the bound PI(s) were eluted with 10 mM HCl and the eluted fractions were quickly neutralized immediately with 1 M Tris. The eluted fractions with trypsin inhibitor activity were pooled and lyophilized for further analysis.

Assay of protease and protease inhibitor activity

The assay of trypsin, *S. litura* midgut protease (SLMP) and *A. janata* midgut protease (AJMP) was carried out according to the method of Erlanger *et al.,* (1961) using BApNA as substrate. Trypsin solution (1 mg/ml) was prepared by dissolving trypsin in 1 mN HCl. Trypsin (10 µg) or SLMP/AJMP of trypsin equivalent activity was mixed with 1 ml of 50 mM Tris-HCl buffer

(pH 8.2) (for commercial trypsin activity) or 50 mM glycine-NaOH buffer (pH 10.5) (for trypsin activity in SLMP and AJMP). The reaction was initiated by the addition of 1 ml of 1 mM BApNA (10% DMSO in 50 mM Tris-HCl, pH 8.2 or 50 mM glycine-NaOH buffer, pH 10.5) and incubated for 30 minutes at 37 ^{0}C. The reaction was terminated by the addition of 200 µl of 30% acetic acid and the absorbance was taken at 410 nm. One unit of trypsin activity was defined as the amount of trypsin that increases absorbance by 1 OD/min.

For the inhibitor assay, a suitable volume of *A. correntina/A. cardenasii* protease inhibitors was mixed with commercial bovine trypsin (10 µg) or with SLMP/AJMP of trypsin equivalent activity and incubated at 37 ^{0}C for 15 min and the assays were conducted at 37 ^{0}C for 30 min. Residual protease activity was then estimated as described above. One protease inhibitor unit was defined as inhibition of 1 unit of protease activity under the given assay conditions.

Thermal stability of A. correntina PIs

An aliquot (30 µl) of the purified *A. correntina* PIs was added to 62.5 mM Tris-HCl buffer (pH 7.4), 10% glycerol, 1% SDS and 0.002 % bromophenol blue in 1.5 ml microfuge tube and placed in boiling water for 15 min or 30 min. The remaining inhibitory activity was determined by zymography assay method.

Partial characterization of S. litura gut proteases

The *S. litura* midgut proteases were partially characterized by the method of Michaud *et al*., (1993) with slight modification. The gut extract (5 µg) was incubated with 1 mM PMSF or 0.3 M TLCK or 0.3 M TPCK in 0.1 M

Tris-HCl buffer (pH 8.2) for 10 min at room temperature. After treatment with the inhibitors, the gut protein extracts were fractionated on 10% SDS-PAGE (Laemmli, 1970). Then the protease activity was visualized in the gel by zymography method.

Coupling of trypsin to CnBr Sepharose

Trypsin was coupled to CnBr activated sepharose according to the following method. 1 g of CnBr activated resin was washed and swelled in several volumes of cold 1 mM HCl for 30 min. A total of 200 ml of HCl (1 mM) was used for each wash and the supernatant was removed by gentle suction with a pipette. Then the resin was washed with 10 ml of distilled water. After which the resin was washed with coupling buffer (0.1 M $NaHCO_3$ buffer containing 0.5 M NaCl, pH 8.3-8.5) and immediately transferred to a solution of the trypsin (30 mg) in coupling buffer (5 ml) in 15 ml capped centrifuge tube. The protein was mixed with the gel for overnight at 4 ^{0}C (on a rocker with end-over-end mixing). Then the unreacted trypsin was washed away using coupling buffer. The unreacted groups in the resin were blocked with 1 M ethanolamine (pH 8) for 2 h at room temperature. Then the coupled resin was washed extensively to remove the blocking solution, first with basic coupling buffer (pH 8.5), then with acetate buffer (0.1 M, pH 4) containing 0.5 M NaCl. This wash cycle of high and low pH buffer solutions was repeated 6 times. Then the resin was stored in 1 M NaCl containing sodium azide at 4 ^{0}C.

Results- Chapter I

Effect of feeding on larval body weight

Feeding bioassays carried out with 4th and 6th instar larvae of *Spodoptera litura* on the detached leaves of *A. cardenasii* showed growth retardation when compared with control larvae fed on JL-24 leaves (Figs. 1 & 2). 4th instar larvae were more affected than the 6th instar larvae.

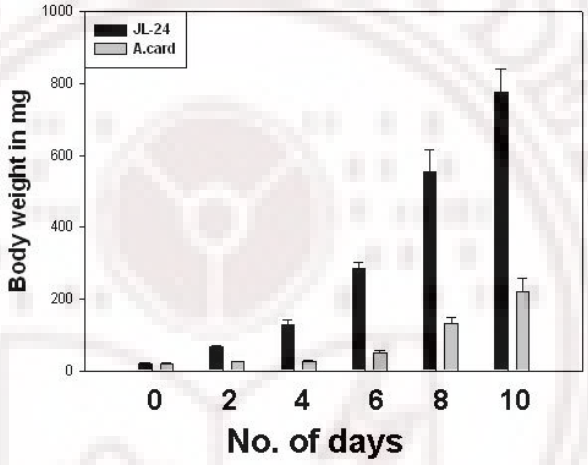


Fig. 1: Effect of feeding of 4th instar *S. litura* larvae on *A. cardenasii* leaves. * Each value is mean ± SD of 5 different experiments and for each experiment 10-15 larvae were used.

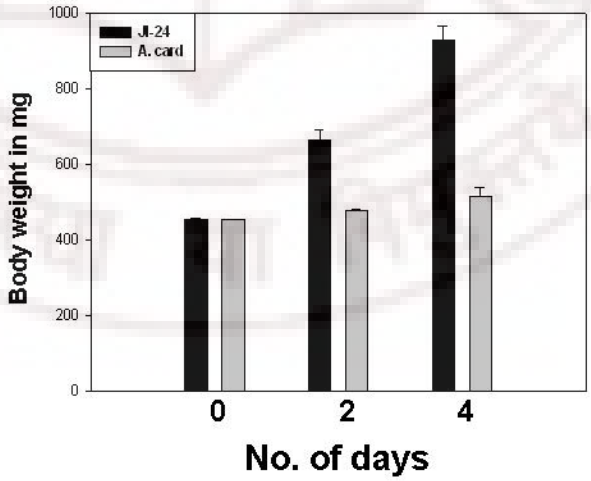


Fig. 2: Effect of feeding of 6th instar *S. litura* larvae on *A. cardenasii* leaves. * Each value is mean ± SD of 5 different experiments and for each experiment 10-15 larvae were used.

Bioassays carried out with the detached leaves of *A. correntina* (Figs. 3 & 4), *A. duranensis* (Figs. 5 & 6) and *A. stenosperma* (Figs. 7 & 8) also showed similar pattern as mentioned above. The 3rd and 4th instar larvae were more affected than late instar larvae (5th or 6th).

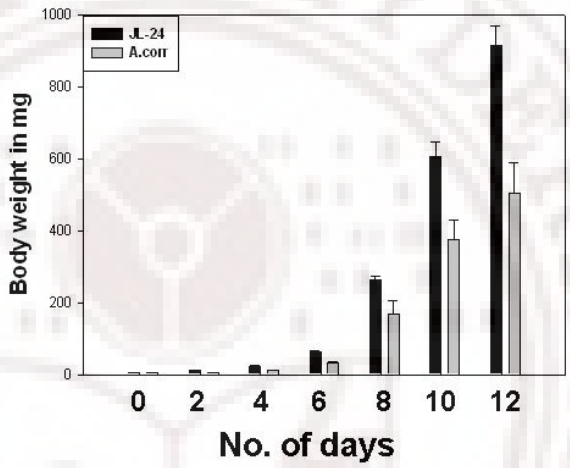


Fig. 3: Effect of feeding of 3rd instar *S. litura* larvae on *A. correntina* leaves. * Each value is mean ± SD of 5 different experiments and for each experiment 10-15 larvae were used.

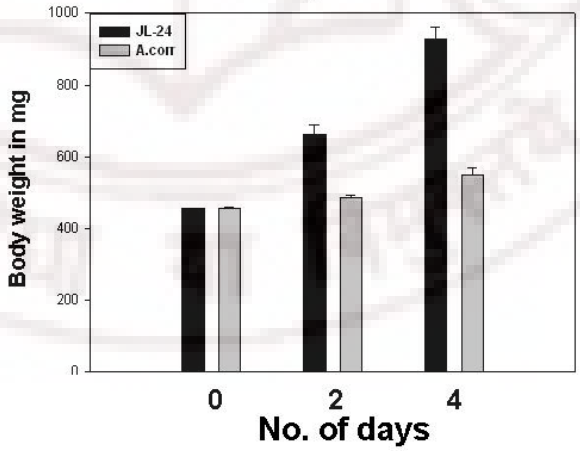


Fig. 4: Effect of feeding of 6th instar *S. litura* larvae on *A. correntina* leaves. * Each value is mean ± SD of 5 different experiments and for each experiment 10-15 larvae were used.

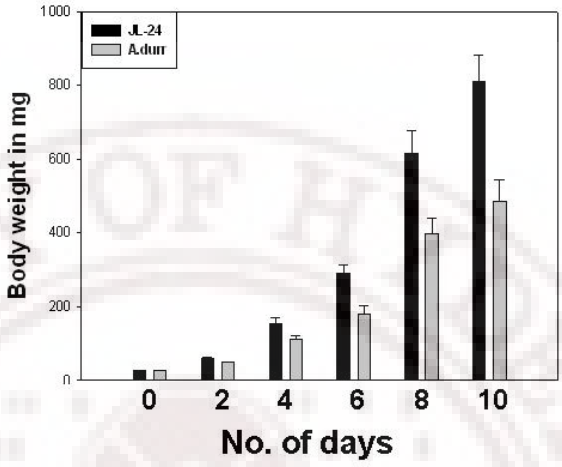


Fig. 5: Effect of feeding of 4th instar *S. litura* larvae on *A. duranensis* leaves. * Each value is mean ± SD of 5 different experiments and for each experiment 10-15 larvae were used.

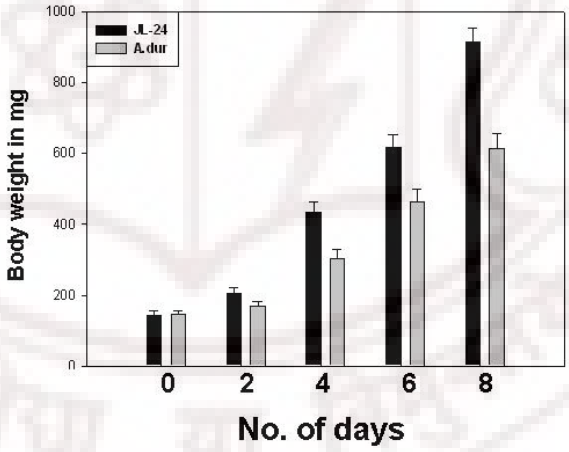


Fig. 6: Effect of feeding of 5th instar *S. litura* larvae on *A. duranensis* leaves. * Each value is mean ± SD of 5 different experiments and for each experiment 10-15 larvae were used.

