BIOCHEMICAL AND MOLECULAR ANALYSIS OF THE DEFENSE MECHANISM IN CHICKPEA AGAINST BIOTIC STRESS

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DECLARATION

Certified that the work incorporated in the thesis entitled '**Biochemical and molecular analysis of the defense mechanism in chickpea against biotic stress**' submitted by Miss Aparna G. Patankar was carried out under my supervision. The material obtained from other sources has been duly acknowledged in the thesis.

(Vidya S. Gupta)

Research Guide

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List of abbreviations

AI	amylase inhibitor
APMSF	4(amidinophenyl) methane sulfonyl fluoride
BA*p*NA	benzoyl-arginyl *p*-nitroanilide
GLUPHEPA	n-glutaryl 1-phenylalanine p-nitroanilide
DAF	days after flowering
EDTA	ethylenediamine tetraacetic acid
HGP	*Helicoverpa armigera* gut proteinases
HGPI	*Helicoverpa armigera* gut proteinase inhibitor
µl	microlitre
ml	millilitre
mM	milimolar
NaOH	sodium hydroxide
nm	nanometre
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PI	proteinase inhibitor
PMSF	phenyl methyl sulfonyl fluoride
rpm	revolutions per min
SDS	sodium dodecyl sulphate
TI	trypsin inhibitor

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CHAPTER 1

Introduction

Chickpea (*Cicer arietinum* L.) is the second most important pulse crop of the world which provides high quality protein especially in a vegetarian diet (Figure 1.1). Apart from human consumption, chickpea is also used as a feed for livestock and contributes substantially to soil nitrogen and phosphate solubilization. It is grown extensively in the Indian subcontinent, Northern Africa and the Mediterranean region. It covers 10.2 million hectares of land and accounts for 7.9 million tons of world's pulse production (Singh 1997). In India, chickpea is the most important pulse crop from agro-economic and nutritional point of view. India is the largest producer of chickpea accounting for 75% and 73%, respectively, of the world's share in terms of the area under cultivation and production (Saxena and Singh 1987). Productivity of chickpea is, however, restricted due to several abiotic and biotic stresses. The abiotic stresses include drought and cold temperatures while the biotic stresses include the fungal diseases such as wilt and blight caused by *Fusarium oxysporum* and *Ascochyta rabei*, respectively. However, the insect pest *Helicoverpa armigera* (Hübner) (Fig 1.2) represents the most important biotic stress to chickpea.

H. armigera (Lepidoptera: Noctuidae) is a serious pest of many important crops and claims a major share in crop losses every year. It is a polyphagous pest of 181 plant species including pigeonpea (*Cajanus cajan*), tomato (*Lycopersicum esculentum*) and cotton (*Gossypium* species), and is expected to become an important pest for other crops such as pearl millet (*Pennisetum glaucum*), maize (*Zea mays*) and tobacco (*Nicotiana tabacum*) (Manjunath et al 1989). Due to extensive agriculture, the host plants are available in close succession and hence the members

Figure 1.1 Chickpea field

Figure 1.2 (a) *Helicoverpa armigera* in the chickpea field
(b) Damaged chickpea pod

of the genus *Helicoverpa* have become a serious pest problem. *H. armigera* feeds on foliage, flowers and particularly on developing seeds with a single larva damaging several pods of chickpea per day leading to severe losses in crop yield.

Chemical pesticides that are used to control *H. armigera* infestation have become increasingly less feasible mainly because of development of pesticide resistance in the insect population and also due to their serious threat to natural ecosystems (Armes et al 1996). Use of *Bacillus thuringiensis* (Bt) toxin for controlling *H. armigera* infestation is an effective alternative to chemical pesticides. However, the lethal effect of this toxin on insect population has led to a rapid development of resistance against it making it essential to have alternative strategies to combat pest attack in the field. A thorough understanding of plant's inherent strengths and manipulating their use will be a promising way to control pest infestation in a sustainable manner (Boulter 1993; Lewis et al 1997). Among several plant defense proteins, proteinase inhibitors (PIs) have been extensively studied for the development of resistance against insect pests (Jouanin et al 1998; Schuler et al 1998).

Since *H. armigera* is the most severe biotic stress to chickpea and Bt strategy has probably only short term benefits, it was thought necessary to explore additional approaches with a view to increase chickpea's resistance against *H. armigera*. Prior to the beginning of my work, biochemical studies were initiated in my laboratory to understand the basic question why chickpea is susceptible to *H. armigera*. It was shown by Giri et al (1998) that though chickpea produced seven isoforms of trypsin

inhibitors (TIs), *H. armigera* gut proteinases (HGP) degraded these TIs thus rendering chickpea TIs susceptible to HGP. My thesis work represents a part of this continuing effort to throw more light on the PIs in chickpea and its wild relatives, complexity of the *H. armigera* gut proteinases; and to identify powerful PIs which will inhibit the gut proteinases and larval growth of *H. armigera* in a sustained manner.

Organization of thesis:

The thesis is organized into five chapters apart from this introductory chapter and the contents of each are as follows:

Chapter 2. Review of literature. Plant proteinase inhibitors: A promising approach of crop protection against insect attack

This chapter reviews the available literature on the insect pests in agriculture and the available biopesticides with special emphasis on plant PIs, their role in plant defense and effect on insect growth and development.

Chapter 3. Diversity of proteinase inhibitors in *Cicer* species and in moisture stress-induced chickpea at various stages of seed development

In this chapter, the results of study on the diversity of PIs in chickpea and its wild relatives and their potential against *H. armigera* gut proteinases are presented. The effect of seed development and moisture stress on the expression of TIs in chickpea seeds has also been included in this chapter.

Chapter 4. Complex nature of gut proteinases of *Helicoverpa armigera* is responsible for its adaptation to chickpea and other host plants

This chapter describes the results of studies on the complexity of specificities of gut proteinases in *H. armigera* fed on four different host plants viz. chickpea, pigeonpea, cotton and okra and during stages of larval development.

Chapter 5. Successive use of non-host plant proteinase inhibitors offers a potential solution for effective inhibition of *Helicoverpa armigera*

In this chapter, the results of study on the potential of PIs from three non-host plants in inhibition of gut proteinases and larval growth of *H. armigera* have been presented. The optimised combination of winged bean PIs and potato PI-2 and their successive use have been proposed as a novel strategy for effective resistance to *H. armigera*.

Chapter 6. Discussion. Proteinase inhibitors as a component of a holistic approach to tackle the problem of insect pests of crops

The results along with suggestions to optimize the PI-based strategies for insect-resistant transgenic plants are summarized in this last chapter.

This is followed by summary and list of references.

CHAPTER 2

Review of literature

Plant proteinase inhibitors: A promising approach of crop protection against insect attack

2.1 Current scenario of crop damage due to insect attack

Insect attack is a major biotic stress to almost all the crop plants causing serious economic losses especially in cereals, legumes and cotton. The annual global losses caused by pests (including diseases) amount to as much as US $ 300 billion (Thomas 1999). Figure 2.1 gives an estimate of the damage caused due to major pests of some important food and fibre crops.

Despite the persistent use of chemical pesticides over a period of 50 years, insect control has not been achieved to a desirable degree. On the contrary, insect pests continue to cause crop losses to the extent of 30-40% globally of the potential production of food, fibre and feed (Thomas 1999). On the background of continuous increase in human population and a growing concern of food security for the next decade, it becomes increasingly critical to develop strategies to control the losses caused by insect pests.

2.2 Use of chemical pesticides creates threat to environment and gives opportunities for insect pests to develop resistance

The use of synthetic pesticides started in 1946 with the commercial introduction of DDT (dichloro diphenyl trichloroethane) (Elzinga 1978) for pest control. The ensuing decades have seen the increasing use, overuse and misuse of various types of synthetic pesticides like DDT, benzene hexachloride, organophosphates and synthetic pyrethroids. As a result, the first disastrous consequence has been in relation to environmental pollution leading to contamination of soil, water and vegetation. Persistent toxic residues have been found to contaminate soils and aquatic sediments in

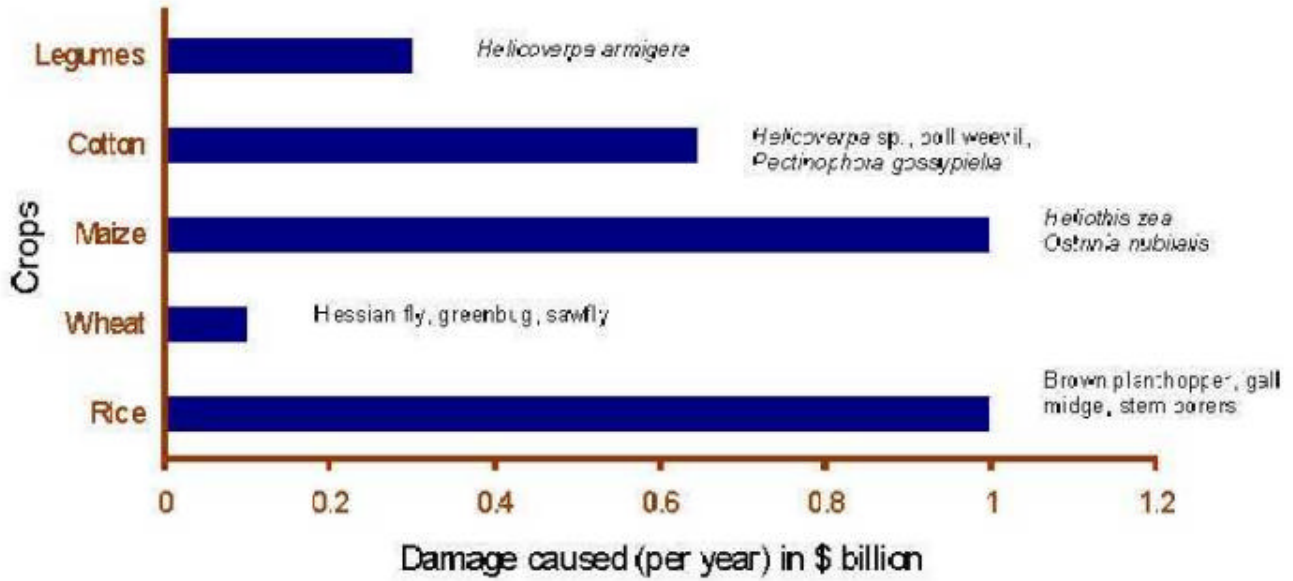

Figure 2.1 Damage caused by insect pests of some important crops. Ref: Romeis and Shanower 1996, Perlak et al 1993, Raina 1997, Hatchett et al 1987 and Duan et al 1996.

water bodies. More importantly, the process has resulted in generation of toxic residues in plants and agricultural produce like foodgrains, fruits and vegetables and even milk. Human consumption of these contaminated foods constitutes an important health hazard. There have been reports of acute and chronic poisoning due to chemical pesticides in many countries (Soon 1997). The health impairments include eye, skin, lung, cardiovascular, immunosuppressive and neurological disorders. In addition, there is a recent evidence about 'gender-warping' chemicals which in the long run may lead to reproductive ailments and declining fertility (Soon 1997).

The second serious problem of unregulated use of chemical pesticides has led to the development of pest resistance and resurgence though chemical pesticides succeeded in controlling pests in the initial phase due to their non-specificity and effectiveness (Thomas 1999). Intensive agriculture of crops coupled with continuous use of chemical pesticides has selected out the resistance alleles in the insect pest population. Even if insecticides kill over 90% of the initial insect population, the rest of the resistant individuals breed among themselves to obtain resistant population which gets selected out with each application. An example of pest resurgence due to use of synthetic pesticide is the emergence of *Helicoverpa armigera* as the dominant pest in the Indian sub-continent though it was not a major pest problem twenty years ago. With the extensive and continuous use of pesticides like organophosphates and synthetic pyrethroids on important crops like cotton and legumes to control *H. armigera* and other pests, *H. armigera* has now emerged as the most

devastating pest relegating some other pests like *Spodoptera litura* to a minor pest status (Jadhav and Armes 1996). Besides, due to their non-specificity, the chemical pesticides also eliminate the other beneficial insects and natural enemies of the pest resulting in a loss of balance in the ecosystem.

The overuse of pesticides has caused the phenomenon of 'pesticide treadmill' which is characterized by increasing use of pesticide, increasing input costs and pesticide-induced outbreaks of insect damage resulting in declining yield and income (Soon 1997). Considering this scenario, there has been an intense requirement for more environment-friendly and sustainable approaches involving use of naturally occurring biological molecules or 'biopesticides'.

2.3 Bt biopesticides: A strong selection pressure on insects due to 'wipeout' effect

Bt biopesticides are the oldest commercially available biopesticides used to control insect attack (Barton et al 1987). Bt biopesticide refers to the insecticidal crystal protein inclusion body present in the spores of the soil bacterium *Bacillus thuringiensis*. On ingestion by the insect, the protoxin solubilizes in the alkaline gut environment and gets activated on proteolysis to yield active toxin. The toxin molecule attaches to receptors on the midgut epithelial cells and leads to disruption of cells' osmotic balance resulting in cessation of feeding and insect death (Frutos et al 1999; Brousseau et al 1999 and the ref. therein).

This biopesticide has a 'wipe out' effect like the chemical pesticides on insects thereby exerting a strong selection pressure on the insect

population for resistance alleles. The unregulated use of Bt has the potential to develop resistance in insects in a short period of time, similar to the chemical pesticides. Reports are available where many insect pests have developed resistance to Bt (reviewed by Frutos et al 1999; Brousseau et al 1999). In general, insects adapt to Bt by many mechanisms which include synthesis of gut proteinase(s) which degrade the toxin (Oppert et al 1996; Forcada et al 1996; Keller et al 1996), absence of an allele of endotoxin-activating protease (Oppert et al 1997), precipitation of toxin (Milne et al 1995) and most commonly due to modification of a receptor site (Frutos et al 1999; Brousseau et al 1999).

Several management strategies including the widely accepted 'refuge' strategy have been proposed so as to delay the onset of resistance to Bt (Alstad and Andow 1995; Gould 1998). However, dominant alleles for resistance to Bt have been reported in *Plutella xylostella* (Liu and Tabashnik 1997), *Heliothis virescens* (Gould et al 1997) and more recently in *Ostrinia nubilalis* (Huang et al 1999) thus questioning the effectiveness of the refuge strategy. More importantly, Liu et al (1999) have shown that Bt-resistant strain of *Plutella gossypiella* on Bt-cotton takes about 6 days longer to develop than the susceptible larvae on non-Bt cotton. This would lead to assortive mating and generate a higher percentage of homozygous resistant insects thus accelerating the evolution of resistant insect genotypes (Liu et al 1999). Besides, Bt has been found to adversely affect non-target insects, for example, Losey et al (1999) have shown that activity of Bt in pollen harmed the monarch larvae. Similarly Saxena et al (1999) have demonstrated that Bt toxin which leached out in the soil from Bt-corn

retained its activity. Thus it is necessary to study the potential adverse effects of Bt strategy on predator insects and soil communities. It also further suggests that over-reliance on Bt may not be advisable and that Bt approach should necessarily be used wisely in conjunction with other pest management approaches.

Some other approaches that have been studied for potential use in pest management include the vegetative insecticidal proteins from bacteria (Estruch et al 1997), genes for enzymes like cholesterol oxidases (Estruch et al 1997), polyphenol oxidase, lipoxygenase (Jouanin et al 1998) and insect chitinase (Kramer and Muthukrishnan 1997); and baculoviruses (Mishra 1998).

2.4 Plant proteinase inhibitors: An opportunity to exploit plants' own defense response

Insect control measures making use of chemical pesticides and Bt are essentially 'therapeutics' with the objective of eliminating the insect pest. However, they do not take into consideration the effect of externally introduced factors on the ecosystem as a whole and hence give only short-term benefits. For long term sustainable solutions to the pest problem, it will be appropriate to study the inherent strengths in the plant system so that they can be used efficiently and holistically (Panda and Khush 1995; Lewis et al 1997).

2.4.1 Plant defense proteins for insect resistance include lectins, amylase inhibitors and proteinase inhibitors

Plants synthesize various proteinaceous and non-proteinaceous compounds against an insect attack. Several plant defense proteins

notably lectins, amylase inhibitors and proteinase inhibitors have been studied for imparting insect resistance (Jouanin et al 1998; Schuler et al 1998). Lectins, which are proteins having affinity for specific carbohydrate moieties, bind to glycoproteins in the peritrophic matrix lining the insect midgut to disrupt digestive processes and nutrient assimilation (Brousseau et al 1999 and ref therein). A lectin from snowdrop (*Galanthus nivalis*) has been found to be toxic to aphids (Hilder et al 1995), the tomato moth *Lacanobia oleracea* (Gatehouse AMR et al 1997) and brown plant hopper (*Nilaparvata lugens*) and green leaf hopper (*Nephotettix virescens*) (Foissac et al 2000). Also wheat germ agglutinin, pea lectin, jacalin and rice lectin have been expressed in plants like tobacco, maize, and potato against mainly aphids (Schuler et al 1998). However, many lectins are toxic/allergenic to mammals (Frutos et al 1999) which might restrict their use.

Amylase inhibitors (AIs) complex with the insect gut amylases and inhibit the growth of insects. Genes for three α-AIs (from common bean, cereals and the bifunctional inhibitor of serine proteinases and α-amylases) (Schuler et al 1998) have been used in transgenic plants. However, the most studied AI is α-AI from common bean (*Phaseolus vulgaris*) which has been used in transgenic pea (Shade et al 1994) and azuki bean (Ishimoto et al 1996) and shown to offer protection against bruchid beetles, *Callosobruchus maculatus* , *C. chinensis* (Shade et al 1994) and also to a field pest pea weevil (*Bruchus pisorum*) (Morton et al 2000). However, Ishimoto and Chrispeels (1996) have demonstrated that Mexican bean weevil overcomes the effect of AI by cleaving it with a serine proteinase.

Another class of plant defense proteins against insect attack is the proteinase inhibitors which has been described in detail in the next section.

2.4.2 Plant proteinase inhibitors in plant defense against insect herbivory

Proteinase inhibitors (PIs) are the most studied class of plant defense proteins. They are abundantly present in seed storage tissues and represent up to 10% of total protein (Casaretto and Corcuera 1995). Proteinases and PIs have been studied in plants for their role in intracellular protein metabolism during seed development, germination, senescence in leaves and root nodules and in conditions of stress such as drought, salinity, wounding and pathogen and insect attack (Brzin and Kidric 1995). In legumes, PIs accumulate in large amounts during seed maturation suggesting their role in deposition and protection of storage protein and in plant defense mechanism (Koiwa et al 1997). Their accumulation in quantities far more than required for inhibiting endogenous proteinases and an absence of inhibitory activity against endogenous plant proteinases in many cases, underline their role as defense proteins against predators. Of all the classes of PIs, the serine PIs are the most abundant and also the most studied proteins and have been categorized into as many as eight families according to their sequence homology (Garcia-Olmedo et al 1987; Ryan 1990) (Figure 2.2).

Green and Ryan (1972) first showed that PIs in potato and tomato were induced upon wounding due to herbivory. Subsequently, Gatehouse and co-workers have demonstrated that the resistance of a cowpea variety to the bruchid beetle was due to the elevated trypsin inhibitor (TI) levels in the seeds (Gatehouse and Boulter 1983). PIs are known to be induced under

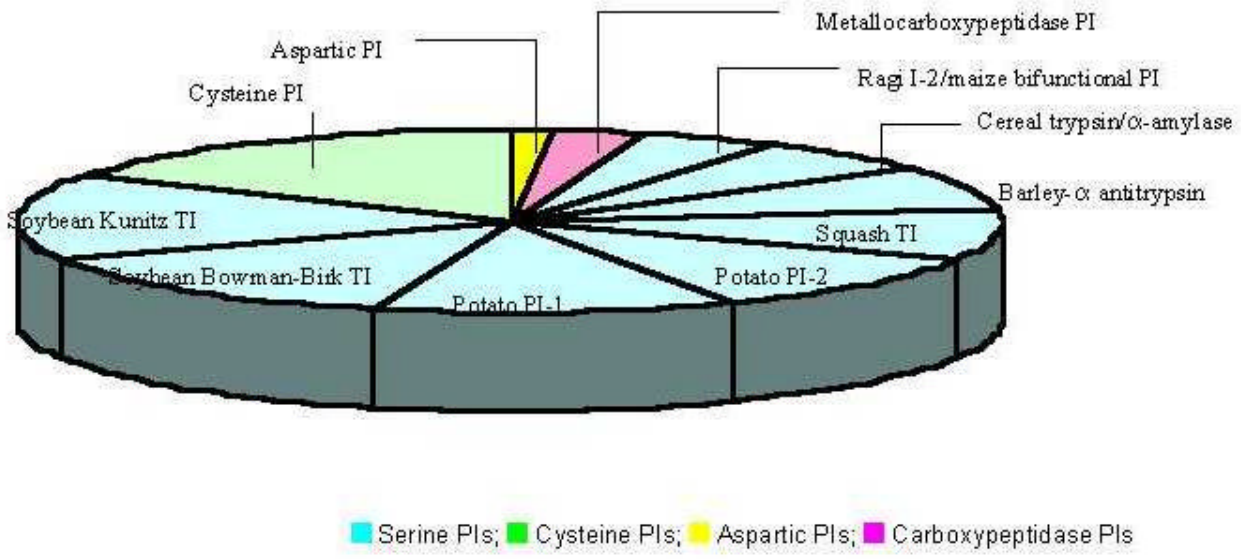

Figure 2.2 Classes of plant PIs

various stress-prone conditions such as insect attack (Jongsma et al 1994; Korth and Dixon 1997; Casaretto and Corcuera 1998; Giri et al 1998; Tamayo et al 2000), mechanical wounding (Pena-Cortes et al 1995; Botella et al 1996; Zhao et al 1996), pathogen attack (Pautot et al 1991; Cordero et al 1994; Jongsma et al 1994) and UV exposure (Conconi et al 1996). PIs are the end products of several defense cascades activated by numerous systemic and non-systemic elicitors such as systemin (Schaller and Ryan 1995), ethylene (Botella et al 1996), methyl jasmonate (Farmer and Ryan 1990; Bolter 1993), abscisic acid (Pena-Cortes et al 1989; Hildmann et al 1992), fungal cell wall oligomers (Doares et al 1995), larval oral secretions (Korth and Dixon 1997), and electrical and hydraulic signals (Wildon et al. 1992; Stankovic and Davies 1997) leading to increased accumulation in local as well as in remote tissues. All these studies have been carried out predominantly for the serine PIs except in cases where cysteine PIs have been studied (Bolter 1993; Botella et al 1996; Zhao et al 1996). Ryan and co-workers have extensively characterized the wound-signalling pathways in tomato and shown that the wound signal is an 18 amino acid polypeptide hormone, systemin (Pearce et al 1991). Systemin travels from wounded parts of the plant to distal organs and induces the synthesis of defense proteins including PIs via the octadecanoid pathway (Schaller and Ryan 1995).

2.4.3 PIs do not kill the insect but exert physiological stress

The mechanism of action of PIs differs from that of Bt. Bt is a toxin whereas PIs carry out physiological change by inhibiting the gut proteinases of the insect pests thus adversely affecting protein digestion.

The insect resorts to overproduction of proteinases to compensate for the inhibited activity leading to deficiency of essential amino acids (Broadway and Duffey 1986a; b). When the induced activity is also inhibited by PIs, the insect is not able to compensate for the inhibited activity. This exerts additional physiological stress resulting in inhibition of growth. PIs also affect a number of vital metabolic processes in the insect including proteolytic activation of enzymes as well as molting and water-balance (Hilder et al 1993; Boulter 1993). This mechanism of action minimizes the possibility of developing resistance in the insects and also ensures less crop damage. Another advantage is that PIs can be used along with many other components of the ecosystem for increased effectiveness. In tritrophic interactions (plants, pests and their predators), the retarded insects become easy targets for greater parasitism by natural enemies (Lewis et al 1997). For example, Heath et al (1997) have reported that *H. armigera* and *Telleogryllus commodus* (black field cricket) which were inhibited by *Nicotiana alata* PI showed increased lethargy and incapability to react to predators, thus increasing their susceptibility to predation. Thus, an outbreak of insect attack can be controlled by retarding the development of the insect pest at the same time sustaining their natural enemies (parasitoids) in the ecosystem. As against this, if 'therapeutic' strategy involving Bt is resorted to, it results in immediate kill (and apparent success) but destroys a reservoir of natural enemies leading to pest resurgence.

Many studies have shown the deleterious effects of ingestion of PIs on insect growth and development (Table 2.1).

Table 2.1. Effects of plant PIs on growth and development of insect pests

Proteinase inhibitor	Insect species	Effect	Reference
Soybean Bowman-Birk inhibitor	*Callosobruchus maculatus*	55-85% M	Gatehouse and Boulter 1983
	Helicoverpa armigera	10-50%WR, 0-70% M	Johnston et al 1993
	Cydia pomonella	No effect	Markwick et al 1995
Soybean Kunitz TI	*Heliothis zea*	30% WR	Broadway and Duffey 1986a
	Helicoverpa armigera	50-99% WR, 20-100% M	Johnston et al 1993
	Spodoptera exigua	15% WR	Broadway and Duffey 1986a
	Ostrinia nubilalis	50% WR	Steffens et al 1978
	Ostrinia nubilalis	10% WR	Larocque and Houseman 1990
	Telleogryllus commodus	50-100% WR	Burgess et al 1994
	Cydia pomonella	0-85% WR	Markwick et al 1995
Potato PI-2	*Heliothis zea*	10% WR	Broadway and Duffey 1986a
	Spodoptera exigua	15% WR	Broadway and Duffey 1986a
	Telleogryllus commodus	50-100% WR	Burgess et al 1994
Potato PI-1	*Telleogryllus commodus*	50-100% WR	Burgess et al 1994
	Cydia pomonella	0-85% WR	Markwick et al 1995
Oryzacystatin I	*Diabrotica undecempunctata*	50% WR, S	Orr et al 1994
	Diabrotica virgifera	50% WR, S	Orr et al 1994
	Leptinotarsa decemlineata	30% WR	Michaud et al 1995b
Carboxypeptidase inhibitors	*Diabrotica undecempunctata*	No effect, S	Orr et al 1994
	Diabrotica virgifera	No effect, S	Orr et al 1994
	Cydia pomonella	25% WR, S	Markwick et al 1995

M: mortality; WR: weight reduction; S: synergism

Adapted from Jongsma and Bolter (1997)

Though PIs inhibit the insect growth and development on feeding, the actual mechanism is not completely clear and has been a focus of number of studies. The effect of PIs as anti-nutritional factors on the regulation of gut proteolytic activity has been earlier studied in animals like rat, chicken and dogs (Ryan 1990). In insects, Broadway and Duffey (1986a; b) first studied the effect of PIs on the gut proteolytic activity and insect growth in *Spodoptera exigua* and *Heliothis zea* and found that there was an increased secretion of trypsin activity on feeding of PIs. They also observed insect growth inhibition at high protein levels in the diet and concluded that there was overproduction of trypsin-like activity to digest the additional protein. This hyperproduction led to the depletion of essential amino acids resulting in inhibited growth. Similar results were observed in *Ostrinia nubilalis* (Laroque and Houseman 1990) and *Spodoptera litura* (McManus and Burgess 1995) on feeding soybean TIs. However, Johnston et al (1993) found a decrease in the trypsin-like activity and retarded growth of *H. armigera* fed on soybean TI. This was attributed to possible overproduction of activity in response to ingested PIs and masking of the increased potential tryptic activity by the excess inhibitor. Similarly Burgess et al (1994) observed 70-90% reduction of gut proteolytic activity accompanying mortality of black field cricket (*Telleogryllus commodus*) fed on potato PI-I and PI-II and concluded *in vivo* inhibition of the gut enzymes by the ingested inhibitors. Broadway (1995) also found retarded growth accompanied by a significant reduction (70%) in the gut proteolytic activity in *Agrotis ipsilon* fed on soybean TI. However, in case of *H. zea* fed on soybean TI, the retarded growth was accompanied by synthesis of some

'inhibitor-resistant' activity (Broadway 1995). Concurrently, Jongsma et al (1995) showed that the insect overcame the effects of PIs by synthesis of inhibitor-insensitive activity. They observed a rise in the inhibitor-insensitive activity in *S. exigua* fed on potato PI-2. Bolter and Latoszek-Green (1997) reported that chronic feeding of the cysteine PI, E-64, by Colorado potato beetle had an adverse effect on insect growth and adult fecundity. They found 2-3-fold rise in the inhibitor-insensitive activity upon ingestion of the PI. This was, however, also accompanied by highly decreased gut proteolytic activity.

The above results suggest that insects resort to increased production of both inhibitor-sensitive and -insensitive proteinases in an attempt to adapt to the ingested PI. Depending upon the efficacy of the ingested PI, the induced proteinase activity may or may not compensate for the inhibited activity. Ultimately the PIs which effect a significant reduction of gut proteolytic activity are able to inhibit insect growth efficiently.

2.5 Insect gut proteinases: Presence of proteinases of different classes and specificity

Since proteinases are responsible for the digestive activity in insect guts, it is necessary to select a candidate PI which has inhibitory activity against the insect gut proteinases. A detailed investigation and characterization of the complement of gut proteinases of the insect pest is, therefore, a pre-requisite in this process.

Proteolytic activity of insect guts comprises many isoforms having diverse properties and specificities (Johnston et al 1991; Terra and Ferreira 1994; Jongsma et al 1996b; Bown et al 1997; Harsulkar et al 1998). The

earlier studies of insect gut proteinases, based on the use of synthetic substrates for bovine and other mammalian trypsins, showed the predominant presence of trypsin-like proteinases (Terra and Ferreira 1994). However, the insect trypsins differ from the mammalian trypsins in (i) their pH optima: they are unstable at acidic pH and have alkaline pH optima (ii) their sensitivity towards inhibitors of plant or chemical origin and (iii) absence of activation or stabilization by Ca^{2+} (Christeller and Shaw 1989; Johnston et al 1991, Purchell et al 1992; Terra and Ferreira 1994). The sequences of many lepidopteran insect trypsins reveal replacement of lysine by arginine which is an adaptation to the highly alkaline environment of the insect gut (Peterson et al 1994; Gatehouse et al 1997). The members of Lepidoptera, one of the most important families of phytophagous insects, have alkaline midguts with pH optima between 8 and 11.5 depending upon the species and they predominantly make use of trypsin-like serine proteinases for digestion (Johnston et al 1991; Christeller et al 1992). For example, *H. armigera*, one of the most important lepidopteran pests, has been shown to possess several families of trypsin-like genes (Bown et al 1997; Gatehouse et al 1997; Mazumdar-Leighton et al 2000). Presence of chymotrypsins and elastase-like proteinases has also been demonstrated in the lepidopteran guts (Christeller et al 1992). The insect chymotrypsins significantly differ in their ability to hydrolyze synthetic substrates and in their interaction with inhibitors (Christeller et al 1992; Peterson et al 1995; Johnston et al 1995; Valaitis et al 1999). In *H. armigera*, presence of at least four multi-member gene families of

chymotrypsin has been demonstrated (Bown et al 1997; Gatehouse et al 1997).