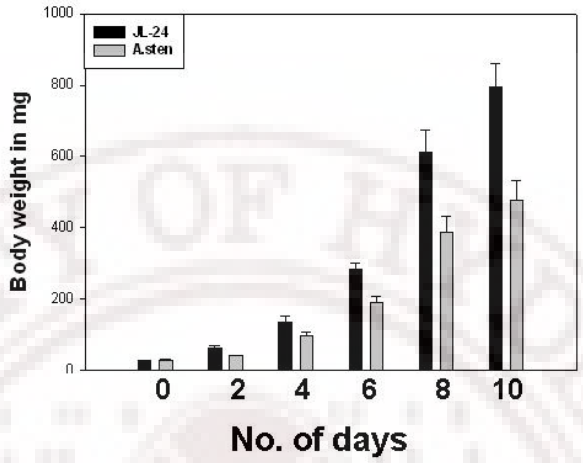


Fig. 7: Effect of feeding of 4th instar *S. litura* larvae on *A. stenosperma* leaves. * Each value is mean ± SD of 5 different experiments and for each experiment 10-15 larvae were used.

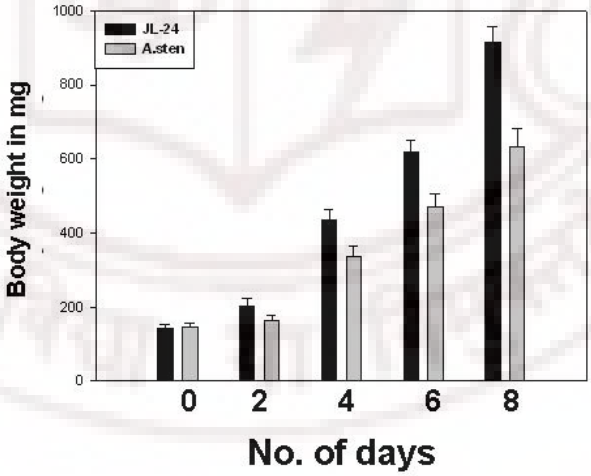


Fig. 8: Effect of feeding of 5th instar *S. litura* larvae on *A. stenosperma* leaves. * Each value is mean ± SD of 5 different experiments and for each experiment 10-15 larvae were used.

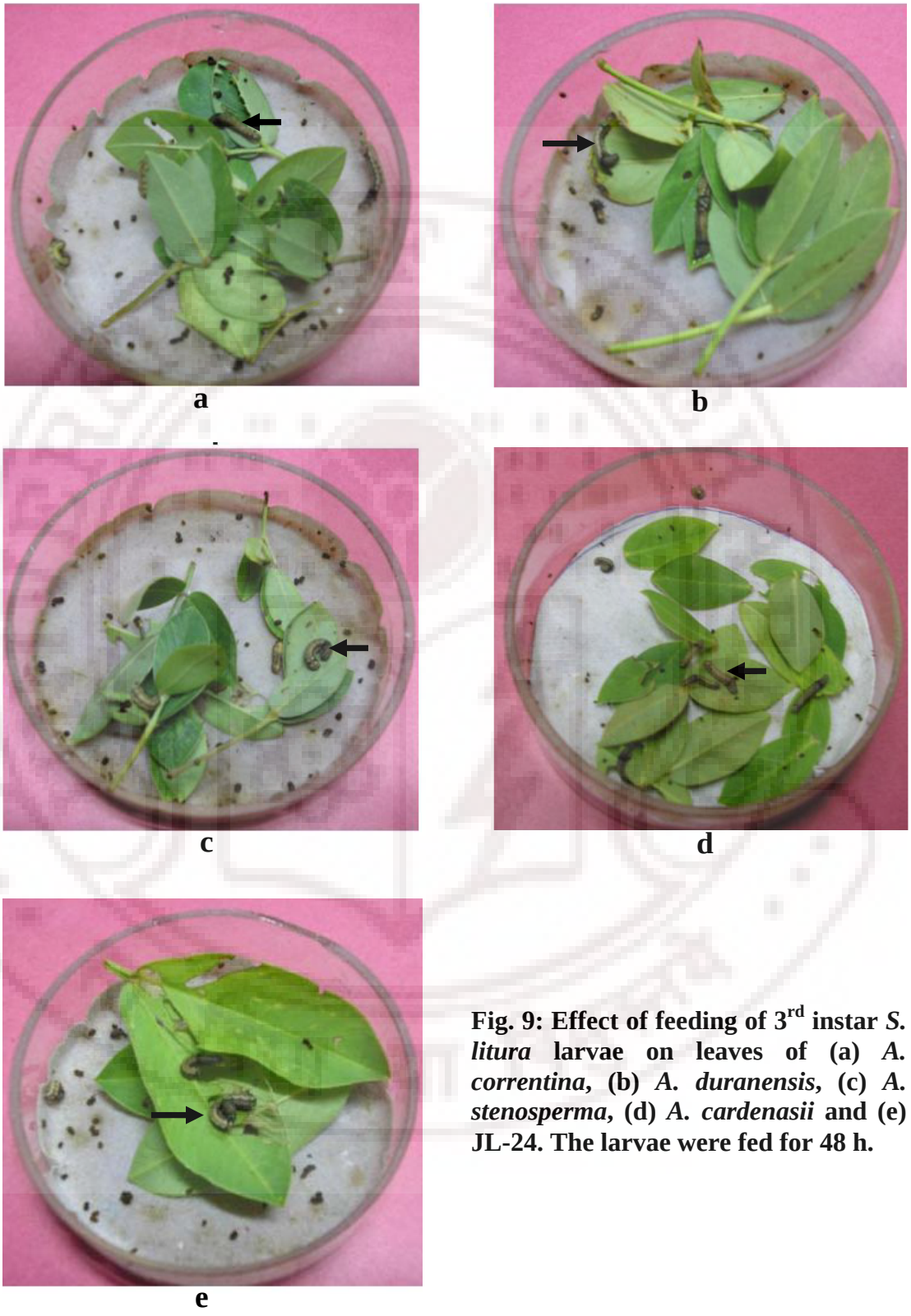


Fig. 9: Effect of feeding of 3rd instar *S. litura* larvae on leaves of (a) *A. correntina*, (b) *A. duranensis*, (c) *A. stenosperma*, (d) *A. cardenasii* and (e) JL-24. The larvae were fed for 48 h.

The effect of feeding of *S. litura* larvae on wild species of groundnut leaves is seen as early as after 48 h, when 3rd instar larvae were used. The larvae which fed on wild groundnut leaves (Figs. 9a, 9b, 9c, 9d) did not grow well when compared with the control larvae fed on JL-24 (cultivated groundnut species) leaves (Fig. 9e). Of the four wild species *A. cardenasii* was found to be most effective in inhibiting the larval growth (Fig. 9d).

Effect of feeding on post-embryonic development and pupal transformation

There was a marked reduction in the transformation of larvae to pupae when they were fed on wild groundnut leaves as compared to JL-24 fed larvae (Fig. 10). The larvae which fed on *A. cardenasii* leaves were the most affected, where the pupal transformation was only 32% followed by *A. correntina* (51%), *A. duranensis* (54%), and *A. stenosperma* (58%) as opposed to control (JL-24).

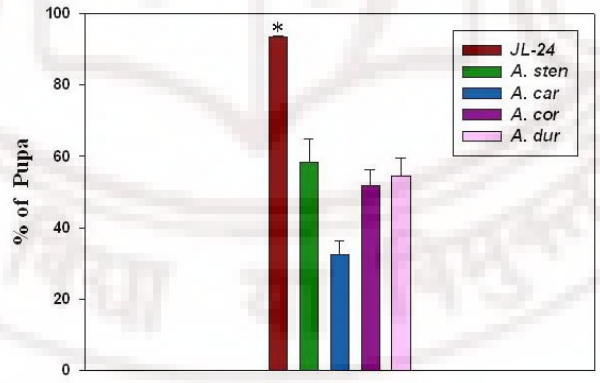


Fig. 10: Effect on larval pupal transformation: 3rd instar larvae of *S. litura* were fed on leaves till they reached pupal stage. Each value is mean ± SD of 5 different experiments and for each experiment 10-15 larvae were used. * After natural mortality.

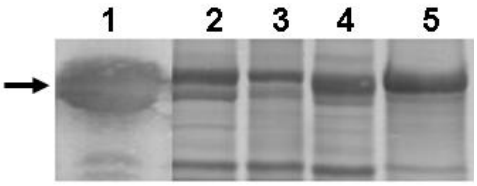
Effect of feeding on protein profile and protein content of the haemolymph

After feeding, when the larvae reached the stage of 6th instar, they were sacrificed and haemolymph was collected and the protein was quantified as well as analysed by SDS-PAGE.

Table 3: Total haemolymph protein content of 6th instar *S. litura.* For this experiment 3rd instar larvae of *S. litura* fed on leaves of different groundnut species and allowed to grow till the 6th instar stage.

Species	Quantity (µg/µl)
JL-24	**80 ± 10.09**
A. correntina	**32 ± 2.95**
A. cardenasii	**28 ± 1.39**
A. duranensis	**43 ± 5.28**
A. stenosperma	**39 ± 3.53**

As shown in table 3, there was significant decrease in the quantity of total protein present in the haemolymph of the larvae that fed on wild groundnut species when compared with the larvae that fed on cultivated species. This reduction was almost 65% *A. cardenasii* while it was 49% in *A. correntina*, 53.75% in *A. duranensis* and 48.75% in *A. stenosperma*.



Lane 1: JL-24
Lane 2: *A. correntina*
Lane 3. *A. cardenasii*
Lane 4: *A. duranensis*
Lane 5: *A. stenosperma*

Fig. 11: Haemolumph protein profile of *S. litura* larvae fed on leaves of different groundnut species. Each lane was loaded with 25 µg of total haemolymph protein and the proteins were visualized with Comassie Brilliant Blue. Note the presence of hexamerin.

Literature survey as well as extensive studies from our laboratory shows that the hexamerins constitute the major component of storage proteins in the haemolymph of lepidopteran insects and play important function as storage proteins which support larva-pupa-adult transformation during post-embryonic development. SDS-PAGE analysis was carried out to check the fate of these hexamerins in the experimental larvae. Results presented in the figure 11 show that the hexamerin content is very much reduced in the case of insects that were fed on leaves of wild groundnut species (lanes 2-5) when compared with the insects that were fed on cultivated groundnut species (lane 1).

Effect of feeding on larval midgut proteases of S. litura

The 3rd instar larvae of *S. litura* were fed either with the leaves of JL-24 or *A. correntina*. When the larvae reached 6th instar the midgut homogenates were prepared and the midgut proteases were analyzed by activity staining using casein diffusion method. The results did not show a major variation

between the protease profile of the larvae that were fed with the leaves of JL-24 and *A. correntina*. But there was an increase in the activity of proteases corresponding to 43, 29, 25 and 20 kDa in the case of larvae that were fed on *A. correntina* leaves (Fig. 12, lanes 3-6).