Cysteine proteinases have been demonstrated to predominate in the guts of Hemiptera and Coleoptera (Murdock et al 1987; Terra and Ferreira 1994) having midguts with a pH in the range of 5-7. The Colorado potato beetle (*Leptinotarsa decemlineata*), one of the most important and studied Coleopteran pests, uses cysteine and aspartic proteinases to digest proteins (Purchell et al 1992; Bolter and Jongsma 1995). It has been hypothesized that use of cysteine proteinases is an evolutionary adaptation by insects to feed on plant tissues rich in serine PIs such as legume seeds (Ryan 1990). However, the presence of serine proteinases has recently been shown in Coleoptera (Girard et al 1998 a; b; c; Girard and Jouanin 1999; Zhu and Baker 2000). Along with the above-mentioned endopeptidases, the presence of exopeptidases (aminopeptidases and carboxypeptidases) has also been reported in insects of phytophagous orders Coleoptera, Orthoptera, Hemiptera, Diptera and Lepidoptera (Christeller et al 1989; Lenz et al 1991; Christeller et al 1992; Terra and Ferreira 1994; Lee and Anstee 1995).

Thus there is a large variation in the proteolytic activity found in the insect guts and it is necessary to characterize the specificity of gut proteinase activity in the insect under study before selecting effective PIs.

2.6. Co-evolution of plant PIs and insect gut proteinases: An attempt to counteract each other's defense mechanism

Presence of isoproteinases of different specificities in the midgut has a great significance for survival and adaptation of phytophagous insects on

several host plants. In a co-evolving system of plant-insect interactions (Ehrlich and Raven 1964; Janzen 1980), insects produce gut proteinases to overcome the effect of ingested plant PIs while plants produce different isoforms of PIs. Both pests and plants have been evolving newer forms of enzymes and inhibitors, respectively, to counteract each other's defense mechanism (Ishimoto and Chrispeels 1996; Bown et al 1997). Laskowski et al (1988) have proposed that structural compatibility between the plant PIs and the insect proteinases determines the level of inhibitory activity against specific proteinases. Structural variation occurring in gut proteinases followed by selection against host plant PIs may modify insect proteinases so that they are insensitive to host plant PIs although of the same class. An alteration in an insect proteinase isozyme may result into less inhibitor binding leading to successful herbivory.

In order to survive, plants also must evolve their inhibitor proteins to effectively inhibit insect proteinases. Plants improve the efficacy of their PIs by producing multi-domain inhibitors exhibiting activity against several proteinases (Jongsma and Bolter 1997). Double headed trypsin-trypsin and trypsin-chymotrypsin inhibitors are common in seeds of leguminous plants (Garcia-Olmedo et al 1987). An eight-domain multicystatin has been described in potato (Orr et al 1994). Some other examples are (i) α-amylase inhibitors of sorghum, maize, ragi and bajra seeds with TI activity (ii) the coix α-amylase inhibitor with endo-chitinase activity and (iii) the lectin-like α-amylase inhibitors of *Phaseolus* (Garcia-Olmedo et al 1987; Ryan 1990). More recently, a novel four-domain member of PI-II family has been described from the stigmas of *Nicotiana alata* (Miller et al 2000).

Moreover, it has been postulated that the reactive sites in the PI genes are the 'hotspots' for mutation (Laskowski and Kato 1980). In fact, a few studies have demonstrated that plant inhibitor genes are prone to mutations (Laskowski et al 1988; Ryan 1990; Kothekar et al 1996). These mutations in PI genes tend to generate an array of allelic variability in plant genome and as a result, the plant gene pool is expected to be rich in PI variants showing diverse properties (Ryan 1990).

2.7 Adaptation of gut proteinases to PIs: A boon in disguise for plant protection?

There are two mechanisms of how insects adapt to plant PIs probably due to the selection pressure acting on an entire insect population when they encounter PIs of their host plants. The first mechanism involves synthesis of inhibitor-insensitive or inhibitor-resistant proteinases (Jongsma et al 1995; 1996b; Broadway 1995; 1997). Jongsma et al (1995) first showed that beet army worm overcomes the effect of dietary PIs by 2- to 3-fold increased secretion of inhibitor-insensitive proteases. Evaluation of trypsin activity in the midgut of larval *H. zea*, *Trichoplusia ni* and *Lymentria dispar* following ingestion of cabbage TIs revealed an enhanced secretion of trypsin-like enzymes which were not susceptible to the inhibitors (Broadway 1995; 1997). Ingestion of soybean Kunitz TI by *H. armigera* larvae induced production of inhibitor-insensitive proteinase activity, which was not due to proteolytic enzymes of different mechanistic classes, but rather due to the variants of the existing enzymes (Bown et al 1997). Wu et al (1997) reported that proteinase activities of *H. armigera* larvae fed on tobacco expressing giant taro TI were insensitive to inhibitor and belonged

to chymotrypsin and elastase classes. More recently, Cloutier et al (1999; 2000) found higher rate of growth of Colorado potato beetle on transgenic potato expressing oryzacystatin I accompanied by increase in insensitive activity. These results suggest that insects possess dynamic abilities to produce inhibitor-insensitive proteinases of the same or different classes in response to their dietary encounter with PIs. However, this kind of adaptation shown by the insects may be limited since the number of PI-insensitive proteinases is less and very often the inhibited proteinase activity is not totally compensated (Jongsma et al 1996b).

The second mechanism by which insects adapt to PIs is through production of proteinases which degrade PIs (Michaud 1997; Giri et al 1998; Girard et al 1998a). Michaud et al (1995b) have reported proteolytic degradation of oryzacystatin I by the gut proteinases of black vine weevil. Similarly, Giri et al (1998) have shown that chickpea defensive TIs are degraded by *H. armigera* gut proteinases. Girard et al (1998a; b) showed that a complex proteolytic system consisting of serine, cysteine, aspartyl proteinases and leucine aminopeptidase in the insect gut conferred a high level of resistance to oryzacystatin I and Bowman Birk inhibitor in beetle larvae.

The proteinase-mediated resistance appears to have multiple advantages for the insects. It is the most effective way to reduce PI concentration in the gut, besides solubilizing and availing of the depot of sulfur-containing amino acids from PIs. Adaptation to PIs by producing PI-digesting proteinases is likely to give cross-resistance to other PIs, since the cleavage sites might be conserved across the families of PIs in plants

(Broadway 1997). The cross-resistance provides wider adaptability to PIs even before exposure, thus strongly supporting polyphagous behaviour of insects. Besides PIs, proteinase mediated resistance also operates against other defense proteins. The Bt resistant strain of tobacco budworm (*H. virescens*) possesses ability to degrade the mature and active toxin protein, while complete hydrolysis of Bt toxin has been found to occur in guts of cotton leaf worm (Oppert et al 1996; Michaud 1997). A midgut serine proteinase of Mexican bean weevil can inactivate toxic effects of high levels of α-AI by limited proteolysis (Ishimoto and Chrispeels 1996).

Thus the insects adapt to ingested PIs by synthesis of proteinases which may be insensitive to and/or which may degrade the PIs. This mechanism of adaptation involving overproduction of proteolytic enzymes is a key to toxicity of PIs to insects as it enforces insects to synthesize new proteinase(s) to compensate the effect of PIs. This demands an additional utilization of essential amino acids and energy besides affecting digestion of proteins and eventually insects suffer considerably due to the presence of PIs.

2.8 Transgenic research involving Bt and plant PI genes

Considering the potential of Bt and PIs in crop protection against insect attack, genes encoding them have been expressed in crop plants with some success in conferring resistance against insects. Figure 2.3 lists the milestones in transgenic research involving Bt and plant PIs. Since 1987, Bt genes have been expressed in various important crops viz. cotton, maize, tomato and soybean. Extensive field trials of these transgenic cultivars have been carried out and they have been commercialized by

Figure 2.3 Some milestones in transgenic research involving Bt and plant PIs.

Year	Reference	Milestone
1998	Wadman 1999	20% of US cotton crop was Bt-cotton and 9% of corn was Bt-corn
1996 onwards	Estruch et al 1997	Bt-transgenic cultivars of maize and cotton released for commercial use
1995	Urwin et al	Used rational protein design to engineer PI with greater inhibitory activity
1991	Perlak et al	Modified the GC content of Bt gene for higher expression in plants
1987	Hilder et al	Expressed cowpea TI gene in tobacco and showed the transgenic plants to be insect-resistant
1987	Vaeck et al; Barton et al; Fishchoff et al	Expressed Bt gene in tobacco and tomato and showed the transgenic plants to be insect-resistant
1981	Schnepf and Whitely	Gene for Bt crystal protein cloned in *E. coli*

various private agri-biotech companies. Bt-transgenic crops have been increasingly planted especially in the United States since 1996. In 1998, as much as 20% of US cotton crop was Bt-cotton and about 9% of corn was Bt-corn (Wadman 1999).

In case of PIs, in 1987, Hilder et al first expressed the cowpea TI gene in tobacco and showed the transgenic plants to be resistant to *H. virescens*. This was a direct evidence of the protective role of PIs against insect herbivory. Subsequently the same gene was expressed in some other plant systems like rice (Xu et al 1996), potato (Gatehouse AMR et al 1997) and strawberry (Graham et al 1995) and these plants showed resistance to other lepidopteran pests like *Lacanobia oleracea* and *H. zea*. Some other inhibitor genes which have been commonly used for transformation include: 1) potato PI-2 expressed in tobacco (Johnson et al 1989; McManus et al 1994, Jongsma et al 1995) and in rice (Duan et al 1996) which were investigated for deleterious effects on lepidopterans *Manduca sexta* (Johnson et al 1989), *Spodoptera litura* (McManus et al 1994), *S. exigua* (Jongsma et al 1995) and *Sesamia inferens* (Duan et al 1996); 2) oryzacystatin I from rice expressed in tomato (Urwin et al 1995), poplar (Leple et al 1995) and oilseed rape (Girard et al 1998a; b; Bonade-Bottino et al 1999) for effect against Coleopteran pests. A list of the various plant PIs that have been used for transformation in other plant species is given in Table 2.2.

Table 2.2. Plant PIs used to produce transgenic plants

Proteinase inhibitor	Target insect	Transformed plant
Cowpea TI	Coleoptera, Lepidoptera	Apple, lettuce, oilseed rape, potato, rice, strawberry,

		sunflower, sweet potato, tobacco, tomato
Soybean TI	Coleoptera, Lepidoptera	Oilseed rape, poplar, potato, tobacco
Soybean Kunitz TI	Lepidoptera	Potato, tobacco, rice[1], poplar[2]
Potato PI-1	Lepidoptera, Orthoptera	Petunia, tobacco
Potato PI-2	Lepidoptera, Orthoptera	Birch, lettuce, rice, tobacco
Tomato PI-1	Lepidoptera	Alfalfa, nightshade, tobacco, tomato
Tomato PI-2	Lepidoptera	Tobacco, tomato
Soybean PI-4	Lepidoptera	Potato, tobacco
Mustard TI	Lepidoptera	Arabidopsis, tobacco[3]
Barley TI	Lepidoptera	Tobacco
Giant taro TI	Lepidoptera	Tobacco[4]
N. alata PI	Lepidoptera	Tobacco, peas[5]
Squash TI		Tobacco
14K-CI (Bifunctional cereal inhibitor of serine proteinases and α-amylases)		Tobacco
Oryzacystatin-1	Coleoptera, Homoptera	Oilseed rape, poplar, tobacco, Arabidopsis[6], potato[7]

Adapted from Schuler et al (1998).

1-7 - Reports of transgenics not covered in review by Schuler et al (1998): [1]Lee et al 1999; [2]Confalonieri et al 1998; [3]DeLeo et al 1998; [4]Wu et al 1997; [5]Charity et al 1999; [6]Walker et al 1999; [7]Cloutier et al 1999, 2000

Table 2.3 lists some of the transgenically expressed PIs and their effects on insect growth and development. Contrary to the transgenic Bt results, transgenic expression of some PIs did not give adequate protection against insect attack (McManus et al 1994; Jongsma et al 1995; Xu et al 1996; Nandi et al 1999; Cloutier et al 1999; 2000).

Table 2.3. Effect of transgenically expressed PIs on insect growth and development

Proteinase inhibitor	Transformed plant species	Insect species	Effect	Reference
Cowpea TI	Tobacco	*Heliothis virescens*	75% WR, 60% M	Hilder et al 1987
	Rice	*Chilo suppresalis*	20-80% R	Xu et al 1996
		Sesamia inferens	20-80% R	Xu et al 1996
	Potato	*Lacanobia oleracea*	50% WR	Gatehouse AMR et al 1997
PI-2	Tobacco	*Manduca sexta*	50% WR	Johnson et al 1989
	Tobacco	*Spodoptera litura*	No effect	McManus et al 1994
	Tobacco	*Spodoptera exigua*	No effect	Jongsma et al 1995
	Rice	*Sesamia inferens*	70% WR	Duan et al 1996
Soybean Kunitz TI	Rice	*Nilaparvata lugens*	40-60% M, 8-fold redn in eggs prodn	Lee et al 1999
	Tobacco	*H. armigera*	No effect	Nandi et al 1999
Oryzacyst - atin I	Tomato	*Globodera pallida*	Empty cysts	Urwin et al 1995
	Poplar	*Chrysomela tremulae*	40% M	Leple et al 1995
	Oilseed rape	*Phaedon cochleariae*	No effect	Girard et al 1998a
	Oilseed rape	*Psylliodes chrysocephala*	Growth stimulation	Girard et al 1998b
	Oilseed rape	*Ceutorhynchus assimilis*	No effect; growth stimulation	Girard et al 1998c
	Tobacco	*Meoidogyne incognita*	55% redn in egg prodn	Vain et al 1998
	Arabidopsis	*Deroceras reticulatum*	30% WR	Walker et al 1999
	Oilseed rape	*Baris coerulescens*	No effect	Bonade-Bottino et al 1999
	Potato	*Leptinotarsa decemlineata*	20% WG, hypertrophic behaviour	Cloutier et al 1999; 2000

Mustard TI (high expression)	Tobacco, *Arabidopsis*	*Spodoptera littoralis*	27% M, 37% WR	DeLeo et al 1998
Mustard TI (low expression)	Tobacco, *Arabidopsis*	*Spodoptera littoralis*	30% WG	DeLeo et al 1998
Giant taro TI	Tobacco	*H. armigera*	22-40% WR	Wu et al 1997
N. alata PI	Tobacco, pea	*H. armigera*	48-53% M, 4-7-fold WR	Charity et al 1999

M: mortality; R: resistance; WR: weight reduction; WG: weight gain

In this review, I have made an attempt to give a brief account of insect gut proteinases and role of Bt and plant PIs for insect resistance in crop plants. However, much more work needs to be done to identify and isolate effective PIs and to express them in transgenic plants for resistance against insect pests to realize the potential of this promising strategy.

CHAPTER 3

Diversity of proteinase inhibitors in *Cicer* species and in moisture stress-induced chickpea at various stages of seed development

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3.1. Introduction

Chickpea (*Cicer arietinum*) is the second most important pulse crop of the world and the most important in India. Its productivity is restricted due to heavy infestation of *Helicoverpa armigera* (Hübner) leading to severe losses in yield. Use of plant proteinase inhibitors (PIs) is one of the promising strategies to impart resistance in plants against insect pests (Ryan 1990).

Serine PIs especially trypsin inhibitors (TIs) are known to be present in chickpea seeds at a very high concentration (Borchers and Ackerson 1947; Sohonie and Bhandarkar 1954). Belew et al (1975) identified three iso-inhibitors from the chickpea seed extract which inhibited both trypsin and chymotrypsin. Further studies on protein sequencing and characterization revealed them to be double-headed inhibitors with a high degree of homology with soybean Bowman-Birk inhibitor (Belew and Eaker 1976). Similar observations were also reported by Smirnoff et al (1976; 1979). Sastry and Murray (1987) showed the presence of 8kD double-headed TIs which did not possess chymotrypsin inhibitory activity in albumin fraction of chickpea seeds. Chickpea TIs contain high amounts of essential sulphur-containing amino acids which contribute to their nutritional value (Sastry and Murray 1987). Additionally they have been found to lose their activity on heat treatment and during germination of seeds (Duranti and Gius 1997) making them safe for consumption after processing.

Though chickpea TIs have been extensively isolated and characterized, very little information is available regarding the biochemical interaction of chickpea PIs with gut proteinases from *H. armigera*. Earlier studies from

our laboratory have shown that chickpea produces seven differentially expressed TIs during the course of seed development which are wound-inducible (Harsulkar et al 1997; Giri et al 1998). However, no efforts have been reported to study and exploit the potential of chickpea germplasm including its wild relatives for effective inhibitors of *H. armigera* gut proteinases (HGP). It is well known that wild germplasm has been used in breeding programs for introgression of genes for desirable traits like disease-resistance and insect pest resistance. For example, *Oryza nivara*, *O. longistaminata*, *O. officinalis*, and *O. brachyantha* have been used in introgression of resistance to brown plant hopper, bacterial leaf blight, white-backed brown plant hopper and yellow stem borer respectively into rice (Brar and Khush 1997). Similar efforts have been made in other crops like wheat, maize, barley, cotton and legumes like bean, cowpea and alfalfa for resistance to insect pests (Khush and Brar 1991). In this study an attempt was made to analyse PIs in wild relatives of chickpea as well as in eight cultivars (*desi* and *kabuli*) for their potential to inhibit HGP. Since chickpea is often grown under water-limiting conditions in the Indian sub-continent (Saxena and Singh 1987) and expression of PIs has been shown to alter under drought conditions (Brzin and Kidric 1995), it was also considered important to study PI expression in developing chickpea seeds exposed to moisture stress.

3.2. Materials and Methods

3.2.1 Materials

Eight cultivars of chickpea (Vijay, Vishal, ICCV2, ICCV10 and PG8505-7: less susceptible cultivars and PG91028, Vishwas and PG8404-1: highly

susceptible cultivars; based on pod damage data) (Anon 1995) were grown in a randomised block design in quadruplicates under irrigated conditions at Pulses Research Station, Mahatma Phule Krishi Vidyapeeth (MPKV), Rahuri. Chickpea flowers were tagged on the day they opened and developing pods were harvested 12, 24, 36, 48 and 60 days after flowering (DAF). Two chickpea cultivars, Vijay and Vishal, were grown under rainfed conditions (without irrigation) and developing pods were collected 5, 10, 15, 20, 25, and 35 DAF. Seeds of wild *Cicer* species were obtained from International Crop Research Institute for Semi-Arid Tropics (ICRISAT), Patancheru, India and from Dr. Fred Muehlbauer of Washington State University (WSU), Pullman, USA. Chemicals used were of analytical grade and were purchased from Qualigens, India or SRL Chemicals, India. BA*p*NA was purchased from Sigma Chemicals, USA.

3.2.2 Preparation of PI extracts

The frozen tissue of the cultivars was ground in a pestle-mortar while the dry seeds were powdered in a mixer-blender. The powder was dehydrated, depigmented and defatted by washing at least six times with acetone followed by hexane. The solvents were removed by filtration and tissue powders were air-dried. The powders were mixed with 10 volumes of distilled water and kept overnight at 4°C for extraction with intermittent shaking. The suspension was centrifuged at 10,000 rpm for 20 min at 4°C and the supernatant was stored in aliquots at -20°C. Protein concentration of these PI extracts was quantified as described by Bradford (1976).

3.2.3 Extraction of HGP

Fifth-instar larvae of *H. armigera* were collected from the chickpea fields at MPKV, Rahuri. The larvae were dissected and the isolated mid-guts were stored frozen at -70^{o}C. As and when required the gut tissue was homogenized in 1 volume of 0.2M glycine-NaOH buffer, pH 10.0 and kept for 2 h at 10^{o}C. The suspension was centrifuged at 10,000 rpm for 20 min and the supernatant was used as a source of HGP.

3.2.4 Estimation of PIs

Inhibitory activity against trypsin and HGP present in PI extracts was measured using the assay described in Giri et al (1998). The assay was carried out using the synthetic substrate benzoyl arginine-*p*-nitroanilide (BA*p*NA) (Erlanger et al 1964). Appropriate volumes of the chickpea tissue extract enough to give 40-60% inhibition of trypsin (20-35% inhibition in case of HGP) were mixed with 15 µg of trypsin or equivalent amount of HGP and allowed to stand for 15 min at 27^{o}C. The residual proteinase activity was measured by incubating the seed extract with 1ml of 1mM BA*p*NA (in 0.2M glycine-NaOH, pH 10.0 for HGP and in 0.1M Tris-HCl, pH 7.8 for trypsin) for 10 min at 37^{o}C. The reaction was terminated by the addition of 200µl of 30% acetic acid. After centrifugation at 10,000 rpm for 10 min, absorbance was measured at 410 nm. One unit of proteinase activity was defined as the amount of enzyme that caused an increase of 1 optical density unit at 410 nm due to the release of *p*-nitroaniline. One PI unit was defined as the amount of inhibitor that inhibited 1 unit of proteinase activity.

3.2.5 Electrophoretic visualization of TIs

TI isoforms were detected by using either gel-X-ray film contact print technique (Pichare and Kachole 1994) or by gelatin-polyacrylamide gel (Felicioli et al 1997). PI extracts were electrophoresed on 12% polyacrylamide gel (Davis 1964). For the X-ray-film-contact-print technique, after electrophoresis, the gel was incubated in 0.1 M Tris-HCl buffer (pH 7.8) for 10 min followed by incubation in 0.1% trypsin for 15 min. The gel was washed and placed on a piece of exposed, unprocessed X-ray film. After 2-5 min, the gel was removed and the X-ray film was washed gently to remove hydrolyzed gelatin. TI activity bands were visible as unhydrolyzed gelatin. The film was then developed and contact printed. For the method of Felicioli et al (1997), 10% polyacrylamide gels with 1% gelatin were used. On electrophoresis, the gelatin-polyacrylamide gel was equilibrated in 0.1M Tris-HCl, pH 7.8 and then incubated in 0.1% trypsin solution for 1 h. It was then stained with Coommassie Brilliant Blue R-250. TI bands were revealed as blue bands against white background.

3.3. Results

3.3.1 Accumulation of PI activity during seed development in different chickpea cultivars

Protein content, TI and HGPI activity of eight different cultivars of chickpea were determined at five developing seed stages *viz.* 12, 24, 36, 48 and 60 DAF (Table 3.1). Protein accumulation took place at a rapid pace after the initial lag phase and increased with seed maturity. The period of maximum protein accumulation for all the cultivars was between 24 and 48 DAF. TI activity was not detectable at 12 DAF stage in any of

Table 3.1. Protein and PI content in the developing seeds of eight different chickpea cultivars. The values are average of four replicates ± SD.

Cultivars	12 DAF	24 DAF	36 DAF	48 DAF	60 DAF
Vijay					
Protein	0.9± 0.48	2.3±1.23	14.5±4.9	37.5±10.6	40.0±16.9
TI units/g	ND[A]	5.4± 0.48	38.9± 2.8	76.5± 2.5	136.7± 3.2
HGPI units/g	ND	5.4± 0.43	5.5± 0.72	5.9± 0.1	5.0± 0.36
Vishwas					
Protein	6.3± 0.28	8.2±0.28	16.2± 3.11	19.42±4.8	28. 6±12.0
TI units/g	ND	25.7± 0.11	74.8± 0.0	118.2± 0.8	104.3± 1.31
HGPI units/g	ND	3.9± 0.14	6.4± 0.39	7.4± 0.15	7.6± 0.26
Vishal					
Protein	2.2±0.84	1.7±0.2	6.6±0.42	14.4±0.28	27.1±1.55
TI units/g	ND	17.2±0.8	37.9± 0.19	81.6± 1.62	130.5± 19.4
HGPI units/g	ND	1.3±0.03	4.4± 0.03	5.3± 0.15	8.7± 0.2
PG91028					
Protein	3.3±0.21	3.7±0.14	7.5±2.12	25.9±8.34	33.4±10.46
TI units/g	ND	13.4± 0.1	48.4± 0.0	101.7± 1.6	127.2± 4.5
HGPI units/g	ND	2.9± 0.04	2.8± 0.5	12. 7± 0.06	11.2± 0.32
ICCV2					
Protein	2.5±0.28	3.0±0.28	32.0±5.65	41.2±7.07	39.6±8.48
TI units/g	ND	43.5± 1.17	67.7± 2.4	60.2± 3.36	198.0± 16.12
HGPI units/g	ND	7.2± 0.3	8.5± 0.52	13.7± 1.4	23.1± 0.1
ICCV10					
Protein	5.5±2.82	3.8± 0.42	16.0±2.82	18.6± 2.82	27.6±0
TI units/g	ND	10.4± 0.49	54.9± 0.15	147.7± 1.27	165.1± 6.72
HGPI units/g	ND	3.5± 0.45	5.3± 0.04	5.4± 0.48	6.7± 0.29
PG8505-7					
Protein	0.9±0.56	2.7±0.49	19.8± 0.28	28.2±5.9	26.2±1.6
TI units/g	ND	15.5±0.49	69.2± 11.27	101.8± 6.16	96.1± 12.26
HGPI units/g	ND	2.3± 0.35	12.8± 0.53	12.2± 0.63	11.6± 0.31
PG8404-1					
Protein	1.2±0.14	1.6±0.28	4.2±0.14	17.1±0.84	28.8±5.37
TI units/g	ND	7.9± 0.18	73.4± 2.89	120.2± 1.13	163.4± 4.23
HGPI units/g	ND	0.86± 0.04	2.4± 0.08	6.8± 0.15	8.9± 0.24

[A]ND-Not detected

the cultivars. It attained detectable levels at 24 DAF with progressive increase till seed maturation. Cv. Vijay, Vishwas, PG91028, ICCV10, PG8505-7, and PG8404-1 showed higher rate of TI accumulation from 24 to 36 DAF. Cv. Vishal showed steady rates of TI increase from 24 to 60 DAF. Cv. ICCV2 was unique to exhibit the highest amount of TI and HGPI activity at 24 and 60 DAF stages among all the cultivars in the present study. As compared to TI activity, HGPI activity was very low but exhibited variation in a wider range in different cultivars through various stages of seed development. At 12 DAF, no HGPI activity was detected in any of the cultivars and at 24 DAF, the cultivars showed HGPI activity ranging from 0.86 to 7.2 units/g. Cv. Vijay was found to have higher HGPI activity at 24 DAF (5.4 units/g) that remained constant till 60 DAF (5 units/g) which was the lowest among all the cultivars. Cv. PG 91028, ICCV2 and PG8505-7 showed higher amount of HGPI activity at 60 DAF as compared to other cultivars.

3.3.2 Electrophoretic profiles of TI isoforms in developing chickpea seeds

Figure 3.1 depicts the electrophoretic patterns of TIs in eight chickpea cultivars through five stages of seed development. All chickpea cultivars showed the presence of two fast-moving TI isoforms specific to early stages of seed development (12 to 36 DAF) and two slow-moving isoforms characteristic of the mature seed stages. Between these two stages, there was a transition stage where the fast- and the slow-moving forms co-existed. Cv ICCV10, Vijay, PG8404-1 and ICCV2, revealed maximum number of TI bands at 24 DAF (Fig. 3.1 A, B, C and E). However, the transition stage was either absent or was too transient to detect in Vishwas

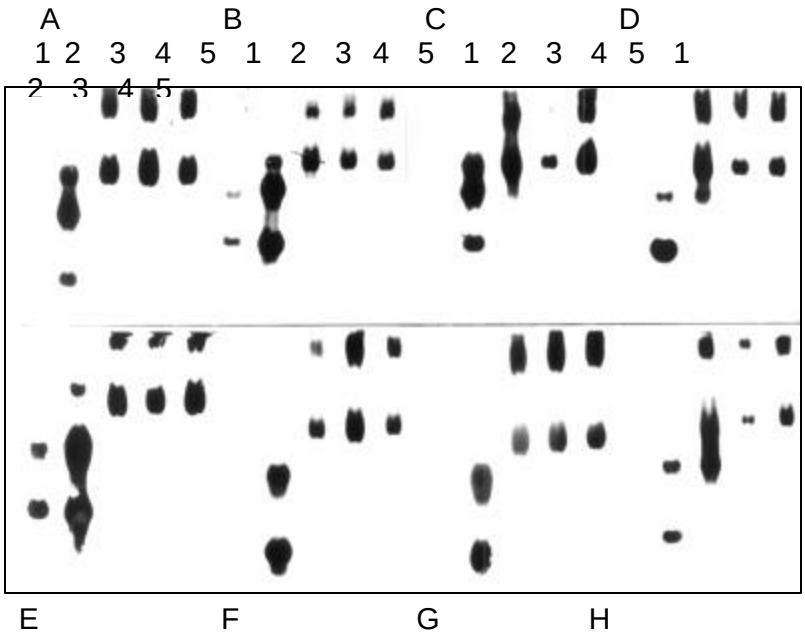

Figure 3.1. TIs in eight chickpea cultivars through five stages of seed development. Electrophoresis was carried out on 12% non-denaturing polyacrylamide gels with discontinuous buffer system. TI activity bands were visualized by gel-X-ray film contact print technique. Equal quantity of protein (30 μg) was loaded in each lane. For 12 DAF extracts, maximum amount of protein (60 μg) was loaded as no activity was detected in spectrophotometric assays. Lanes 1, 2, 3, 4, 5 correspond to 12, 24, 36, 48 and 60 DAF. **A**-ICCV10, **B**-Vijay, **C**-PG8404-1, **D**-Vishal, **E**-ICCV2, **F**-Vishwas, **G**-PG8505-7 and **H**-PG91028.

and PG8505-7 (Fig. 3.1F and G). Cv. Vijay and ICCV2 (Fig. 3.1B and E) could be distinguished from other cultivars as they exhibited TI activity bands at 12 DAF stage, although, no TI activity was detected in solution assays. This can be attributed to the higher sensitivity of the gel-X-ray film contact-print technique over spectrophotometric assay. Ambekar et al (1996) have reported the presence of TI activity bands in pigeon-pea during seed development using the same technique although there was no detectable TI activity in caseinolytic assay. None of the other chickpea cultivars showed any detectable TI activity at 12 DAF by either of the assay methods.