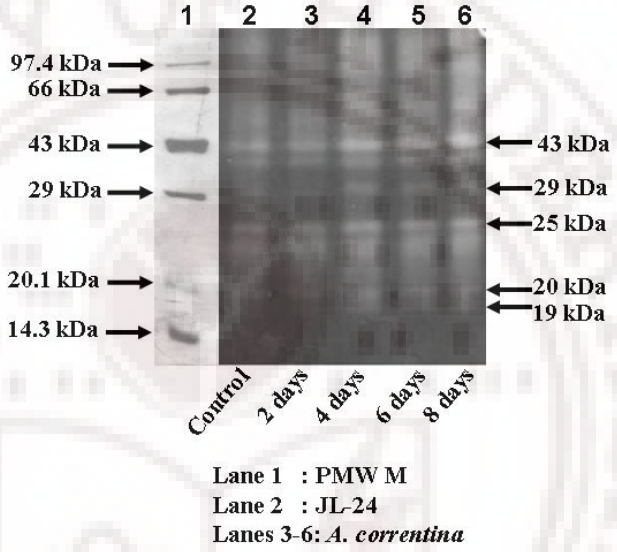


Fig. 12: Zymograph showing digestive proteinase activity of *S. litura* larvae fed on leaves of *A. correntina* and control (JL-24) plants. Lanes 2-6 were loaded with 5 µg protein of larval midgut extract. For this experiment 4th instar larvae were fed for 4 days.

Characterization of S. litura larval midgut proteases

The gut protease profile from 6th instar larvae which were grown on castor leaves was analyzed by substrate gel electrophoresis using casein diffusion method. For this experiment freshly prepared midgut homogenate was treated with different protease inhibitors and their effect on the proteases was analyzed (Fig. 13). Each lane was loaded with 5 µg of the midgut homogenate protein. The results show the inhibition of the four major

proteases (25, 32, 34 and 43 kDa) by PMSF (lane 3). Firthermore the inhibition was fairly high for 32 and 34 kDa proteases, which were almost completely inactivated. TLCK inhibited completely the protease corresponding to 43 kDa (lane 4) but had no effect on other proteases. None of the proteases were affected by the treatment with TPCK (lane 5)

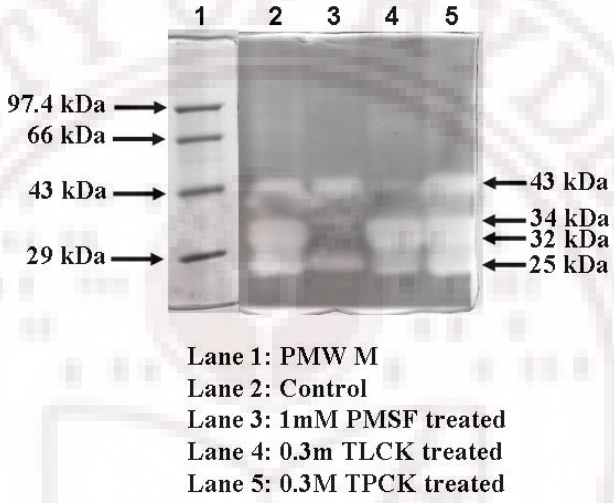


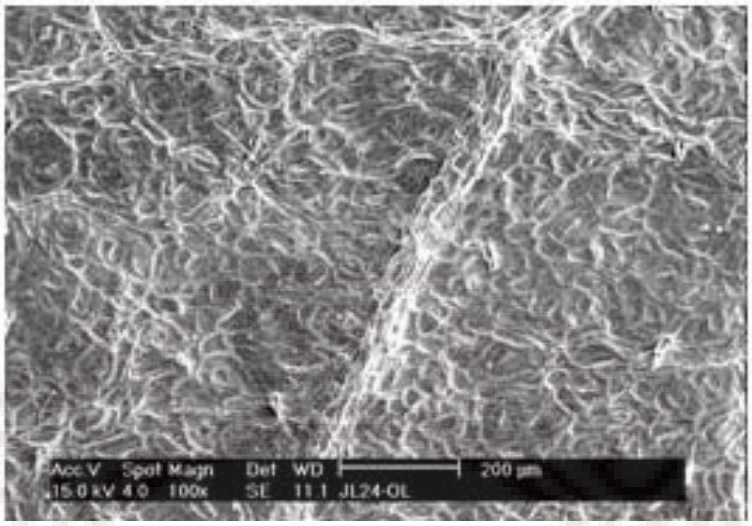
Fig. 13: Zymogram of *S. litura* midgut proteases showing the effect of different protease inhibitors. 5 µg of gut protein extract was fractionated in 10% SDS-PAGE.

Results- Chapter II

Correlation of leaf surface and feeding inhibition-

As we have seen the retarded growth of the larvae that were fed with the leaves of wild groundnut species, detailed scanning electron microscopic studies of leaf surface was carried out to analyze the physical structures which might be responsible for defenses including the feeding inhibition in the larval forms of *S. litura*.

Parameters relating to physical defenses like trichomes were compared across young and old leaves of wild and cultivated groundnut plants. The results presented in figures 14-17 clearly show a significant difference between adaxial and abaxial surface of the leaves. The trichomes are present abundantly on the abaxial surface of the leaves (Figs. 14 & 15) except for *A. duranensis* which has very few numbers of trichomes (Fig. 14c). The adaxial surface of the leaves show little or no trichomes (Figs. 16 & 17). The density of trichomes was also notably different between the old and young leaves (Figs. 14 & 15). *A. cardenasii* possessed the highest density of trichomes (Figs. 14b & 15b) followed by *A. stenosperma* (figs. 14d & 15d), *A. correntina* (Figs. 14e & 15e), *A. duranensis* (Figs. 14c & 15c) and JL-24 (Figs. 14a & 15a). The adaxial surface of both young and old leaves of *A. cardenasii* (Figs. 16b & 17b), *A. stenosperma* (Figs. 16d & 17d), *A. duranensis* (Figs. 16c & 17c) and JL-24 (Figs. 16a & 17a) do not contain any trichome.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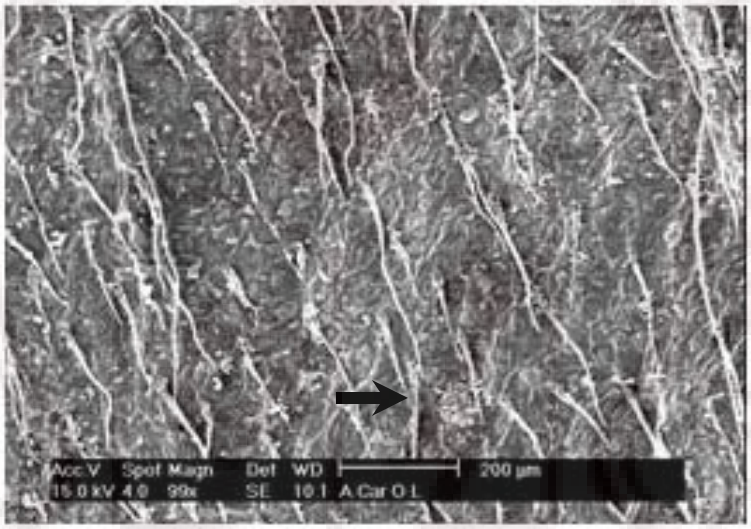
15.0 kV 4.0 100x SE 11.1 JL24-OL

14a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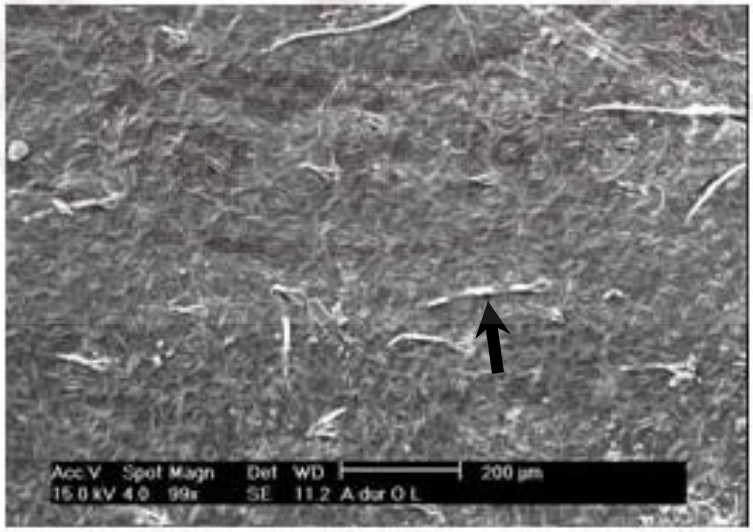
14b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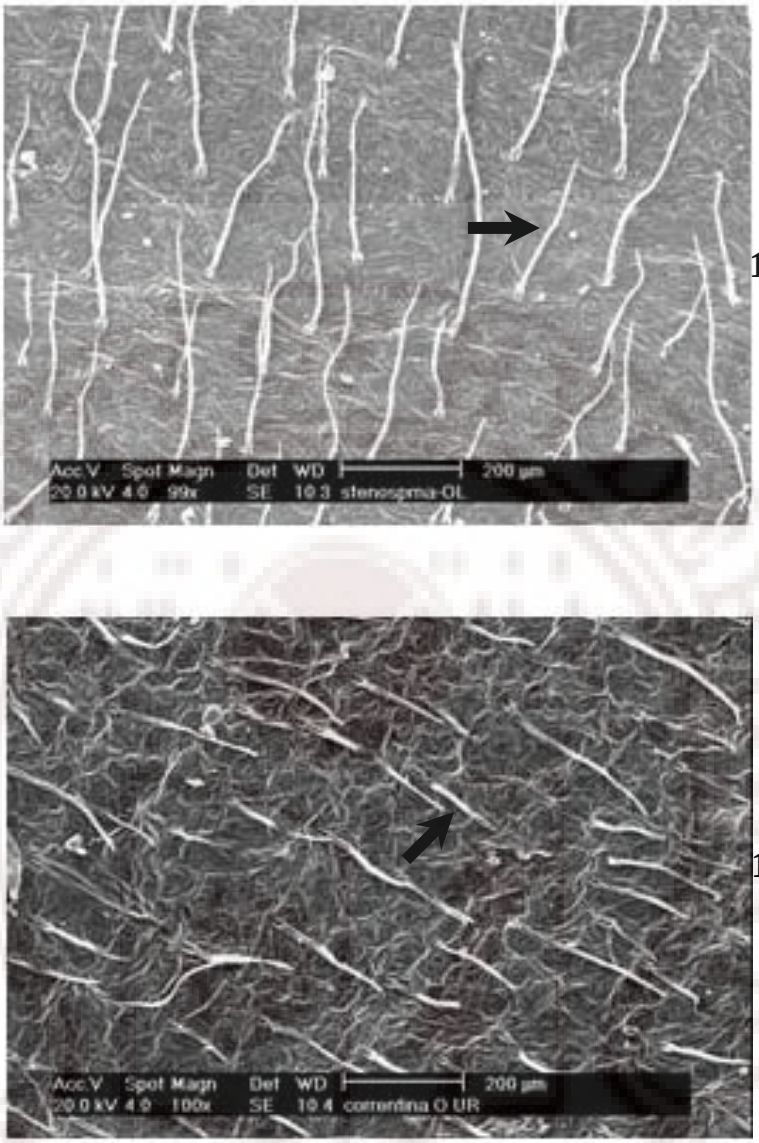
15.0 kV 4.0 99x SE 11.2 A.dor O.L

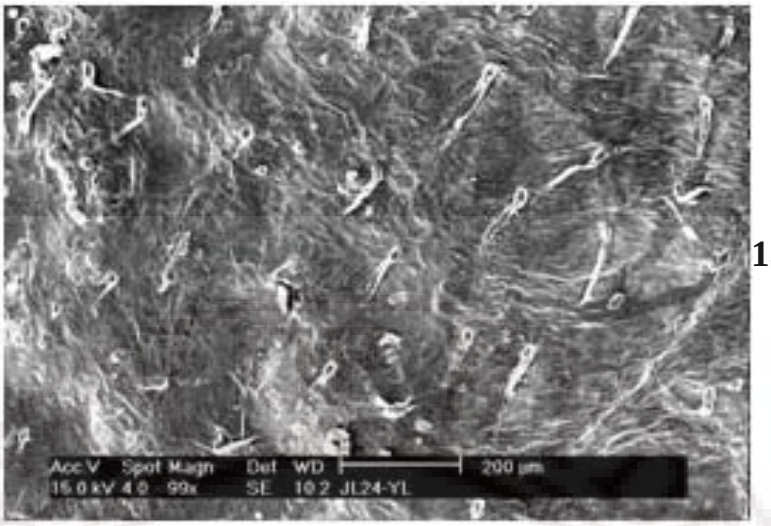
14c



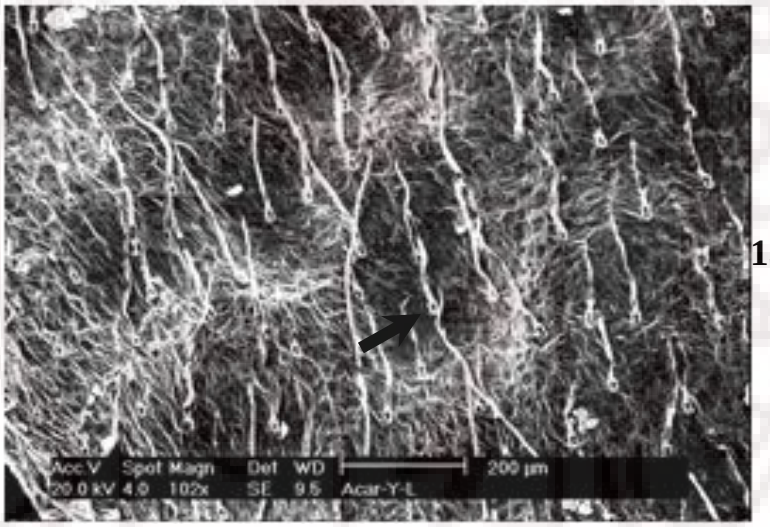
14d

14e

Fig. 14: Scanning electron micrograph of abaxial surface of old (30 days) leaves of JL-24 (14a), *A. cardenasii* (14b), *A. duranensis* (14c), *A. stenosperma* (14d) and *A. correntina* (e). Each micrograph was taken at 100x magnification. (➡ Trichome)


Acc.V Spot Magn Det WD 200 µm
15.0 kV 4.0 99x SE 10.2 JL24-YL

15a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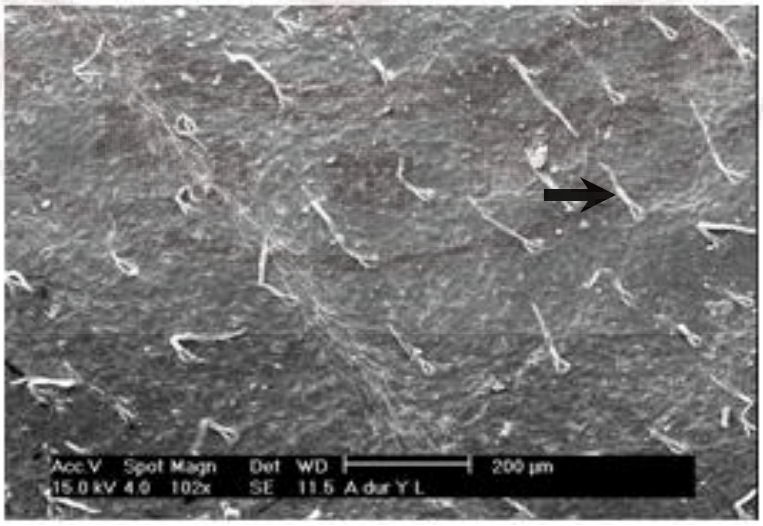
20.0 kV 4.0 102x SE 9.5 Acar-Y-L

15b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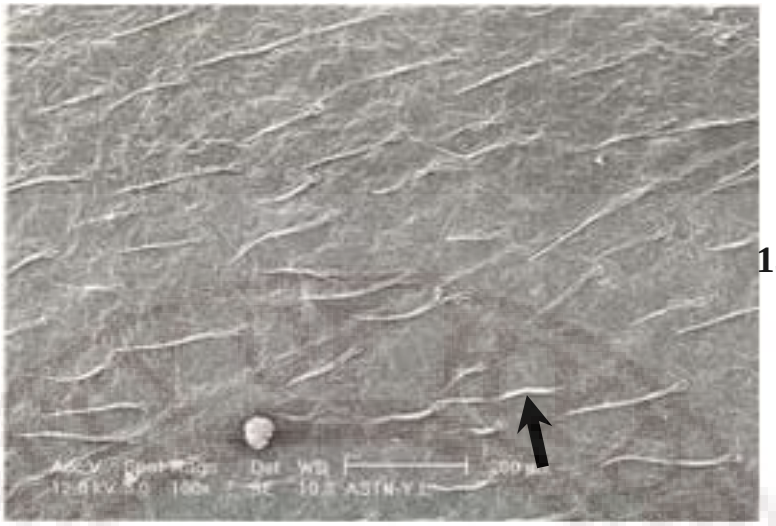
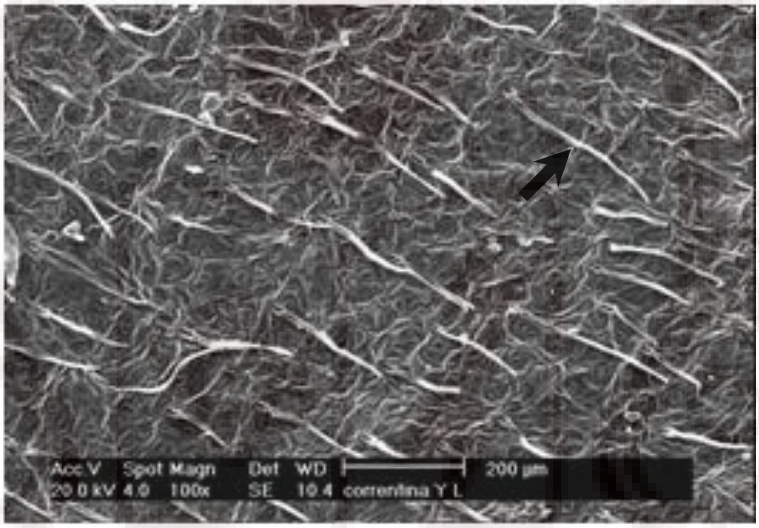
l5c

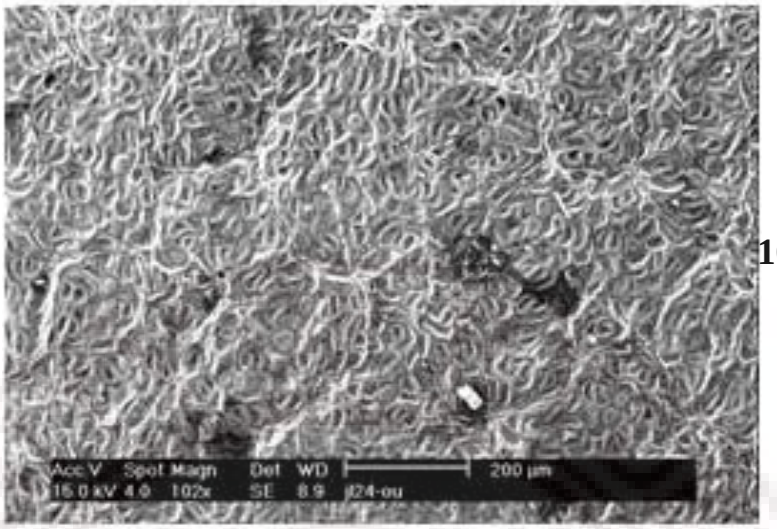


15d



15e

Fig. 15: Scanning electron micrograph of abaxial surface of young (3 days) leaves of JL-24 (15a), *A. cardenasii* (15b), *A. duranensis* (15c), *A. stenosperma* (15d) and *A. correntina* (15e). Each micrograph was taken at 100x magnification. (➡ Trichome)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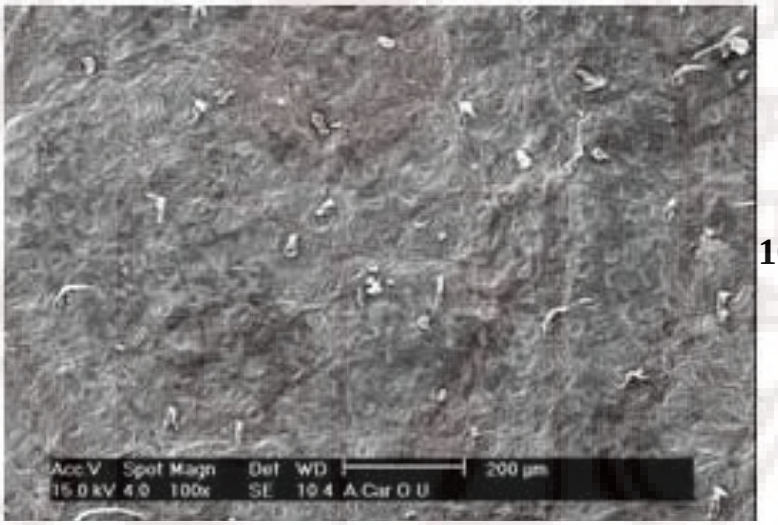
15.0 kV 4.0 102x SE 8.9 j24-ou