3.3.3 Distribution of TI activity in seed organs

The distribution of TI activity and storage proteins was studied in different seed organs of mid-mature and mature seeds of Cv. Vijay (Table 3.2). The embryo-axis exhibited considerably higher protein than the cotyledon at mid-maturation. More importantly, the embryo showed about nine-fold more TI and HGPI activities than the cotyledon at this stage. However, the rate of increase in PI activity in the cotyledon was remarkably more than that in the embryo-axis as the seed matured (Table 3.2). At mid-maturation, 52% TI activity was localized in the cotyledon and 48% in the embryo-axis (Fig. 3.2). However in mature seed, the cotyledon contributed to 91% of the TI activity as against only 9% by the embryo. When the seed organs were measured for their contribution of fresh weight, at mid-mature stage shares of cotyledon, seed coat and embryo axis were 68%, 31.3% and 0.7%, respectively. In mature seed, however, the cotyledon contributed to 84% of fresh weight with the share of seed coat and embryo

Table 3.2. Distribution of PI activity and storage proteins in different organs of developing and mature seeds of chickpea cv. Vijay. The values are the average of three replicates ± SD

Seed organ	Protein (mg/g)		TI units/g		HGPI units/g	
	Mid-mature	mature	mid-mature	mature	mid-mature	mature
Pod-cover	0.24±0.0	0.187 ± 0.02	ND[A]	ND	ND	ND
Seed-coat	0.21±0.0 4	0.375 ± 0.04	ND	ND	ND	ND
Cotyledon	11.25±3. 0	31.87±3. 8	21.54 ± 0.25	237.5 ± 3.75	10.6 ± 1.4	57.8± 0.3
Embryo-axis	52.15±2. 43	42.85±3. 0	181.62 ±5.8	336.68 ±11.01	87.0 ± 1.4	104.6 ± 2.8

[A]ND Not detectable

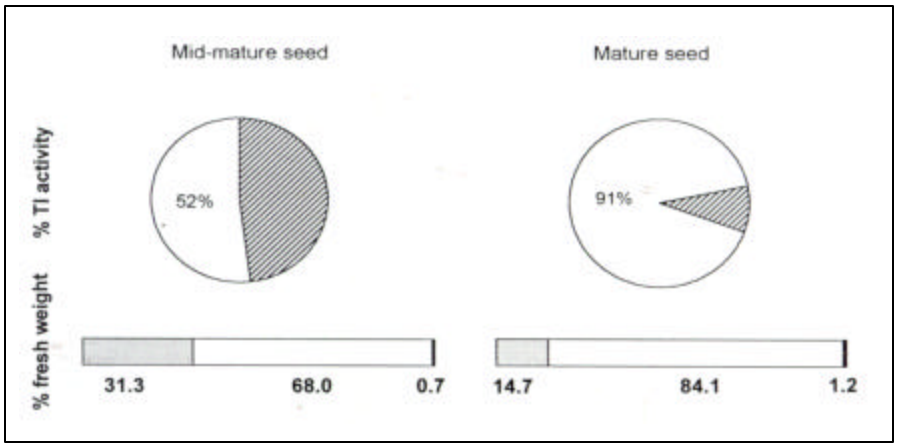

Figure 3.2. Distribution of TI activity in organs of mid-mature and mature seed of chickpea Cv. Vijay. The percent values of TIs were calculated as per the fresh weight contributed by the respective seed organs. TI activity in the cotyledon (□) and embryo axis (□). The bar graphs show the percent distribution of fresh weight of seed organs. Seed coat (□), cotyledon (□) and embryo (□) of mid-mature and mature seeds were separated and the percent fresh weight contributed by each of them was calculated.

axis at 14.7% and 1.2%, respectively (Fig 3.2). Thus the embryo-axis showed higher specific activity for PI than cotyledon indicating higher PI deposition in embryo-axis.

3.3.4 Changes in PI activity under the influence of moisture stress

When the two chickpea cultivars viz. Vijay and Vishal were grown under rain-fed conditions, the following specific changes were observed: (i) shortening of the seed maturation period from 60 DAF to 35 DAF, (ii) greater accumulation of protein between 25 to 35 DAF (iii) higher protein content in mature seeds and (iv) decrease in TI and HGPI activity as compared to irrigated plants (Table 3.3).

3.3.5 TI variants in Cicer species

Figure 3.3 shows the variability in TI isoforms in seeds of wild *Cicer* species. The wild species exhibited diversity in TI isoforms with respect to number and activity as compared to chickpea cultivars. Accessions of the same *Cicer* species obtained from WSU (USA) and ICRISAT (India) exhibited differences in TI profiles. For example, *C. pinnatifidum* (WSU) showed inhibitor activity bands (Fig. 3.3, lane 4) while same species obtained from ICRISAT did not possess any TI band (lane 17). Similarly, *C. echinospermum* obtained from WSU and ICRISAT individually showed variation in TI profiles (lanes 2 and 15). Three accessions of *C. bijugum* obtained from ICRISAT and one accession from WSU also revealed variation in TI patterns. In case of *C. bijugum* (WSU) two major and one minor activity bands were detected (lane 3). Accession ICCW 42# 200 exhibited at least three TI bands of high intensity (lane 10) while accessions ICCW 72 LWC 42-2 and ICCW 42# 201 showed one major and

Table 3.3. Effect of moisture-stress on protein and PI content in developing seeds of chickpea cv. Vijay and Vishal grown under rainfed conditions. The values are average of three replicates ± SD.

Stage (DAF)	Protein (mg/g)		TI units/g		HGPI units/g	
	Vijay	Vishal	Vijay	Vishal	Vijay	Vishal
5	5.7± 0.28	4.62±0.17	ND[A]	ND	ND	ND
10	8.3±0.14	6.25±3.6	ND	ND	ND	ND
15	9.4±0.98	6.37±0	ND	ND	ND	ND
20	15.32±4.6	16.97±0.38	11.05±0.16	17.73±0.35	1.3±0.04	1.58±0.03
25	17.58± 2.68	18.55±0	17.58±0.49	22.86±0.23	2.7±0.5	2.84±0.02
30	37.80±6.7	39.37±9	32.96±0.84	65.37±0.37	3.7±0.14	6.60±0.142
35	58.70±12.3	39.87±2.6	54.10±0.43	77.21±1.33	3.2±0.07	5.11±0.01

[A]ND- Not detectable.

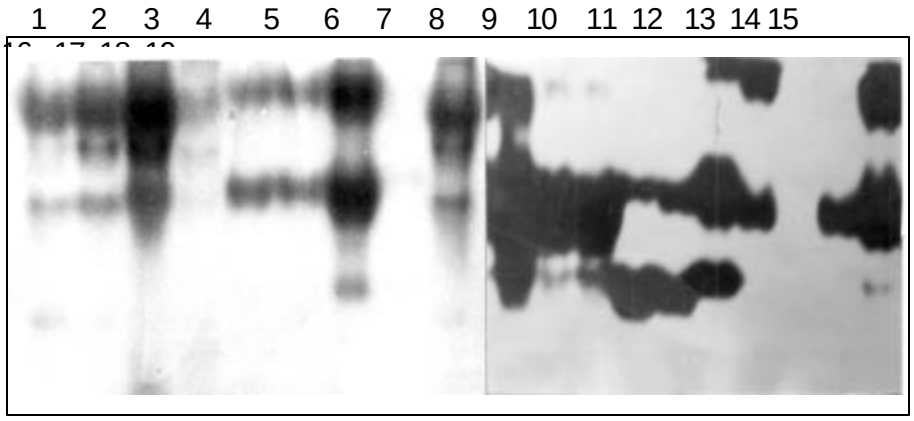

Figure 3.3. TI profiles of wild relatives of chickpea. In each lane 30 µg of seed protein was loaded while in case where less or no TI activity was detected 50 µg of protein was loaded. Lanes 1 to 9 (*Cicer* wilds obtained from WSU) and lanes 10 to 19 (wilds from ICRISAT). Lanes 1 and 16 *C. reticulatum*; lanes 2 and 15 *C. echinospermum;* lane 3 *C. bijugum*; lane 10 *C. bijugum* (ICCW 42# 200), lane 11 *C. bijugum* (ICCW 72 LWC 42-2); lane 12 *C. bijugum* (ICCW 42#201)*;* lanes 13 and 14 *C. judaicum*; lanes 4 and 17 *C. pinnatifidum;* lane 5 *C. oxydon;* lane 6 *C. microphyllum;* lane 7 *C. anatolicum;* lane 8 *C. songaricum;* lanes 9 and 19 *C. arietinum;* lane 18 *C. cuneatum.*

one minor TI band (lanes 11 and 12). *C. oxyodon* (lane 5) and *C. microphyllum* (lane 6) showed very similar TI band patterns. *C. reticulatum* (lanes 1 and 16) resolved into two bands similar to that in chickpea (lane 9) with the addition of a minor fast-migrating band. *C. anatolicum* (lane 7) and *C. echinospermum* (lane 15) showed three TI bands of the same mobility whereas TI band was not detected in *C. songaricum* (lane 8). Both the accessions of *C. judaicum* revealed two TI activity bands differing in their mobility (lanes 13 and 14) while *C. cuneatum* (lane 18) showed a single TI activity band.

3.3.6 Cicer PIs are ineffective inhibitors of HGP

To check the potential of *Cicer* PIs in inhibiting HGP, the amount of seed extract required to inhibit 100% trypsin activity was titrated against HGP. The results are summarized in Table 3.4. The highest inhibition of HGP (36%) was effected by *C. bijugum* PIs (Accession ICCW-72 LWC-42-2) followed by 33% in *C. echinospermum* and *C. arietinum* (Cv. Vijay). The HGP inhibition by other *Cicer* species ranged between 2 to 30%. The lowest HGP inhibition was detected in seed extract of *C. cuneatum* (2%). *C. pinnatifidum* obtained from WSU showed 23% inhibition of HGP while the accession from ICRISAT showed only 7% inhibition. Increasing the net amounts of PI extracts did not change these percentage activities.

3.4. Discussion

3.4.1 Variation in PIs of chickpea cultivars during seed development and in Cicer species

In this study, the variation observed at the transitory stage during seed development among the chickpea cultivars (Fig. 3.1) points to considerable

Table 3.4. Inhibition of *H. armigera* gut proteinase activity by PIs from mature seeds of chickpea (*C. arietinum*) and its wild relatives. The amount of inhibitor, which showed 100% inhibition of trypsin, was chosen for assessing the inhibition of HGP. The values are average of four replicates ± SE.

Genotype	Source	Inhibition of HGP (%)
C. arietinum (cv Vijay)	ICRISAT	33±2
C. bijugum (ICCW41 #200)	ICRISAT	29±0.3
C. bijugum(ICCW72 LWC42-2)	ICRISAT	36±1
C. bijugum (ICCW42 #201)	ICRISAT	28±0.6
C. judaicum (ICCW 33)	ICRISAT	23±0
C. judaicum (ICCW92 LR126)	ICRISAT	24±1.5
C. echinospermum (ICCW44 #204)	ICRISAT	33±0.5
C. reticulatum	ICRISAT	28±0.3
C. pinnatifidum	ICRISAT	7±0.8
C. cuneatum	ICRISAT	2±0.1
C. pinnatifidum	WSU	23±1
C. oxyodon	WSU	21±2
C. anatolicum	WSU	25±1
C. songaricum	WSU	12±1
C. microphyllum	WSU	18±0.5
C. echinospermum	WSU	5±1
C. reticulatum	WSU	21±1.2
C. bijugum	WSU	30±2

variability in the process of expression and/or modification of TI proteins. The differential expression of TI forms can be attributed to temporal expression of gene groups or to post-translational modifications of PIs (Domoney et al 1995; Giri et al 1998). Domoney et al (1995) have reported generation of multiple TI forms in pea from two primary gene products. Giri et al (1998) have shown that the chickpea TI-5 is the proteolysis product of chickpea TI-1. Further in our studies, the PI content of chickpea cultivars at different stages of seed development did not show strong correlation with the pod-damage data of the same cultivars reported by Anon (1995). This could be attributed largely to the insensitivity of HGP towards chickpea PIs and strong feeding preference of *H. armigera* in the field. The latter is evident when *H. armigera* has a choice of host, and this might explain the inconsistency revealed in the pod damage data of the same cultivars in three consecutive years (Anon 1993, 1994, 1995). The HGPI activity of less susceptible and highly susceptible cultivars towards *H. armigera* also did not show any strong correlation. Interestingly, HGPI activities varied widely among the cultivars with ICCV2 showing the highest HGPI activity in mature seeds (Table 3.1). Obviously, however, this level of HGPI activity is not sufficient to accord protection to chickpea against *H. armigera* in the field.

The TI isoforms of wild *Cicer* species revealed significant variation as against a greater conservation of TI isoforms in the mature seeds of the chickpea cultivars (Fig. 3.3). A similar observation existed in pigeonpea where TIs and chymotrypsin inhibitors were found to be conserved in mature seeds of the cultivated pigeonpea while a high level of diversity

existed in the uncultivated species of *Cajanus* (Kollipara et al 1994; Pichare and Kachole 1996). *C. reticulatum* and *C. arietinum* showed similar TI band patterns which suggested that *C. reticulatum* was genetically closer to *C. arietinum*. This agreed well with the karyotype and crossability data, seed storage protein-analysis and isozyme variation reported earlier which showed that *C. reticulatum* is the presumed progenitor of *C. arietinum* (Ladizinsky and Adler 1976; Kazan and Muehlbauer 1991; Tayyar and Waines 1995). The variation observed in the *Cicer* wilds can be considered significant, as the TIs are known to be defense proteins against herbivores (Ryan 1990).

When effectiveness of the wild *Cicer* PIs was assessed against HGP by *in vitro* assays, none of them showed more than 36% inhibition (Table 3.4). *H. armigera* is a polyphagous pest and possesses a population of proteinases in its gut (Bown et al 1997; Harsulkar et al 1998). HGP activity is not only insensitive but also possesses the ability to digest chickpea TIs (Giri et al 1998). The ineffectiveness of *Cicer* wild PIs indicated that *H. armigera* is adapted to a wide range of PIs. Field screening for resistance to *H. armigera* in chickpea and its wild relatives did not yield any good candidates offering substantial resistance against the insect pest.

3.4.2 Tissue specificity of PIs

Various reports have shown that the seed expresses an unique set of seed-specific proteins (Gatehouse et al 1986; Goldberg et al 1989), which are expressed almost exclusively during embryogenesis and are temporally and spatially regulated in the seed organs. The seed-specific proteins include embryo-axis-specific proteins (expressed throughout

embryogenesis) and seed-storage proteins, lectins and PIs that are expressed from mid-maturation to late-maturation stages of embryogenesis (Goldberg et al 1989). PIs accumulate during seed/tuber maturation suggesting that they facilitate accumulation of seed-storage proteins (Koiwa et al 1997). The present study showed that the embryo-axis possessed a very high level of PI activity even in immature seeds as compared to the cotyledons. The PI activity of seed increased with maturity, however, the increase was more in cotyledons than in other tissues. Recently, Welham et al (1998) showed the immunolocalization of TI activity in embryo-axis in developing as well as germinating seeds in *Pisum.* The high localization of PI activity in the embryo-axis might be attributed to their defensive properties, which are utilized for protection of embryo from insect pests.

3.4.3 Moisture-stress vis-a-vis synthesis of PIs

Chickpea is grown in dry areas on conserved soil moisture and in the areas where extremes of temperature persist during the lifecycle of the plant (Singh 1997). When chickpea cultivars were grown under rainfed conditions (as against irrigated), seed maturation period decreased from 60 to 35 DAF (our field observation). Kollipara et al (1994) observed that the seed-maturation period decreased by growing long-duration pigeonpea cultivars at locations different from their area of adaptation. Early maturity allows the plant to escape from drought and is found to be beneficial for chickpea cultivation in peninsular India. Response to supplemental irrigation by chickpea is substantial and often doubles the yield under optimum soil conditions (Saxena and Singh 1987). The present work

revealed increase in seed protein content and lowered PI activity in both the cultivars, which were subjected to moisture-stress. This was, however, accompanied by a lowered yield of the crop (data not shown). The increase in protein content in both the cultivars of chickpea under rain-fed conditions may be a response to the stress stimulus. In legume plants exposed to terminal drought conditions, there is a rapid mobilization of carbohydrates from the leaves and stem towards seed (Subbarao et al 1995) which is possibly diverted to a greater protein synthesis. However, further investigations are needed to arrive at any definite conclusion. Moreover, it has been demonstrated that under conditions of stress, drought-sensitive cultivars of *Phaseolus* and *Vigna* showed increase in proteolytic activity possibly depleting protein deposits whereas the drought-resistant cultivars, on the other hand, showed decrease in proteolytic activity and increase in PI contents (Brzin and Kidric 1995). Increase in PI activity in the seeds of the pigeonpea cultivars grown in different locations from their area of adaptation was also reported by Kollipara et al (1994).