16a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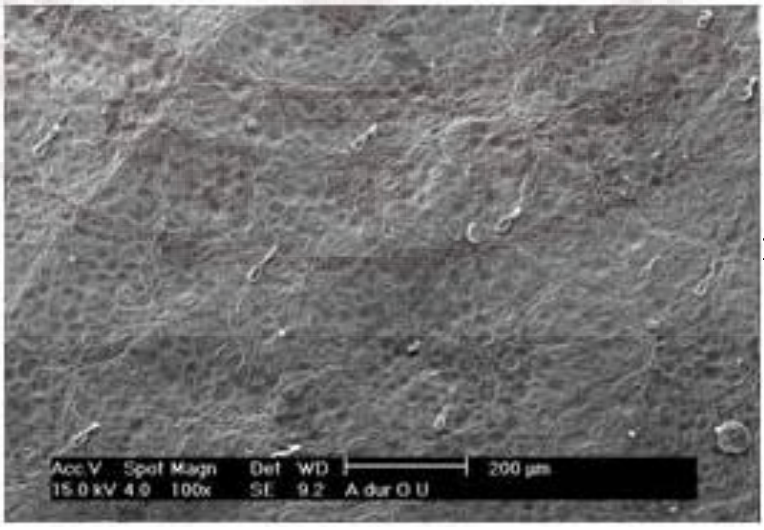
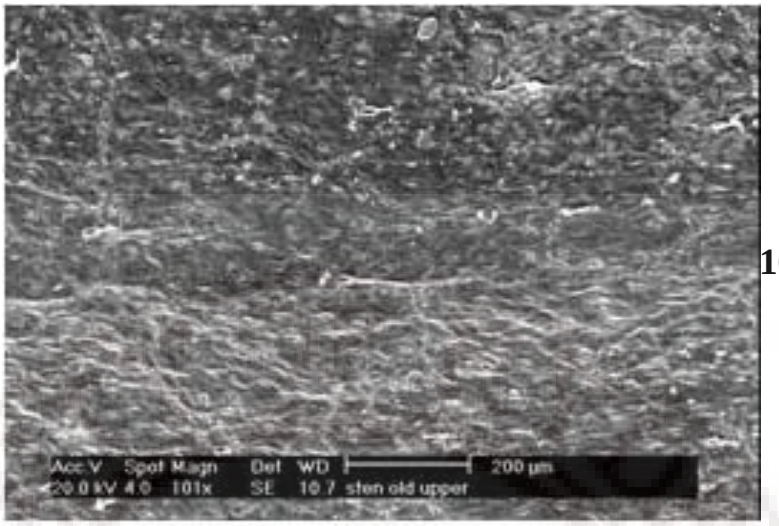
15.0 kV 4.0 100x SE 10.4 A.Car 0 U

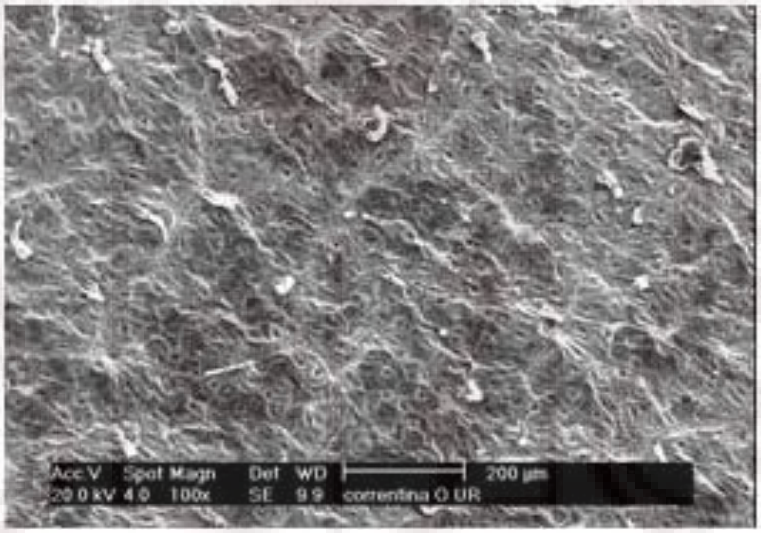
16b


Acc.V Spot Magn Det WD 200 µm
15.0 kV 4.0 100x SE 9.2 A dur 0 U

16c

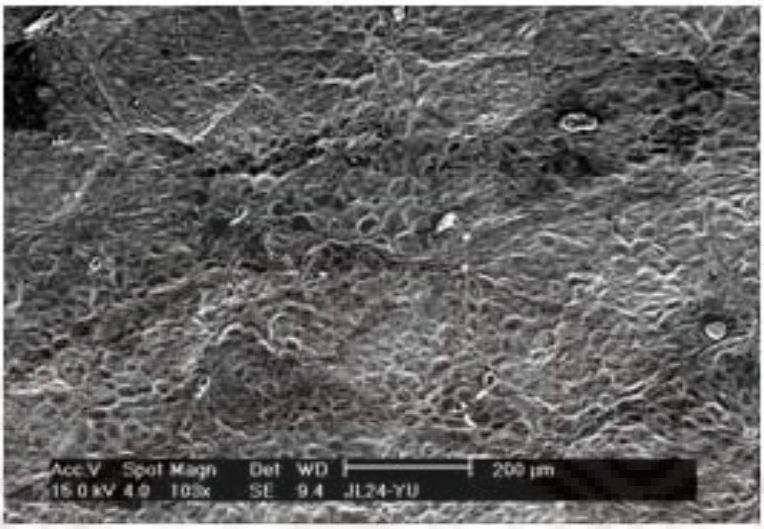


16d

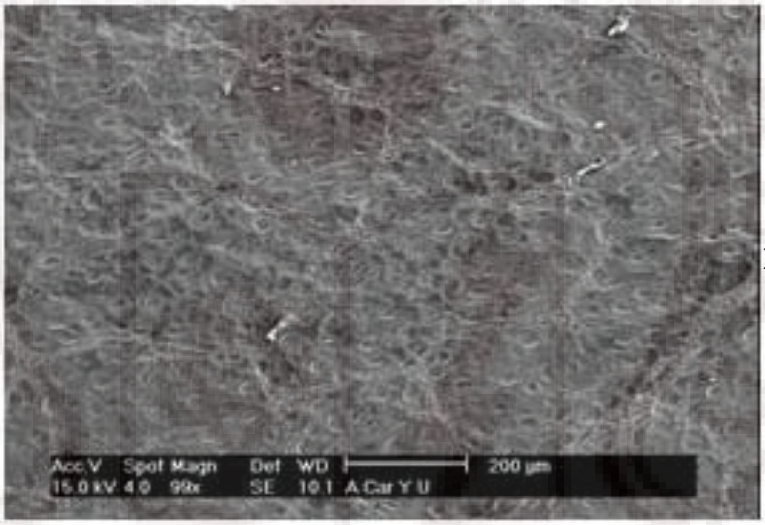


16e

Fig. 16: Scanning electron micrograph of adaxial surface of old (30 days) leaves of JL-24 (16a), *A. cardenasii* (16b), *A. duranensis* (16c), *A. stenosperma* (16d) and *A. correntina* (16e). Each micrograph was taken at 100x magnification.


Acc.V Spot Magn Det WD 200 µm
15.0 kV 4.0 103x SE 9.4 JL24-YU

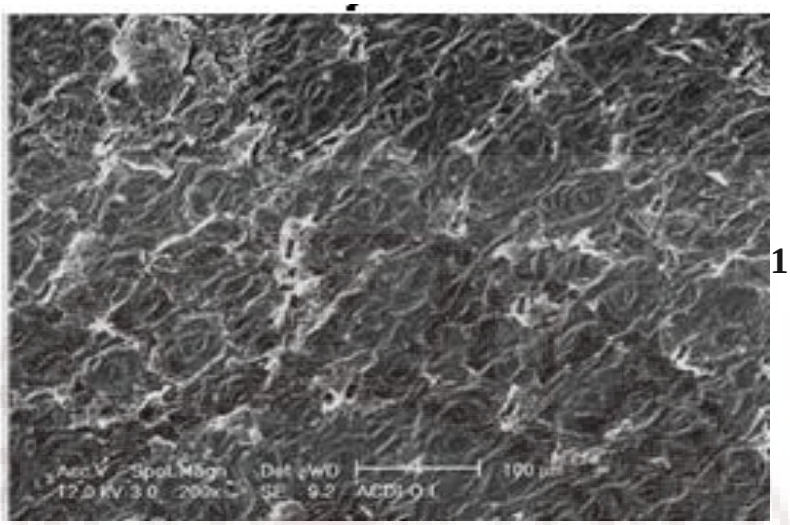
17a


Acc.V Spot Magn Det WD 200 µm
15.0 kV 4.0 99x SE 10.1 A Car Y U

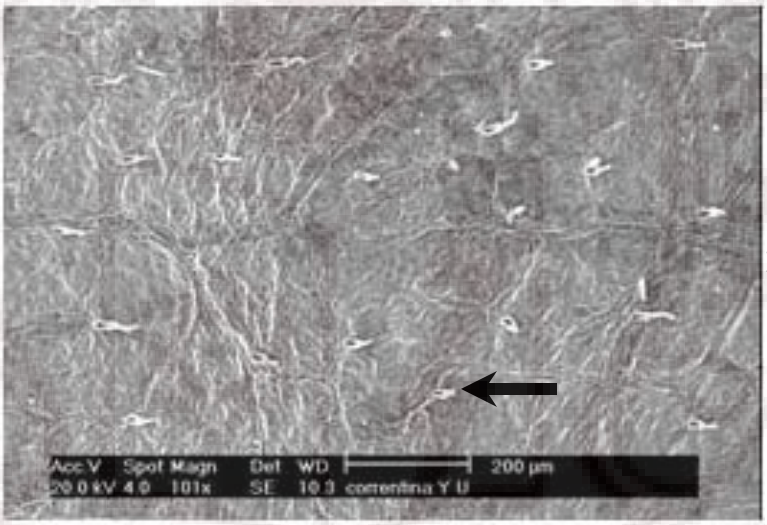
17b


Acc.V Spot Magn Det WD 200 µm
15.0 kV 4.0 98x SE 11.0 A dur Y U

17c



17d



17e

Fig. 17: Scanning electron micrograph of adaxial surface of young (3 days) leaves of JL-24 (17a), *A. cardenasii* (17b), *A. duranensis* (17c), *A. stenosperma* (17d) and *A. correntina* (17e). Each micrograph was taken at 100x magnification. (➡ Trichome)

Results- Chapter III

A careful observation of the leaf surface reveals variation in the length of trichomes across the species (Figs. 14 & 15). The results presented in the figure 18a show that trichome is the longest in *A. stenosperma*, followed by *A. cardenasii*, *A. correntina*, *A. duranensis* and JL-24. However, no correlation was found between the trichome length and the percentage of gain in body weight of larvae that fed on the leaves of evaluated groundnut species (Fig. 18b).

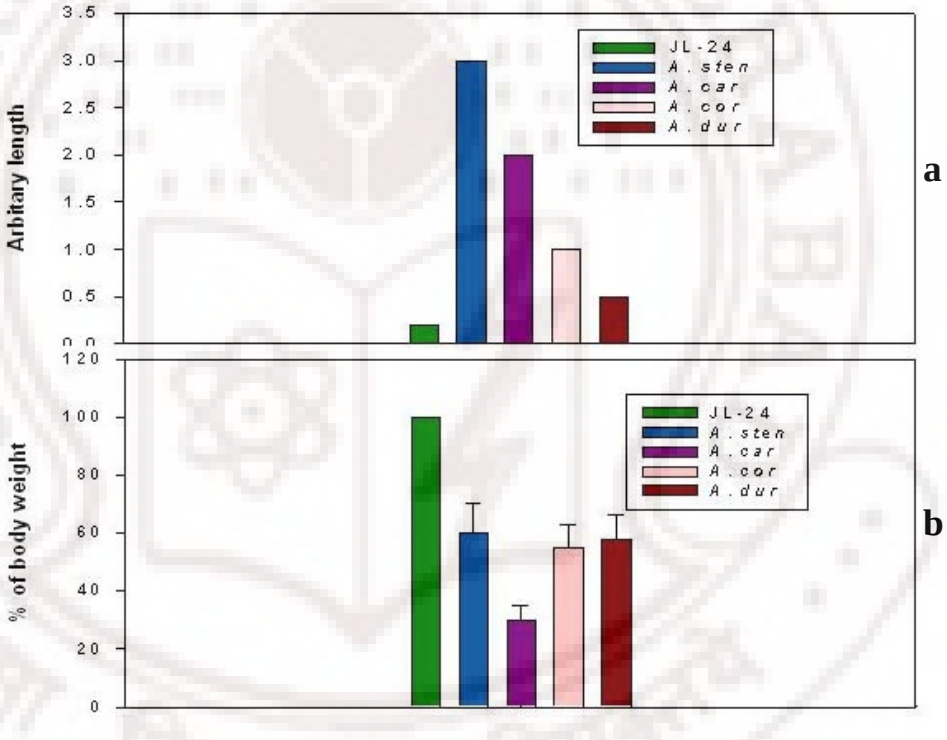


Fig. 18: Correlation between the length (arbitrary units) of trichome and % gain in body weight of *S. litura* larvae. 3rd instar larvae were allowed to feed on different species of groundnut leaves. When the larvae reached 6th instar, their body weight was noted. (a) Length of trichome (b) % gain in body weight. For each experiment 8-10 larvae were used and each value is mean ± SD of four independent experiments.

Purification and characterization of protease inhibitors from A. correntina and A. cardenasii

In recent years there is a growing interest in the identification of novel PIs because of their potent antiproliferative activity and in prevention of carcinogenesis in a wide range of *in vivo* and *in vitro* systems/models, as well as their use in developing pest resistance in otherwise susceptible plants (Qi *et al.*, 2005). Here we describe the purification and some properties of protease inhibitors from wild *Arachis* species *A. correntina* and *A. cardenasii*.

Purification and characterization of protease inhibitors from A. correntina

The crude extract prepared from the thoroughly washed and air dried leaves (5 g) of *A. correntina* was used for the purification of protease inhibitors. All the purification steps were carried out at room temperature unless otherwise specified. The purification of the PIs is summerised in table 4.

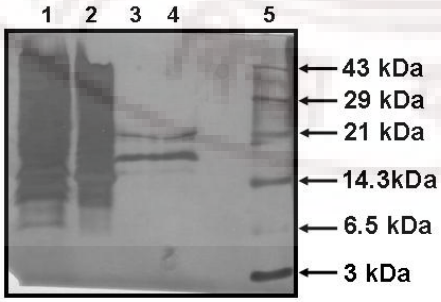
Table 4: Summary of purification of *A. correntina* PIs

Purification step	Quantity of protein (mg)	Activity against bovine Trypsin (Inhibitory units, IU)	Specific activity
Crude extract of leaf	40.00	43.50	1.08
Dialysate after 60% $(NH_4)_2SO_4$ fractionation	12.80	28.30	2.21
Affinity purified	0.825	15.88	19.24

PIs from leaf of *A. correntina* were purified to homogeneity using $(NH_4)_2SO_4$ precipitation and trypsin-Sepharose affinity chromatography. The specific activity of the crude extract towards bovine trypsin was 1.08 TIU/mg. The 60% $(NH_4)_2SO_4$ fraction was applied to a trypsin-Sepharose column. The bound inhibitor eluted as a single peak, on reducing the pH with 10 mN HCl. Affinity chromatography proved to be a very convenient step for the isolation of PIs from leaves, although the possibility of limited digestion of the inhibitor by the immobilized trypsin during purification cannot be excluded. The resulting affinity purified PIs have fairly high specific activity (19.19 IU/mg).

Homogeneity and size of the A. correntina PIs

The homogeneity and apparent molecular mass of the *A. correntina* PIs were estimated by SDS-PAGE. When subjected to 12% SDS-PAGE (Schagger and Jagow, 1980) under reducing conditions, the purified protease inhibitor preparation showed the presence of three polypeptides with molecular weights of 16, 18 and 20 kDa (Fig. 19).

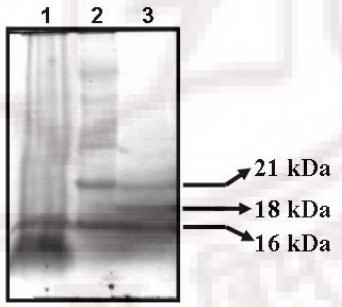


Lane 1: Crude
Lane 2: 60% $(NH_4)_2SO_4$ frac
Lane 3: Purified PI (Elute 2)
Lane 4: Purified PI (Elute 3)
Lane 5: PMW-L

Fig. 19: Purification profile of *A. correntina* PIs Lanes 1 & 2 were loaded with 15 µg of protein and lanes 3-5 were loaded with 5 µg of protein.

Specificity of A. correntina PIs

The purified *A. correntina* PIs were tested for their inhibitory activity against trypsin and chymotrypsin (serine proteases), pepsin (aspartic protease) and papain (cysteine protease) using gelatin-embedded 12% SDS-PAGE. These PIs inhibited trypsin and among these three PIs, the 16 kDa PI showed maximum inhibitory activity which is evident by the intensity of the corresponding band with 16 kDa (Fig. 20). *A. correntina* PIs also inhibited chymotrypsin and the 18 kDa inhibitor appeared to have maximum chymotrypsin inhibitory activity which is once again evident by the thickness of the corresponding band (Fig. 21a). However, these PIs failed to inhibit either pepsin (Fig. 21b) or papain (Fig. 21c) as is evident by the clear gel without any inhibitor protein bands.



Lane 1: Crude
Lane 2: 60% $(NH_4)_2SO_4$ fraction
Lane 3: Affinity purified

Fig. 20: Zymographic study- the gelatin gel containing *A. correntina* PIs was incubated with trypsin (see materials and methods). Lanes 1 & 2 were loaded with 15 µg of protein and lane 3 was loaded with 10 µg of protein. Proteins were stained with Comassie Brilliant blue.

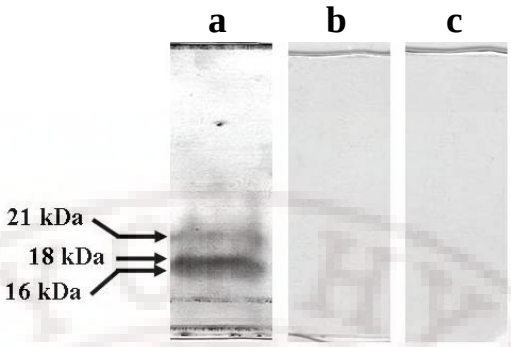


Fig. 21: Zymographic study- The gelatin gel containing *A. correntina* PIs was incubated with chymotrypsin (a), pepsin (b) and papain (c) (see materials and methods). Each lane was loaded with 5 µg of purified protein. Proteins were stained with Comassie Brilliant blue.

Thermal stability of A. correntina PIs

The thermal stability of *A. correntina* PI activity in 62.5 mM Tris-HCl buffer (pH 7.4) containing 10% glycerol, 1% SDS and 0.001% Bromophenol blue was investigated. Results presented in figure 22 clearly show that the PIs are relatively stable at boiling temperature of the water for 30 min (lane 2)

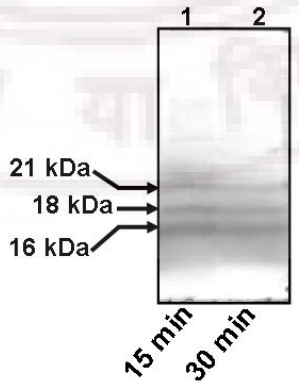


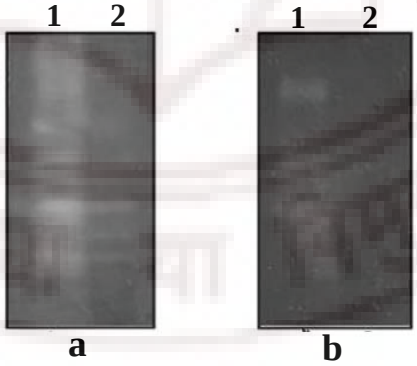
Fig. 22: Heat stability of inhibitory activity of *A. correntina* PIs at boiling temperature of water for 15 and 30 min. Each lane was loaded with 5 µg of the purified protein.

Inhibitory activity of A. correntina PIs on insect gut proteases

The inhibitory activity of *A. correntina* PIs was tested using gelatin-embedded SDS-PAGE as well as enzyme assay using synthetic substrates for the proteases.

(i) Inhibitory activity of A. correntina PIs on S. litura proteases

Midgut homogenate as well as midgut luminal enzyme preparation proteins were run in a gelatin SDS-PAGE. The gel was initially incubated with 40 mg of *A. correntina* crude extract and then incubated in Tris-HCl buffer (pH 7.4) (Fig. 23b). For control, the gel was incubated with buffer only (Fig. 23a). The remaining assay of protease activity was carried out as mentioned in materials and methods. Figure 23b clearly shows that the degree of inhibition of protease activity was much higher towards the midgut luminal enzyme preparation (lane 2) than the midgut homogenate (lane 1).



Lanes 1: Midgut homogenate
Lanes 2: Midgut luminal enzyme preparation

Fig 23: Effect of *A. correntina* leaf protein extract on larval gut proteases of 6th instar *S. litura* larva. Each lane was loaded with 5 µg of protein. (a) Treated with Tris-HCl buffer (pH 7.4) and (b) treated with plant protein extract.

(ii) Inhibitory activity of A. correntina PIs on S. litura and A. janata gut proteases

A. correntina PIs inhibited around 25% activity of the 6th instar *S. litura* larval midgut proteases while the inhibition was almost 73% with 6th instar larvae of *A. janata* (Fig. 24).

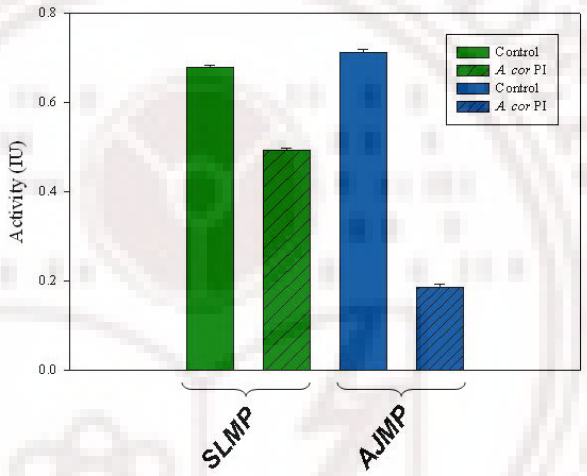


Fig 24: Inhibition of *Spodoptera litura* (SLMP) and *Achaea janata* midgut protease (AJMP) activity by purified *A. correntina* PIs. Inhibition assay was conducted using BApNA as substrate.

Purification and characterization of protease inhibitor from A. cardenasii

The crude extract prepared from the thoroughly washed and air dried leaves (5 g) of *A. cardenasii* was used for the purification of protease inhibitors. All the purification procedures were carried out at room temperature unless otherwise specified. The purification of the PIs is summerised in table 5.