In summary, this work indicates that considerable variability exists in the chickpea PIs (i) during seed maturation in different cultivars (ii) in response to moisture stress, (iii) with respect to localization in different seed organs and (iv) in wild *Cicer* species. It is also evident that although there is a significant variability in the inhibitory activity against HGP in chickpea and in its wild relatives, none of them can inhibit HGP activity totally to offer

protection against *H. armigera*. The potential of PIs of chickpea and its primary gene pool to develop resistance against *H. armigera* is limited thus emphasizing the need for genetic transformation of chickpea with suitable heterologous PI(s) to counteract the mid-gut proteinases of *H. armigera*.

CHAPTER 4

Complex nature of gut proteinases of *Helicoverpa armigera* is responsible for its adaptation to chickpea and other host plants

4.1. Introduction

Helicoverpa armigera (Hübner) (Family: Lepidoptera) is a serious pest of many important crops and claims a major share in crop losses every year (Manjunath et al 1989). Studies on *H. armigera* have focussed on the digestive proteinases secreted in its gut. Earlier studies on the *H. armigera* gut proteinase (HGP) activity have shown that it is predominantly trypsin-like with high pH optima (Johnston et al 1991) although presence of chymotrypsin, carboxypeptidase and elastase-like activities has also been reported recently (Bown et al 1997; 1998; Wu et al 1997). Screening of cDNA library prepared from mid-guts of *H. armigera* reared on high protein and inhibitor-free diet has revealed 18 genes encoding trypsin-like proteinases, 14 genes of chymotrypsin-like proteinases and 2 genes of elastase-like proteinases (Gatehouse et al 1997). More recently, Mazumdar-Leighton et al (2000) have shown the presence of two more transcripts encoding distinct trypsin-like proteinases in *H. armigera*. Additionally, the specificity of trypsin- and chymotrypsin-like proteinases towards proteinase inhibitors (PIs) has been found to vary with the iso-inhibitors (Bown et al 1997; Harsulkar et al 1998). Furthermore, Wu et al (1997) showed that when PIs inhibiting total proteinase activity *in vitro* were fed to larvae of *H. armigera*, the insect altered its midgut proteinase composition and the newly synthesized proteinases were insensitive to the PIs fed. Considering these results, it is evident that insects have complex mechanism(s) of regulating gut proteinases and therefore, a detailed investigation of *H. armigera* gut proteinases may prove to be very fruitful.

To date there is little information available regarding the sensitivity of gut proteinase activity of *H. armigera* feeding on various host plants and during the larval development. This information will be important for identifying effective PIs from different plant sources for their subsequent transgenic expression in host plants.

In the present study the diversity and the specificity of *H. armigera* gut proteinases of larvae growing on chickpea was assessed and compared with that of larvae fed on three other host plants viz. pigeonpea, cotton and okra using specific substrates and inhibitors and visualizing the isozymes on electrophoretic gels. Further, changes in the proteinase activities during the different stages of larval development and proteinase activity in the fecal matter of larvae growing on natural or artificial diet were analysed.

4.2. Materials and Methods

4.2.1 Materials

Seeds of chickpea (*Cicer arietinum* cv. Vijay), pigeonpea (*Cajanus cajan* cv. BDN-2) and cotton (*Gossypium arboreum* cv. K-32) were obtained from the Pulse Research Station and Oilseed Research Station, Mahatma Phule Krishi Vidyapeeth (MPKV), Rahuri. Okra seeds (*Abelmoschus esculentum* cv. Parbhani Kranti) were commercially obtained from Damani Seeds, India. All chemicals were of analytical grade and purchased from Qualigens, India or SRL Chemicals, India. Azocasein, BA*p*NA and GLUPHEPA were from Sigma Chemicals, USA. Chemical inhibitors were purchased from Boehringer Mannheim, Germany.

4.2.2 Preparation of plant PI extracts

Dry seeds were processed for preparation of PI extracts as detailed in Section 3.2.2. These were assayed for trypsin inhibitor (TI) activity (as described in section 3.2.4) and used as source of plant PIs.

4.2.3 Rearing H. armigera larvae

H. armigera larvae were reared on an artificial diet under controlled conditions to collect the larvae of various developing stages. Composition for one litre of the artificial diet was as follows: chickpea seed meal 140 g, yeast extract 14 g, Bavistin™ 0.4 g (BASF, Mumbai, India), formalin 0.2 ml, ascorbic acid 4.3 g, sorbic acid 1.3 g, methyl benzoate 2.6 g, tetracycline 0.5 g, one tablet of vitamin B-complex, two drops of vitamin E and agar 17 g (Giri and Kachole 1998). Newly hatched larvae were reared on cubes of artificial diet for all instars. Mid-guts of larvae of each instar were harvested and frozen till used for proteinase analysis.

Fifth instar larvae were separately collected from fields of chickpea, pigeonpea (Pulse Research Station) and cotton (Oilseed Research Station) at MPKV, Rahuri and their mid-guts were isolated by dissection. In case of okra, newly hatched larvae were reared separately on developing seeds of okra till fifth instar and then dissected for midgut isolation.

4.2.4 Preparation of HGP

20 midguts of the fifth instar larvae were used per experiment for extraction of HGP. For the lower instars, upto 50 larvae were used for extraction. The midgut contents were extracted as detailed in Section 3.2.3.

4.2.5 Proteinase assays

Total gut proteinase activity was measured by azocaseinolytic assays (Brock et al 1982). For azocaseinolytic assay, 60µl of diluted enzyme was added to 200µl of 1% azocasein (in 0.2M glycine-NaOH, pH 10.0) and incubated at 37^{o}C for 30 min. The reaction was terminated by addition of 300µl of 5% trichloroacetic acid. After centrifuging at 10,000 rpm for 10 min, an equal volume of 1N NaOH was added to the supernatant and absorbance was measured at 450nm. Trypsin and chymotrypsin-like activities were estimated using chromogenic substrates benzoyl-arginyl *p*-nitroanilide (BA*p*NA) (Erlanger et al 1964) and n-glutaryl 1-phenylalanine p-nitroanilide (GLUPHEPA) (Mueller and Weder 1989). For trypsin assay, diluted enzyme (150µl) was added to 1ml of 1mM BA*p*NA (in 0.2M glycine-NaOH pH 10.0) and incubated at 37^{o}C for 10 min. The reaction was terminated by addition of 200µl of 30% acetic acid and absorbance was measured at 410nm. For chymotrypsin assay, gut extract was added to different tubes and volume made up to 700µl with 0.2M glycine-NaOH buffer, pH 10.0. 25µl of GLUPHEPA (10 mg/ml in dimethyl formamide) was added to each tube and reaction mixture was incubated at 37^{o}C for 1 hour. The reaction was terminated by adding 200µl of 30% acetic acid and absorbance was checked at 405nm. One proteinase unit was defined as the amount of enzyme that increases absorbance by 1 OD under the given assay conditions.

Eight chemical inhibitors viz. antipain, leupeptin, pefabloc, aprotinin, chymostatin, E-64, pepstatin and EDTA were used in the range of 1.8 µM to 10 mM concentration for maximum inhibition of the enzyme in assays.

Inhibitors were dissolved in water (antipain, leupeptin, pefabloc, EDTA and aprotinin) or DMSO (chymostatin) or methanol (pepstatin) or water/ethanol (1:1) (E-64) as per the manufacturer's instructions. For the inhibitor assays, suitable volume of seed extract or chemical inhibitor required for maximum inhibition of the enzyme was added to the gut proteinase extract and incubated at room temperature (27^{o}C) for 15 min. The residual proteinase activity was then estimated using casein as substrate (Belew and Porath 1970). The proteinase-inhibitor mixture was added to 0.5ml of 0.5% casein (in 0.2M glycine-NaOH, pH 10.0) and kept at 37^{0}C for 20 min. The reaction was terminated by addition of 750 µl of 5% trichloroacetic acid. After centrifugation at 10,000 rpm for 10 min, absorbance of the supernatant was checked at 280nm. For every assay, suitable controls were co-incubated with the test samples.

4.2.6 Visualization of HGP isoforms

Visualization of HGP isoforms after native polyacrylamide gel electrophoresis was carried out using the gel-X-ray film contact print technique (Harsulkar et al 1998). After electrophoresis, the gel was equilibrated in 0.2 M glycine-NaOH buffer, pH 10.0 and then overlaid on unprocessed X-ray film. After 45 min to 1 h the gel was removed and the X-ray film was washed with hot water (45-50^{o}C) to reveal the proteinase activity bands as hydrolyzed gelatin. The X-ray film was developed and then contact-printed.

4.2.7 Fecal matter analysis

Ten fifth instar larvae collected from the fields of the host plants were fed upon the respective developing seeds in vials for 2 overnights and the

resulting fecal matter was collected everyday for 2 days. Similarly fecal matter was collected from the fifth instar larvae fed on artificial diet. The fecal matter samples were extracted similarly as gut contents in 0.2M glycine-NaOH buffer, pH10.0. These extracts were used for proteinase analysis either on gel or in solution assays.

4.3. Results

4.3.1 Activity and visualization of gut proteinases of H. armigera fed on various host plants

To assess the gut proteinase composition of the larvae fed on various host plants, total proteinase (azocaseinase), trypsin-like (BA*p*NAase) and chymotrypsin-like (GLUPHEPAase) activities of *H. armigera* growing on four host plants viz. chickpea, pigeonpea, cotton and okra were assayed (Table 4.1). Azocaseinase (25.17 units per 10 guts) and BA*p*NAase (29.33 units) activities were the highest in the larvae fed on chickpea while GLUPHEPAase activity was the highest in those fed on cotton (0.63 units). *H. armigera* fed on chickpea showed more than 2.5 to 3-fold overall gut proteinase activity as against the activities shown by *H. armigera* feeding on pigeonpea, cotton and okra.

When the gut extracts of *H. armigera* larvae were resolved on 12% native polyacrylamide gel for visualization of proteinase activity bands, several major and minor bands were detected (Figure 4.1). To visualize minor activity bands, more activity (0.5 BA*p*NAase units) was loaded on gel. Because of high loading concentration, the major proteinase bands merged in case of isoforms having closer mobilities. Inspite of this, some of the minor activity bands detected on the X-ray film could not be

Table 4.1 Gut proteinase activity of *H. armigera* larvae fed on various host plants. The values are average of three replicates ± SD.

Host plant	Target tissue	Proteinase activity (U/10 guts)		
		Azocaseinase	BApNAase	GLUPHEPAase
Chickpea	Seed	25.17± 1.48	29.33± 1.27	0.04±0
Pigeonpea	Seed	7.62± 1.23	7.95± 0.67	0.39±0.03
Cotton	Boll	6.66± 0.18	5.88± 0.06	0.63±0.08
Okra	Capsule	9.58± 0.31	11.68±0	0.16±0.01
Artificial diet	--	22.45± 0.84	19.29±1.35	0.05±0

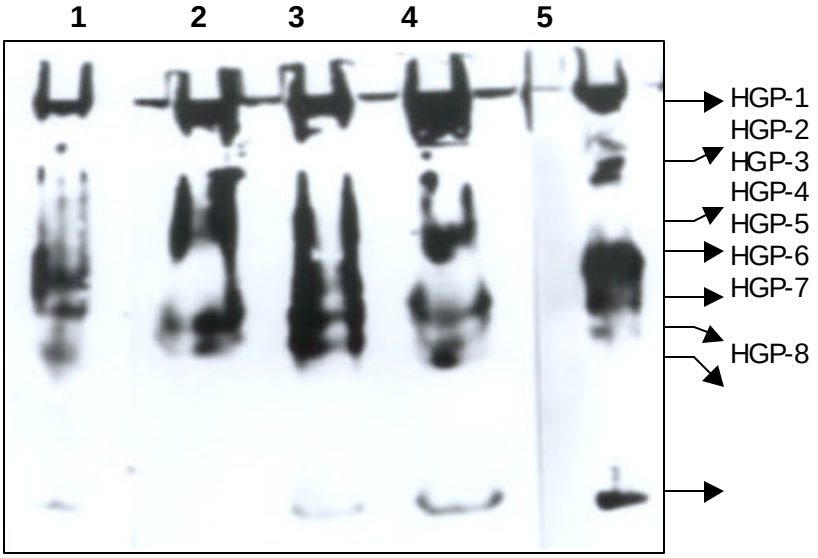

Figure 4.1. Gut proteinase profiles of *H. armigera* fed on different hosts and diet. Gut proteinase activity of *H. armigera* fed on chickpea (lane 1), pigeonpea (lane 2), cotton (lane 3), okra (lane 4) and artificial diet (lane 5) was loaded on 12% polyacryamide gel and visualized by the gel-X-ray film contact print technique.

reproduced in photographic contact print because of their low activity. Gut proteinases from larvae fed on chickpea and artificial diet showed several bands with close mobilities leading to smear. Hence for these samples, 0.25 units of activity was loaded. Gut proteinases from larvae feeding on chickpea showed the presence of bands 1, 5, 6 and 8 (Figure 4.1, lane 1) with some slow migrating proteolytic activity observed which could not be contact-printed. Gut proteinase activity of larvae fed on artificial diet exhibited expression of more bands of isoproteinases than that observed in case of larvae fed on chickpea (Figure 4.1, lane 5). A few diet-dependent proteinase bands were evident in the HGP of larvae fed on pigeonpea, cotton and okra (Figure 4.1, lanes 2, 3 and 4).

4.3.2 Inhibition by chemical inhibitors and plant PIs of gut proteinase activity of H. armigera feeding on different hosts

In order to examine specificity of HGP of larvae feeding on chickpea as well as on the other hosts, the gut extracts were assayed for inhibition by chemical inhibitors of different specificity (Table 4.2). The studies revealed very interesting results in the specificity of HGP to these inhibitors. HGP of larvae fed on chickpea were inhibited strongly by serine PIs antipain (80%), leupeptin (80%), pefabloc (85%) and aprotinin (70%) but not by E-64, pepstatin or EDTA pointing to the absence of cysteine proteinases, aspartic proteinases and metalloproteinases, respectively, in the gut. Similarly, HGP of larvae fed on cotton and okra showed 60-100% inhibition by serine PIs. However, partial inhibition by chymostatin (39% in cotton and 45% in okra) suggested the presence of chymotrypsin-like activity in the gut which was also observed as GLUPHEPAase activity (Table 4.1). HGP of larvae

Table 4.2 Effect of chemical inhibitors on gut proteinase activity of *H. armigera* fed on various host plants. Inhibition of gut proteinase activity was measured using casein as a substrate. The values are average of three replicates ± SE.

Chemical inhibitor	Specificity of the inhibitor	Effective concn.	% inhibition of gut proteinase activity of larvae fed on:			
			Chickpea	Pigeonpea	Cotton	Okra
Antipain	Serine proteinases	265µM	80±4	55±2	63±10	60±3
Leupeptin	Serine proteinases	21µM	80±10	70±4	100±0	63±5
Pefabloc	Serine proteinases	10mM	85±3	93±7	100±0	95±1
Aprotinin	Serine proteinases	1.8µM	70±8	86±10	86±14	56±1
Chymo-statin	Chymotrypsin	264µM	30±10	78±6	39±9	45±8
E-64	Cysteine proteinases	111µM	00	23±7	12±2	00
Pepstatin	Aspartic proteinases	5.8µM	00	22±5	00	00
EDTA-Na_2	Metallo-proteinases	1mM	00	59±7	18±4	00

fed on pigeonpea showed inhibition of activity by the serine PIs antipain (55%), leupeptin (70%), aprotinin (86%) and pefabloc (93%). However, the proteinase activity was significantly inhibited by chymostatin (78%) and EDTA (59%) and to a lesser extent by E-64 (23%) and pepstatin (22%) indicating the existence of proteinases with highly complex specificities in the gut of *H. armigera* fed on pigeonpea.