Table 5: Summary of Purification of *A. cardenasii* PIs

Perification step	Quantity of protein (mg)	Activity against bovine Trypsin (Inhibitory units, IU)	Specific activity
Crude extract of leaf	61.2	51.25	0.83
Dialysate after 60% $(NH_4)_2SO_4$ fractionation	19.2	35.6	1.88
Affinity purified	0.94	17.9	18.9

The *A. cardenasii* protease inhibitor was purified to homogeneity using $(NH_4)_2SO_4$ precipitation and trypsin-Sepharose affinity chromatography. The specific activity of the crude extract towards bovine trypsin was 0.837 TIU/mg. The 60% $(NH_4)_2SO_4$ fraction was applied to a trypsin-Sepharose column. The bound inhibitor was eluted as a single major peak on reducing the pH with 10 mN HCl. Although affinity chromatography gave reasonably good result the possibility of limited digestion of the inhibitor by the immobilized trypsin during purification cannot be excluded. Affinity purified PIs have fairly good specific activity (18.9 IU/mg).

Homogeneity and size of the A. cardenasii PI

The homogeneity and apparent molecular mass of the *A. cardenasii* PI was estimated by SDS-PAGE. When subjected to SDS-PAGE under reducing conditions, the gel pattern of the purified protease inhibitor preparation from *A.*

cardenasii showed the presence of a single polypeptide with a molecular mass of around 18 kDa (Fig. 25).

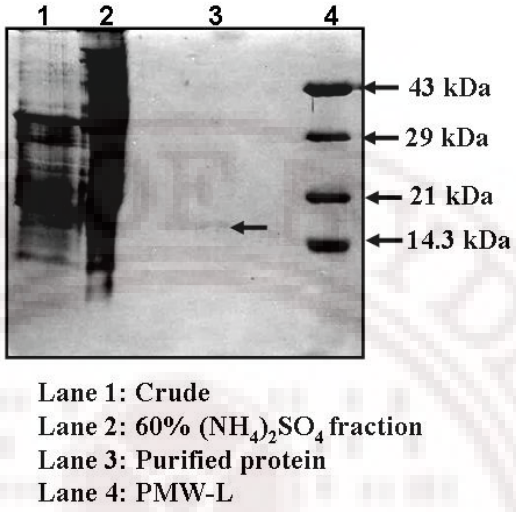


Fig. 25: SDS-PAGE analysis of purified *A. cardenasii* PI. Lanes 1 & 2 were loaded with 15 µg of protein and lane 3 was loaded with 5 µg of protein.

Stability of A. cardenasii PI

Result presented in figure 26 shows that the inhibitory activity *A. cardenasii* PI is not affected at a temperature up to 100°C for 30 min.

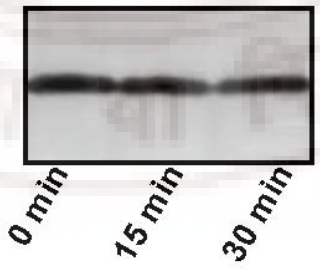


Fig. 26: Heat stability of inhibitory activity of *A. cardenasii* PI at boiling temperature of water for 15 and 30 min. Each lane was loaded with 5 µg of the purified PI.

Immunological similarity between soyabean Bowman-Birk inhibitor (BBI) and A. cardenasii PI

Antibodies were generated against commercially available soyabean Bowman-Birk inhibitor. Soyabean BBI antibodies cross reacted with the *A. cardenasii* PI (Fig. 27) indicating that the soyabean BBI and *A. cardenasii* PI have immunological similarity.

Lane1: *A. cardenasii* PI
Lane2: Soyabean BBI

Fig. 27: Western blot analysis of purified *A. cardenasii* PI. The PIs were fractionated by SDS-PAGE, blotted onto nitrocellulose, probed with anti-soyabean BBI antiserum and detected using a secondary alkaline phosphatase-coupled antibody (Materials & methods).

Inhibitory activity of A. cardenasii PI on S. litura and A. janata gut proteases

A. cardenasii PI inhibited around 29% activity of the 6th instar *S. litura* larval gut proteases on the other the same PIs inhibited almost 80% of the total gut protease activity of 6th instar larvae of *A. janata* (Fig. 28).

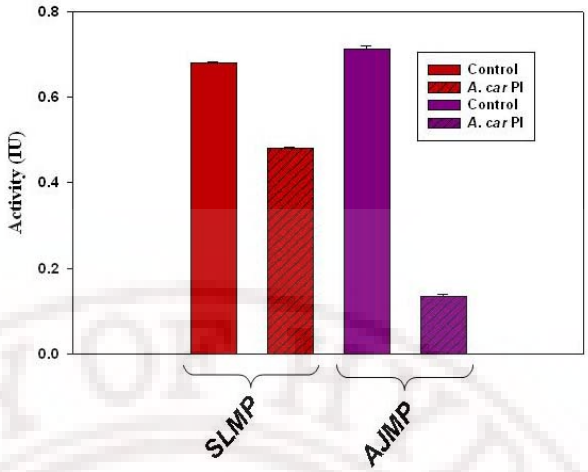


Fig. 28: Inhibition of *Spodoptera litura* and *Achaea janata* midgut protease activity by purified *A. correntina* PIs. Inhibition assays were conducted using BApNA as substrate.

Discussion

The major objective of the present study was to evaluate the anti-feedant and growth inhibitory activity of different wild groundnut species, and identify few potent insecticidal species for detailed analysis. This is mainly to isolate/purify and characterize the anti-feedant and growth inhibitory factor(s) from the wild species of groundnut. To this end, the anti-feedant and growth inhibitory activity of *A. correntina, A. cardenasii, A. duranensis* and *A. stenosperma* was evaluated using feeding bioassays. Further, the protease inhibitors from *A. correntina* and *A. cardenasii* were purified and characterized using different analytical and preparative methods. Midgut proteases of *S. litura* larvae were also characterized using substrate gel electrophoresis technique.

The result of this study showed that the middle (3^{rd} & 4^{th}) instar larvae of *S. litura* were more affected upon feeding on a potent groundnut species than the late (5^{th} & 6^{th}) instar larvae. When larvae of *S. litura* were allowed to develop on artificial diet containing soybean (Kunitz) trypsin inhibitor (SBTI), the neonate larvae were more susceptible to the effects of protease inhibition than the older larvae (McManus and Burgess, 1995). Similar results were obtained earlier when rats were fed with diets containing PIs, where younger animals had higher growth inhibition (Grant *et al*. 1993). Further, Bhattacharyya *et al.,* (2007) also reported that the PIs of *Archidendron ellipticum* greatly affected the survival of early instar larvae of *S. litura*. Later Charity *et al.* (1999) reported that *Helicoverpa armigera* and *H. punctigera,* when fed on the transgenic tobacco and pea plants expressing *Nicotiana alata*

PI, showed increased mortality within first 8 days of the feeding. The observation that the feeding on SBTI containing diet, transgenic plants expressing *Nicotiana alata* PI and feeding on wild groundnut species which might contain protease inhibitors clearly show that they have a greater influence on neonate larvae (in terms of mortality) and middle instar larvae (in terms of growth rate), respectively. This also suggests the usefulness of the PIs as a resistant factor in transgenic plants. Such an effect will have maximum benefit if the transgenic plant acts as a host for adult females to lay eggs, and where the newly hatched larvae feed on the plant tissue.

In contrast to the study presented here, SBTI supplied in artificial diet did not initially affect survival of *Helicoverpa armigera* larvae but affected their growth in the first 14 days and it was only after this the mortality of the larvae increased (Johnston *et al*., 1993). However, Charity *et al.* (1999) reported that *H. armigera* larvae, after ingesting Na-PI, not only developed slowly but were also lethargic. A recent study demonstrated significant reduction in growth of H. armigera larvae after feeding on winged bean chymotrypsin inhibitor incorporated in artificial diet (Telang *et al.,* 2009). For practical reasons, in the field, if the larval stage is prolonged, and has slow mobility the exposure to environmental stress as well as predators also would increase and such insects may not protect themselves as vigorously as the normal ones.

Hexamerins are the most important molecules which serve as nitrogen and aminoacid pool to support *de novo* synthesis of proteins during

metamorphosis in insects (Burmester and Scheller, 1995, 1996; Pan and Telfer, 1996). In the present study, the feeding of leaves of wild groundnut species to *S. litura* larvae resulted in retarded larval growth as well as decline in the transformation of larvae into pupae. This could be due to the reduced storage protein levels in the larvae, because of the anti-feedant activity of the wild groundnut plants. The synthesis of hexamerins including arylphorins (storage proteins) is dependant on nutrient supply (Tojo *et al.* 1985; Webb and Riddiford, 1988). In holometabolous insects including lepidopterans the hexamerins are predominantly synthesized by the fat body and released simultaneously into the surrounding body fluid (hemolymph). In the haemolymph their concentration increases gradually from penultimate larvae to final instar larvae, where they may account for 80% of the total haemolymph proteins by weight (Kramer *et al.,* 1980; Tojo *et al.,* 1980; Palli and Locke, 1987; Kanost *et al.,* 1990; Telfer and Kunkel, 1991; Hannerland, 1996; Kirankumar *et al.,* 1997). Previous studies from our laboratory showed that hexamerins are utilized during metamorphosis as well as adult reproduction (Ismail and Dutta-Gupta, 1990; Ismail and Dutta-Gupta, 1991; Kirankumar *et al.,* 1997; Arif *et al.,* 2007, 2008).

Results obtained in the present study clearly show a significant increase in the larval gut protease activity as well as synthesis of new proteases after feeding on the leaves of wild groundnut plants when compared with the larvae that fed on leaves of JL-24 (control) plants. These results corroborate with the findings of McManus and Burgess (1995) where in a significant stimulation of

tryptic and elastase activities in the digestive proteases extracted from larvae fed on artificial diet containing SBTI. The hyper-production of trypsin with the ingestion of SBTI was shown to have deleterious consequences to variety of organisms including insects (Broadaway and Duffy, 1986). Present study further suggests that the larvae actively mobilize genomic resources in the digestive tract to mitigate the impact of ingested plant protease inhibitor (Chi *et al.,* 2009). However, to further ascertain whether this increase in stimulation of protease (trypsin like) activity is the primary mode of action of the ingested inhibitors and possibly crucial for insect survival, more definitive experiments have to be carried out.

In the next part of the work, the relation between the physical defenses (mainly trichomes) of the plants and the feeding inhibition of the larvae was evaluated to clarify whether the observed growth inhibition was anyway associated with physical hindrance. Hence, various parameters such as number and size of trichomes were compared across young and old leaves of wild and cultivated groundnut plants. In this study it was observed that the abaxial surface of both young and old leaves possess considerable numbers of trichomes in all wild groundnut species analysed in present study, except for *A. duranensis,* which has few trichomes. On the other hand the adaxial surface of young as well as old leaves possess fewer trichomes. This is in contrast to the findings of Matsuki *et al.,* (2004), who reported a significant difference in physical defences between early and late leaves of *Betula ermanii* and *Betula platyphylla,* where trichome density was significantly greater in early (young)

leaves than on late (old) leaves. In the present study no correlation was found between the feeding and the number/size of the trichome, suggesting that the observed feeding inhibition in *Spodoptera litura* larvae is most likely not related to physical structures of leaf surface.

As the observed effects of wild groundnut leaf feeding was comparable to that of feeding of protease inhibitors (PIs) and keeping in view that the members of the leguminaceae family contain protease inhibitors, we planned to isolate protease inhibitors from the leaves of wild groundnut species. The strategy for the isolation was based on the removal of the interfering DNA by treating the leaf extract with polyethyleneimine and interaction between the inhibitors and their cognate proteases, apart from using different chemicals to avoid the oxidation of the proteins by phenols and quinones. In this procedure a differential pH was utilized for the elution of these inhibitors from immobilized trypsin Sepharose columns by virtue of their different degree of association with the enzyme. Utilizing this strategy we isolated three PIs from *A. correntina* and one PI from *A. cardenasii*. All the protease inhibitors showed inhibitory activity on substrate gel electrophoresis.

The isolated PIs from *A. correntina* and *A. cardenasii* inhibited trypsin as well as chymptrypsin, however, at present, it is not clear whether the inhibitors have two active sites (one for trypsin and the other for chymotrypsin) or a single active site (*i.e.*, only one active site interacting with both trypsin and chymotrypsin). They did not inhibit either pepsin (an aspartic acid protease) or papain (a cysteine protease), indicating that they belong to the

class of serine protease inhibitors. They are also resistant to heat treatment which is a characteristic feature of the Bowman-Birk PIs (DiPietro and Liener, 1989). Western blot and immunodetection studies revealed the cross reactivity of *A. cardenasii* PI with antibodies raised against Bowman-Birk inhibitor (BBI) of soybean. This suggests that the *A. cardenasii* PI is immunologically related to soybean BBI. The inhibitory activity of both *A. correntina* PIs and *A. cardenasii* PI was higher towards *A. janata* proteases than *S. litura* proteases. This observation is in agreement with the results obtained by Harsulkar *et al.*, (1999) where they reported that the PIs from non-host plants were more potent inhibitors of *Helicoverpa armigera* gut proteases than the PIs of host plants. More than one hundred insect species have been reported as pests on groundnut in India (Amin, 1988). However, of these, only a few of them cause economic loss and *S. litura* is one among them while *A. janata* is not among the major pests of groundnut (Ghewande and Nandagopal, 1997).

The PIs isolated from *A. correntina* and *A. cardenasii* leaves are found to be different in their molecular mass from those which have been reported from *A. hypogaea*. Tur-Sinai *et al.,* (1972) have purified a 7.5 kDa trypsin and chymotrypsin inhibitor from groundnut seeds, which was stable in water when heated at 100 ^{0}C for 15 min. Later Norioka *et al.,* (1982) purified five Bowman-Birk type trypsin and chymotrypsin PIs (double headed) from *Arachis hypogaea* seeds. The molecular masses of these inhibitors were 7.4, 7.6, 6.9, 6.8 and 6.7 kDa. However, about fourty years ago, Hochstraser and his co-workers (1969) isolated two PIs from peanut seeds (*A. hypogaea*). These

inhibitors had a molecular weight of 17 kDa and were tetramers of a subunit, which consisted of 48 amino acid residues.

All the above reports along with the results obtained in the present study suggest that probably different types of PIs are synthesized/stored in different tissues/storage organs of plants including groundnut. Further, the amino acid sequence studies reveal that the different molecular mass PIs could be the product of a single gene (Norioka and Ikenaka, 1983).

The proteases have been reported to be present in midgut lumen of *Spodoptera litura* (Ahmad *et al,* 1976). An attempt was made to characterize the proteases of the midgut of *S. litura,* and results reveal the presence of four different proteases which belong to the serine class of proteases, which is typical to lepidopteran larvae (Terra, 1990; Christeller *et al,* 1992; Hegedus *et al,* 2003). Using substrate gel electrophoresis assays the presence of trypsin like enzyme in *S. litura* larval midgut is clearly demonstrated by its inhibition with trypsin specific chemical inhibitor TLCK. Further, no chymotrypsin like activity was found, as there was no inhibition of the enzyme activity when treated with TPCK, a chymotrypsin specific inhibitor. These results can be correlated with findings that the *S. litura* larval midgut has predominently trypsin like activity and no chymptrypsin like activity which was reported by Ahmad *et al.,* (1980) using enzyme assays. This also suggests that the enzymes which are synthesized in the gut epithelial cells and the enzymes that are secreted into the midgut lumen are similar in nature (Terra and Ferreira, 1994). In addition, Down *et al,* (1999) to their surprise, detected the soybean Kunitz

trypsin inhibitor (SKTI) in the haemolymph of *Lacanobia oleracea* following ingestion in the diet. The exact mechanism by which the inhibitor was transported to the haemolymph from the gut is not known.

Finally, the analysis of inhibitory activity of *A. correntina* PIs and *A. cardenasii* PI on *S. litura* and *A. janata* midgut proteases reveal a significant inhibition of enzymatic activity in non-host insect (*A. janata*).

There are already reports that the phytophagous insects can adapt to the presence of either transgenic or endogenous serine PIs of its host plants by over-expressing PI-insensitive proteases (Bolter and Jongsma, 1995; Jongsma *et al.,* 1995; Broadway, 1996). This problem can be overcome by using PIs from non-host plants (Duan *et al.*, 1996; Harsulkar *et al.,* 1999; Telang *et al.,* 2005) and/or by using PIs of different classes that work synergistically (Amirhusin *et al.*, 2007). In a second approach PIs from sources other than plant could also be used (Brunke *et al.,* 1995; Thomas *et al.,* 1995; Christeller *et al.*, 2002; Christy *et al.,* 2009). In another approach PIs engineered by structural modeling can be used (Urwin *et al*, 1995a, 1995b).

In conclusion the present work demonstrates the presence of various moderate molecular mass (16-21 kDa) PIs in the wild species of groundnut, which play a protective role in insect defense and could be exploited for management of lepidopteran pests.

Summary and Conclusions

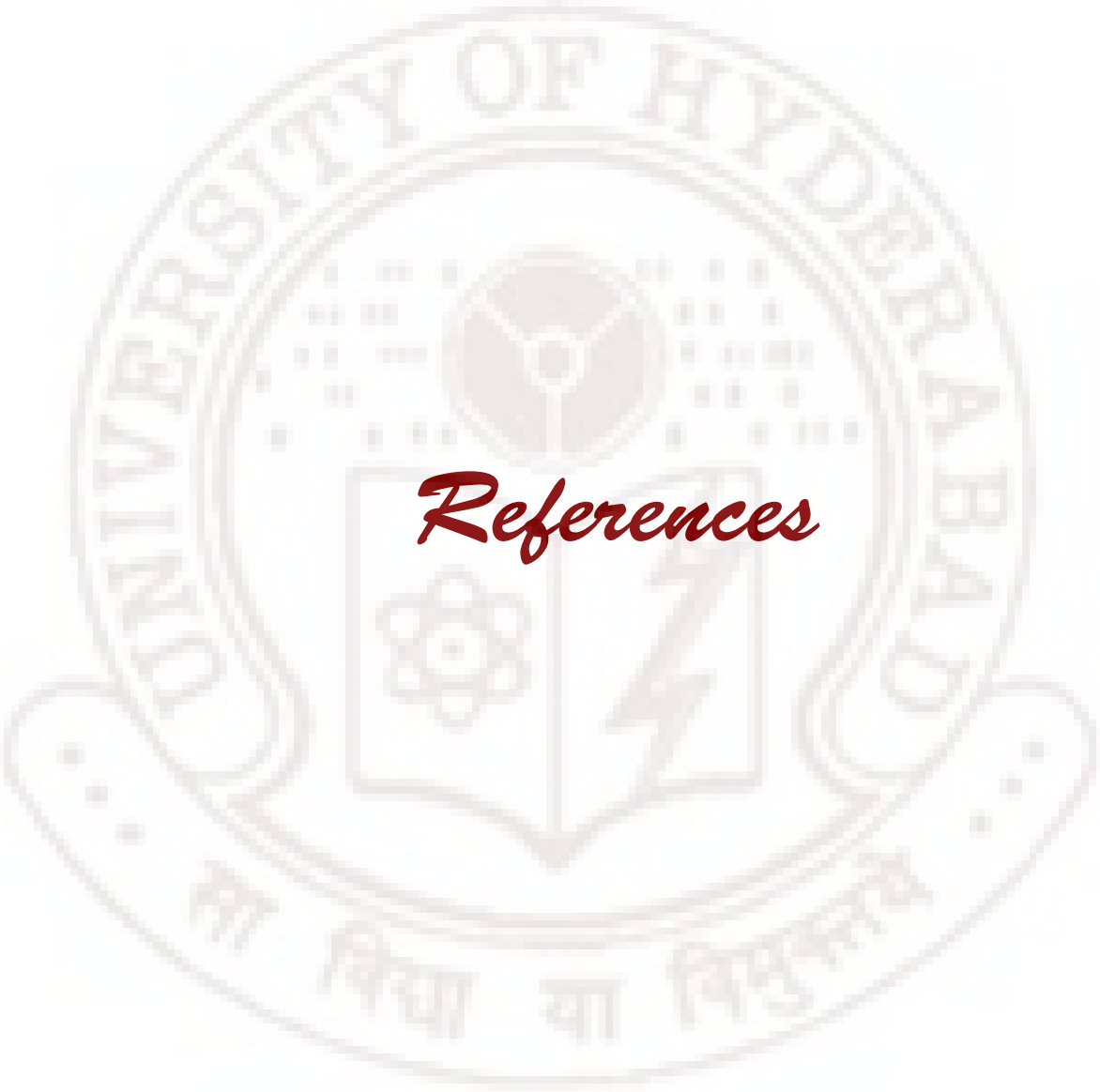
Summary

- The present study showed that the early instar larvae of *S. litura* were more affected by the feeding on leaves of wild groundnut species than the late instar larvae. Further, it also resulted in the decrease in transformation of larvae into pupae
- There was hyper-production of protease activity and synthesis of new proteases in the larvae that fed on leaves of wild groundnut plants when compared with the larvae that fed on JL-24 leaves (control).
- The abaxial surface of both young and old leaves possessed considerable numbers of trichomes except for *A. duranensis*, which showed fewer trichomes. The adaxial surface of young and old leaves showed the presence of few trichomes. The feeding of larvae on these leaves was not related to the number and the arbitrary size of trichome.
- Three protease inhibitors of molecular masses 16, 18 and 20 kDa were purified and characterized from *A. correntina*. They were heat resistant and belonged to the class of serine protease inhibitors.
- The inhibitory activity of *A. correntina* PIs was much higher towards trypsin than chymotrypsin. Further the PIs inhibited around 25% of the 6^{th} instar *S. litura* larval midgut protease activity, while the inhibition was almost 73% with 6^{th} instar larvae of *A. janata.*
- One PI of molecular mass 18 kDa was purified from *A. cardenasii* leaves. This was also heat resistant, belonged to the class of serne protease inhibitors and was immunologically related to soyabean BBI.

- *A. cardenasii* PI inhibited around 29% of the 6th instar *S. litura* larval gut protease activity on the other hand the same PIs inhibited almost 80% of the total gut protease activity of 6th instar larvae of *A. janata*

Conclusion

In short, screening of wild groundnut species was carried out by feeding bioassays for the selection of insect resistant ones. The relation between the leaf morphology and feeding inhibition was studied. Three protease inhibitors from *A. correntina* and one protease inhibitor from *A. cardenasii* was purified and partially characterized. The inhibitory activity of both *A. correntina* PIs and *A. cardenasii* PI was more towards *A. janata* midgut proteases than the *S. litura* midgut proteases.



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