To visualize the inhibition of specific major proteinases, HGP of larvae fed on chickpea was treated with type-specific chemical PIs viz. 4(amidinophenyl) methane sulfonyl fluoride (APMSF), phenyl methyl sulfonyl fluoride (PMSF), EDTA, E-64 and pepstatin and then loaded on 12% native polyacrylamide gel (Figure 4.2). PMSF, a general inhibitor of serine proteinases inhibited all the isoproteinases (Figure 4.2, lane 3). APMSF, an inhibitor of serine proteinases (Figure 4.2, lane 2), EDTA (Figure 4.2, lane 4) and pepstatin (Figure 4.2, lane 6) showed partial inhibition of the slower moving minor band but completely inhibited the fast moving minor band. HGP treated with E-64 showed an apparent shift in the migration of the three isoproteinase bands and inhibition of the minor isoproteinase completely (Figure 4.2, lane 5).

The inhibition potential of PIs from different hosts (chickpea, pigeonpea, cotton and okra) against HGP of larvae fed on these host plants was determined (Table 4.3). Chickpea PIs inhibited 45-54% of HGP of larvae fed on chickpea, cotton, and okra, but did not inhibit that of larvae feeding on pigeonpea. Pigeonpea PIs exhibited less than 50% inhibition of HGP of larvae reared on the four host plants. Cotton PIs inhibited nearly half the HGP activity of larvae growing on chickpea (55%), cotton (57%) and okra

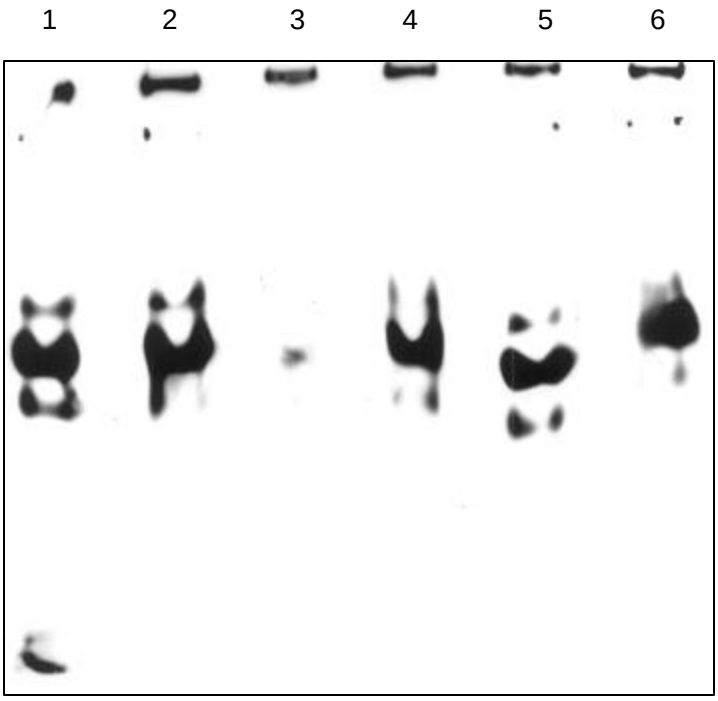

Figure 4.2. Inhibition of HGP isoforms by chemical inhibitors. *H. armigera* isoproteinases fed on chickpea (lane 1) were treated with chemical inhibitors APMSF (lane 2), PMSF (lane 3), EDTA (lane 4), E-64 (lane 5) and pepstatin (lane 6), loaded on 12% PA gel and visualized by the gel-X-ray film contact print technique.

Table 4.3. Effect of plant PIs on gut proteinase activity of *H. armigera* feeding on various host plants. Inhibition of gut proteinase activity was measured using casein as a substrate. The values are the average of three replicates ± SE

Source of HGP	% inhibition of gut proteinase activity by plant PIs [a]			
	Chickpea	Pigeonpea	Cotton	Okra
Chickpea	45±2	45±0	55±3	85±1
Pigeonpea	0	33±0	23±2	53±2
Cotton	54±5	18±6	57±5	80±0
Okra	50±2	25±2	65±4	83±1

[a]Maximum possible inhibition due to inhibitor extract given

(65%); however could inhibit only 23% of the activity of larvae fed on pigeonpea. Okra PIs showed maximum inhibition (80-85%) of HGP activity among all host PIs studied. However, only 53% HGP inhibition was detected in the larvae feeding on pigeonpea.

4.3.3 Expression of HGP during the development of H. armigera larvae

To assess the expression of gut proteinases during the larval development, total proteolytic (azocaseinolytic) and trypsin- (BA*p*NAase) and chymotrypsin-like (GLUPHEPAase) activities of *H. armigera* larvae (fed on artificial diet) during their development through six instars were estimated (Figure 4.3). Proteolytic activities were barely detected in the first instar larvae but they increased continuously up to fifth instar giving maximum activity in the fifth instar larvae. The activity declined drastically in the sixth instar. As against this, very low chymotrypsin activity was detected at all stages of larval development (data not shown).

The gut extract of the larvae at various instars when loaded on 12% native polyacryamide gel showed the differential expression of isoproteinases (Figure 4.4). In the first instar larvae, isoproteinases were not detected due to very low activity. The second instar larvae showed the presence of five bands of isoproteinases viz. 1, 2, 7, 8 and 10 (Figure 4.4, lane 1) while the third instar showed the presence of proteinase bands 1, 2 and 10 with additional new bands 4 to 6 (Figure 4.4, lane 2). In the fourth and fifth instars, increased expression of proteinase activity was observed compared to the earlier instars. The fourth instar showed presence of all the bands of proteinase activity except band 8 (Figure 4.4, lane 3) and exhibited a unique band 9 which was absent in all the other instars. In the

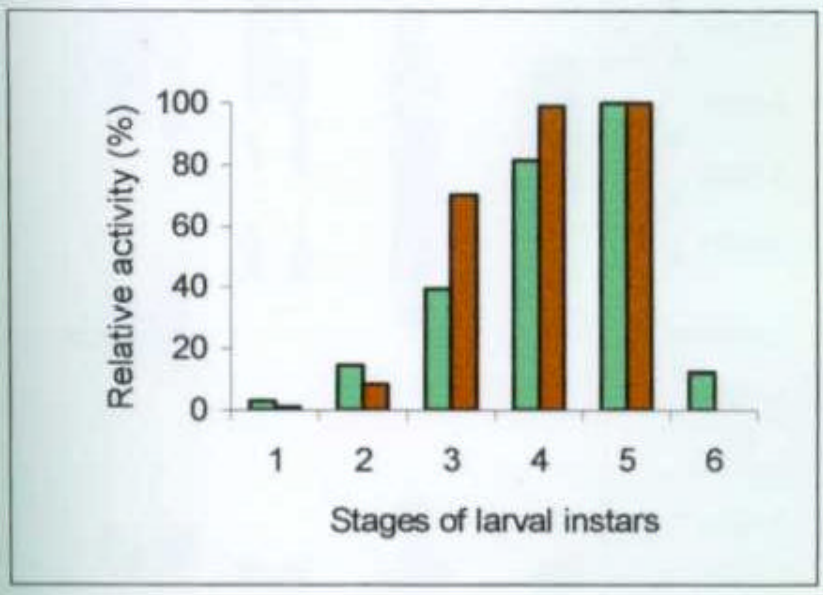

Figure 4.3. Gut proteinase activity of *H. armigera* during the stages of larval development. Total gut proteinase activity was measured using azocasein as a substrate (■) and trypsin-like activity was measured using BA*p*NA as a substrate (■).

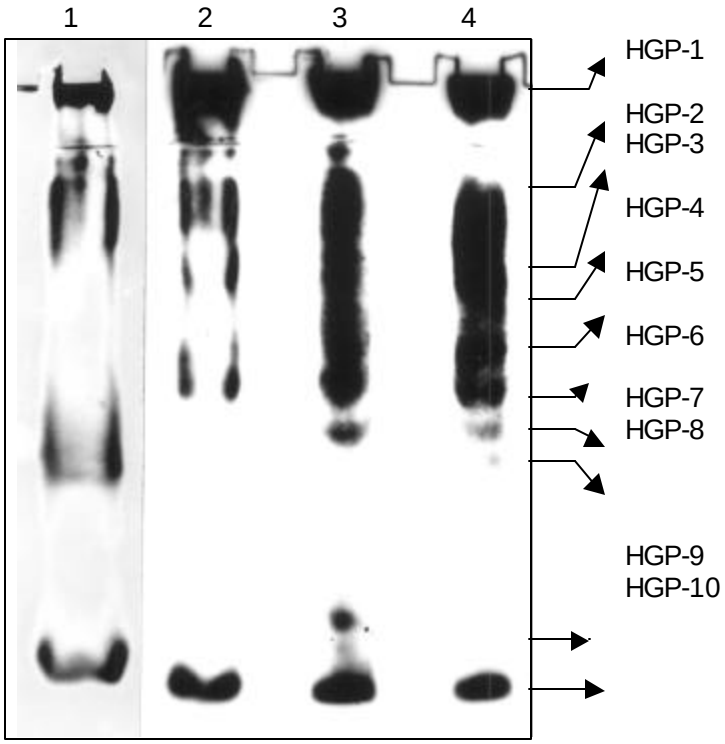

Figure 4.4. HGP profiles of the various larval instars. One unit BA*p*NAase activity of larvae of second instar (lane 1), third instar (lane 2), fourth instar (lane 3) and fifth instar (lane 4) were loaded on the gel and visualized as described in Materials and Methods. High amount of activity was loaded to visualize accumulation of minor bands during larval development.

fifth instar, HGP activity was distributed into nine bands in which bands 7 and 8 were seen as minor bands (Figure 4.4, lane 4). No activity band of HGP was detected in the sixth instar which corroborated well with the solution assay results.

4.3.4 Inhibition of HGP of second and fifth instars by chemical inhibitors

When HGP of second and fifth instars of *H. armigera* larvae fed on artificial diet were assessed for their inhibition by chemical inhibitors (Table 4.4), HGP of second instar larvae showed about 60 to 65% inhibition by serine PIs leupeptin, pefabloc and aprotinin with the exception of antipain which inhibited only 39% of HGP activity. Interestingly, significant inhibition was shown by E-64 (37%), pepstatin (39%) and EDTA (25%) indicating the presence of specificity other than serine proteinases in the second instar larval gut. Chymostatin did not show inhibition, which was also confirmed by the total absence of GLUPHEPAase activity (data not shown). HGP of larvae of fifth instar showed inhibition by all the serine PIs viz. antipain (63%), leupeptin (61%), pefabloc (87%) and aprotinin (50%); and the chymotrypsin inhibitor chymostatin (45%) but no inhibition by inhibitors of cysteine proteinases (E-64), aspartic proteinases (pepstatin) and metalloproteinases (EDTA) suggesting the increased dominance of serine proteinases with the instar development.

The differential susceptibilities of the proteinases to chemical inhibitors observed in the two larval instars suggests the dynamic nature of expression of the gut proteinases possessing different specificity during the course of larval development.

Table 4.4 Effect of chemical inhibitors on gut proteinase activities of second and fifth instar of *H. armigera* larvae fed on artificial diet. Inhibition of gut proteinase activity was measured using casein as a substrate. The values are average of three replicates ± SE

Chemical inhibitors	Specificity of the inhibitor	Effective concentration	% inhibition of HGP of	
			Second instar	Fifth instar
Antipain	Serine proteinases	265µM	39±5	63±0
Leupeptin	Serine proteinases	21µM	65±4	61±2
Pefabloc	Serine proteinases	10mM	60±2	87±2
Aprotinin	Serine proteinases	1.8µM	59±4	50±9
Chymostatin	Chymotrypsin	264µM	00	45±3
E-64	Cysteine proteinases	111µM	37±2	00
Pepstatin	Aspartic proteinases	5.8µM	39±3	00
EDTA-Na_2	Metallo proteinases	1mM	25±5	00

4.3.5 Analysis of proteinases in the fecal matter

To study the putative hyperproduction of proteinases in response to dietary PIs, the fecal matter of larvae feeding on different hosts or on artificial diet was collected and assessed for proteinase activity (Table 4.5). Interestingly, fecal matter from larvae feeding on different host plant PIs showed the presence of estimable total and trypsin-like proteinase activity. The fecal matter of larvae reared on artificial diet showed high total proteinase activity (114.3 units per g). Comparatively the fecal matter of larvae fed on natural diet (hosts chickpea and okra) showed less than half the proteinase activity of that in the diet-fed larvae. No proteinase activity was detected in the fecal matter of pigeonpea-fed larvae. The fecal matter from larvae fed on natural diet (hosts chickpea, pigeonpea and okra) showed five-fold less BA*p*NAase activity.

To visualize the possible differences in proteinases, the fecal matter of larvae growing on three hosts viz. chickpea, pigeonpea and okra and of those reared on artificial diet was resolved on 12% native polyacrylamide gel by loading equal BA*p*NAase units (Figure 4.5). In the fecal matter of larvae fed on chickpea, two major activity bands were detected with minor activity at the top of the well (Figure 4.5, lane 1). No activity bands were detected in fecal matter of the pigeonpea fed larvae (Figure 4.5, lane 2). The fecal matter of the larvae fed on okra capsules or artificial diet showed the presence of high activity bands along with detectable fast moving proteinase isozymes (Figure 4.5, lanes 3 and 4).

Table 4.5 Proteinase activity present in the fecal matter of *H. armigera* fed on different diets. The values are the average of three replicates ± SD

Host plant	Proteinase activity (U/g)	
	Azocaseinase	BA*p*NAase
Chickpea	43.7± 1.42	15.33± 0.46
Pigeonpea	0.0	0.638± 0.05
Okra	45.3± 3.4	21.06± 0.79
Artificial diet	114.3± 2.8	108.2± 1.4

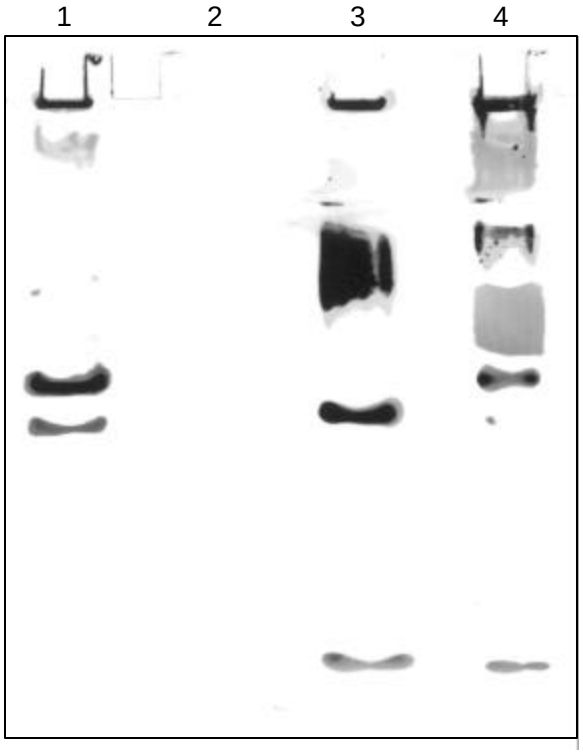

Figure 4.5. Proteinase profiles in the fecal matter of larvae feeding on host plants or on artificial diet. Proteinase profiles of the fecal matter of larvae reared on chickpea (lane 1), pigeonpea (lane2), okra (lane 3) and artificial diet (lane 4).

4.4. Discussion

In recent years, insect gut proteinases have been purified and characterized using chemical inhibitors, substrates of several specificities and other activators or stabilizers (Christeller et al 1989; Johnston et al 1991; Valaitis 1995; Jongsma et al 1996a; Denolf et al 1997; Marchetti et al 1998; Valaitis et al 1999). However, it is also essential to study the composition of their mid-gut in order to plan PI-based strategies for insect resistance. Moreover since the insect has the capacity to alter mid-gut composition within the same generation for neutralizing the effect of PIs, understanding of the nature of gut proteinase activity together with the activity induced upon ingestion of PIs will be important for selecting PIs or combination of PIs for developing insect-resistance.

As shown by earlier studies, insects rapidly alter their gut composition by up- and down-regulation of proteinases in response to ingested PIs (Jongsma and Bolter 1997). This process of altering the secretion of proteinases may be more complicated in generalized (polyphagous) feeders than the specialized feeders. The generalized feeders are shown to be more adapted to several classes of inhibitors as compared to the specialized feeders (Broadway and Villani 1995). Diet-related plasticity has been shown in the Coleopteran Colorado potato beetle (Overney et al 1997) and in lepidopterans *Spodoptera exigua* (Broadway and Duffey 1988), *Ostrinia nubilalis* (Larocque and Houseman 1990), *H. zea* (Lenz et al 1991) and *Choristoneura fumiferana* (Valaitis et al 1999). The focus of our studies has been on *H. armigera*, a polyphagous insect pest on several crop plants such as cotton and the legumes, chickpea and pigeonpea. A

few earlier studies have reported the effects of various inhibitors added to the artificial diet on the regulation of gut proteinases and growth of *H. armigera* (Johnston et al 1993; Bown et al 1997; Gatehouse et al 1997). This is the first study to understand the nature of HGP activity of insects fed on four different host plants, chickpea, pigeonpea, cotton and okra and also during larval development and their reponse to different chemical and plant PIs.

4.4.1 Quantitative changes in gut proteinases of H. armigera fed on different host plants

In the present study, larvae fed on chickpea showed more than 2.5 to 3-fold total as well as trypsin-like proteinase activity than the larvae fed on the other host plants (Table 4.1). Higher HGP activities in larvae fed on chickpea and artificial diet may be a result of either high protein content of the diet or response of the insect to the dietary PIs which partially inhibit HGP activity. Hyperproduction of proteinases in response to ingested PIs has been shown to exert an extra load on the insect for energy and essential amino acids resulting in retardation of the insect growth (Broadway and Duffey 1986a; b). However, in the present study, secretion of high activity in response to chickpea seeds (in the field) and chickpea flour-containing artificial diet did not correlate with the retardation of insect growth. Larvae of *H. armigera* grew normally (uninhibited) on developing chickpea seeds (in the field) and on the chickpea flour-containing artificial diet; thus, the significance of the high proteinase activity observed remains to be explained. On the other hand, the proteolytic activity was observed to be low in larvae fed on pigeonpea. A possible explanation may be that

chickpea PIs are synthesized very early in the developing seed (Giri et al 1998) while in pigeonpea, PIs are synthesized later during seed maturation (Ambekar et al 1996) resulting in delayed exposure of the insects to these PIs.

Analysis of fecal matter showed that fecal matter of insects feeding on natural plant diet had lower proteinase activity than that of larvae fed on artificial diet (Table 4.5). This might be due to the insects' response to ingested PIs which were abundant in artificial diet (chickpea flour has high PI content).

4.4.2 Diverse specificity of gut proteinases in H. armigera fed on different host plants

The gut proteinase complement of *H. armigera* has been demonstrated to contain predominantly trypsin-like proteinase activity (Johnston et al 1991). Although, the insect trypsins are similar to bovine trypsin in their catalytic properties, they differ in their pH optima (Johnston et al 1991, Purchell et al 1992) and in their sensitivity towards inhibitors of plant or chemical origin (Christeller and Shaw 1989; Terra and Ferreira 1994). In our data, the differences in the extent of inhibition by the four serine PIs towards HGP of larvae fed on the four different hosts pointed to the different specificity in the trypsin-like activities in the *H. armigera* gut. Bown et al (1997) also reported that though the serine PIs antipain, leupeptin and benzamidine inhibited 98% of HGP activity, PMSF, a general serine proteinase inhibitor, could inhibit only 28% of HGP activity larvae reared on artificial diet. Further, presence of at least 28 genes for trypsin- and chymotrypsin-like proteinases in *H. armigera* with 90% homology and minor

differences near the active site of these proteinases was shown by Bown et al (1997). Recently, Mazumdar-Leighton et al (2000) reported the presence of two transcripts for trypsin-like proteinases in *H. armigera*. However, interestingly, they were quite distinct from those reported by Bown et al (1997) and Gatehouse et al (1997). The authors attributed their presence to the fact that the larvae were reared on corn and not on artificial diet.

In the present study, moderate to high levels of chymotrypsin-like activity was detected in the larvae feeding on okra, pigeonpea and cotton whereas there was very low activity in case of larvae fed on chickpea (Tables 4.1 and 4.2). Lot of ambiguity exists regarding chymotrypsin-like proteinases in lepidopteran insect guts. Several workers have reported either absence or very low levels of chymotrypsin-like activity among the lepidoptera (Christeller et al 1992). Christeller et al (1992) also have identified elastase-like proteinases to be important enzymes in lepidopteran guts. Elastase and chymotrypsin show several overlapping properties, especially with respect to their sensitivity towards inhibitors, presenting a possibility that some of the chymotrypsin-like activity may actually be contributed by elastase-like enzymes or vice versa. Wu et al (1997) have also reported secretion of chymotrypsin-like and elastase-like activities in *H. armigera* gut in response to giant taro TI.

An important result of our study is the presence of several additional specificity of proteinases (other than serine proteinases) in the *H. armigera* gut of larvae fed on four different hosts (Table 4.2). The gut composition of larvae fed on pigeonpea contrasted with that of *H. armigera* larvae fed on

another susceptible legume, chickpea. *H. armigera* feeding on pigeonpea revealed the presence of metalloproteinases and low percentage of aspartic and cysteine proteinases which were totally absent in the guts of larvae fed on chickpea. This is the first report to our knowledge showing the presence of specificities other than serine proteinases in *H. armigera* gut.

4.4.3 Changes in the gut proteinase activity during larval development

H. armigera larvae are foliar feeders at early instars and later they shift to developing seeds or bolls. This change in the nature of diet may also effect a corresponding change in the gut proteinase complement. However, we have studied the gut proteinase activity of the various larval instars fed on the same diet (artificial diet) in order to bring out the qualitative and quantitative differences in gut composition during larval development. Earlier Michaud et al (1995a) have demonstrated constitutive expression of nine cysteine proteinases with quantitative differences during advancing larval stages in Colorado potato beetle. Our study revealed qualitative as well as quantitative differences during the larval development of *H. armigera* (Fig 4.3 and 4.4; Table 4.4). The second instar larvae showed a wider range of proteolytic activity belonging to different classes other than serine proteinases. However, the larvae of the fifth instar showed a predominance of the serine proteinases and absence of other classes. Novillo et al (1997) have shown that the cysteine PI E-64 inhibited serine proteinases of several lepidopteran species. In the present study, it may be possible that serine proteinases in the gut of the second instar larvae are sensitive to E-64 or the larvae might be producing cysteine

proteinases. Zhao et al (1998) reported the presence of cysteine proteinase in the eggs of *H. armigera*. However, the synthesis of these proteinases is switched off during the larval development to fifth instar. Differential expression of proteinases in *Spodoptera littoralis* during larval development was found to be responsible for inactivation of Bt toxin in fifth instar larvae (Keller et al 1996). Whitworth et al (1998; 1999) reported the highest proteinase activity with selective expression of an elastase in the fourth instar of the imported fire ant *Solenopsis invicta.* In conclusion, gut proteinases are regulated not only by the nature of the diet (including PIs) but are also governed temporally during larval developmental stages.

4.4.4 Diversity in proteinases: Basis for polyphagous nature of H. armigera

A polyphagous insect like *H. armigera* which is reported to feed on as many as 181 plant species (Manjunath et al 1989) would be expected to have a complement of gut proteinases that is capable of overcoming the effect of the PIs of all these host plants. The results reported here reveal the existence of diverse specificities of proteolytic enzymes present in *H. armigera* gut. Although, serine proteinases are predominantly present in *H. armigera*, they are complemented by the presence of cysteine proteinases, aspartic proteinases and metalloproteinases in varying proportions depending upon the chronic ingestion of different host PIs. The presence of such a genetic complement and diversity in the expression of proteinases possessing different specificity may be responsible for the polyphagous nature of the insect pest.

In conclusion, the diversity in the proteinase activity observed in *H. armigera* gut and the flexibility in their expression during developmental

stages and depending upon diet should be considered and the insect's response to the PIs should be essentially studied for selection of the appropriate PIs and their transgenic expression for insect resistance.

CHAPTER 5

Successive use of non-host plant proteinase inhibitors offers a potential solution for effective inhibition of *Helicoverpa armigera*

This chapter has been accepted as a full-length paper in Plant Physiology (1999) 121: 497-504

5.1. Introduction

Plant proteinase inhibitors (PIs) have been extensively studied for their role in plant protection (Ryan 1990). Incorporating their genes for resistance against insect pests has been advocated as a promising strategy as the PIs minimize the possibility of development of resistance in insect pests. In our studies, chickpea and its wild relatives did not yield any effective PIs against the diverse gut proteinases of *H. armigera*, therefore, it was thought essential to search for sources of effective PIs in other plants. In this effort, several non-host plants were screened and winged bean, groundnut and potato PIs (PI-I, II and III) were found to be promising candidates. The work presented here aimed at evaluating *in vitro* and *in vivo* effects of host and non-host plant PIs on *H. armigera*. The host group of plants selected for this study included chickpea, *Cicer echinospermum* (wild relative of chickpea), pigeonpea, *Cajanus scaraboides* (wild relative of pigeonpea) and cotton (*Gossypium arboreum*). The activity and *in vitro* stability studies of host and non-host plant PIs, on the basis of their inhibition towards *Helicoverpa armigera* gut proteinases (HGP), were carried out. Feeding assays were performed to ascertain the potency of the inhibitors in inhibiting the growth of *H. armigera* larvae. The results provide basis for selection of a few non-host PIs and present an optimized combination for developing *H. armigera*-resistant transgenic plants.

5.2. Materials and Methods

5.2.1 Materials

Seeds of chickpea (*Cicer arietinum* cv. Vijay), pigeonpea (*Cajanus cajan* cv. BDN-2), groundnut (*Arachis hypogea*) and cotton (*Gossypium*

arboreum cv. K-32) were obtained from the Pulse Research Station and Oilseed Research Station, Mahatma Phule Krishi Vidyapeeth (MPKV), Rahuri. *Cicer echinospermum* and *Cajanus scaraboides* seeds were procured from International Crop Research Institute for Semi Arid Tropics, Patancheru, India while winged bean seeds (*Psophocarpus tetragonolobus* cv. iiHp Sel 21) were obtained from National Bureau of Plant Genetic Resources, Regional Station, Panjabrao Krishi Vidyapeeth Campus, Akola. Purified potato PI-I, II (Bowman-Birk type) and III (Kunitz type) were provided by Prof. C. A. Ryan, Washington State University, Pullman, USA. All chemicals used were of analytical grade and were purchased from Qualigens, India or SRL Chemicals, India. Azocasein, BApNA and GLUPHEPA were from Sigma Chemicals, USA.

5.2.2 Preparation of plant PI and HGP extracts

The plant PI extracts were prepared from dry seeds using the method detailed in section 3.2.2.

Fifth-instar *H. armigera* larvae were collected from chickpea fields of Pulse Research Station, Rahuri and their midguts were dissected out. Midguts were also isolated from the larvae fed on artificial diet with or without added PIs (see section 5.2.6). Mid-guts isolated by dissecting the larvae were stored at -20^{o}C till further use. Extraction of HGP was carried out as detailed in section 3.2.3. HGP from larvae fed on three other host plants, pigeonpea, cotton and okra was prepared as detailed in section 4.2.3.

5.2.3 Proteinase and PI assays

Total gut proteinase activity was measured by caseinolytic (Belew and Porath 1970) and azo-caseinolytic assays (Brock et al 1982). Trypsin and chymotrypsin-like activities were estimated using chromogenic substrates benzoyl-arginyl *p*-nitroanilide (BA*p*NA) (Erlanger et al 1964) and n-glutaryl 1-phenylalanine p-nitroanilide (GLUPHEPA) (Mueller and Weder 1989). The assays were similar to that described earlier in section 4.2.5. For the inhibitor assays, suitable volume of seed extract was added to the HGP extract or to the respective proteinase and incubated at room temperature (27^{o}C) for 15 min. The residual proteinase activity was then estimated. One proteinase unit was defined as the amount of enzyme that increases absorbance by 1 OD under the given assay conditions.

5.2.4 Visualization of isoforms of proteinases and PIs

The inhibitory activity of plant PIs against isoforms of proteinases was visualized by the gel-X-ray film contact print technique (Pichare and Kachole 1994; Harsulkar et al 1998). After electrophoresis of HGP, the gel was equilibrated in 0.2M glycine-NaOH, pH 10.0. Individual lane strips were incubated in PI extracts of host and non-host plants, washed briefly and then placed upon a piece of unprocessed X-ray film. After incubation for 30 min-1 h, the gel was removed and the X-ray film was washed with hot water to remove the hydrolysed gelatin. Proteinase bands which were uninhibited by the PIs were visible as hydrolysed gelatin.

Trypsin inhibitor (TI) and HGPI bands from host and non-host plants were detected using gels co-polymerised with 1% gelatin (Felicioli et al 1997). After electrophoresis, the gels were equilibrated in 0.1 M Tris-HCl

buffer, pH 7.8 for TI activity and in 0.2M glycine-NaOH buffer, pH 10.0 for HGPI activity. The respective gels were transferred to solutions containing 0.1% trypsin or HGP of equivalent activity and incubated for 1-2 h with constant shaking. The gels were then washed with warm water, fixed in 10% trichloroacetic acid, stained with Coomassie Brilliant Blue R-250 and destained. Dark blue bands of unhydrolysed gelatin appeared at the site of PI activity against the faint blue background.

5.2.5 Treatment of PIs with HGP

To confirm the stability of PIs, equal units of inhibitor from host or non-host plants were treated with HGP at 37^{o}C for 30 min and for 3 h and PI activity was estimated as described above in solution assays. HGP-treated seed extracts were also analyzed on 12% polyacrylamide gel by the gel-X-ray film contact print technique (Pichare and Kachole 1994; Giri et al 1998) to detect the trypsin iso-inhibitors stable to HGP.

5.2.6 Feeding assays

Bioassays were conducted by feeding *H. armigera* larvae on host or non-host PIs incorporated in an artificial diet (Giri and Kachole 1998). Artificial diet was prepared as given in section 4.2.3. Cubes of diet (2 g) were cut and used for feeding experiment. The basic diet was supplemented with the seed extracts of host or non-host plants in appropriate quantities so as to give equal TI units (3 units/g of diet). Forty larvae each of early second instar were reared on the diets and gain in weight was meticulously recorded on every second day, till pupation. The experiment was repeated at least three times. Mdguts were isolated by dissecting the larvae and were stored at -20^{0}C for proteinase analysis.

5.2.7 Fecal matter analysis

The fecal matter of larvae fed on artificial diet with or without PIs was collected. It was extracted similarly as the excised guts and this extract was used for proteinase analysis.

5.3. Results

5.3.1 Inhibition of HGP by host and non-host plant PIs

Several host and non-host plants were analyzed for inhibition of HGP activity out of which three non-host plants whose PIs showed total inhibition of HGP were selected for further analysis. Inhibition of HGP was studied at pH 7.8 and pH 10 because two groups of proteinases showing activity at specific pHs were identified earlier in the HGP complement (Harsulkar et al 1998). Table 5.1 gives an account of efficiency of inhibition of HGP activity by various plant PIs. A close examination of data in Table 5.1 revealed that PIs from host group of plants comprising chickpea, pigeon pea and cotton along with *C. echinospermum* showed between 33% and 55% inhibition of HGP activity at pH 7.8 and 10.0 while *Cajanus scaraboides* PIs did not inhibit HGP activity. PIs from the non-host plants of *H. armigera* viz. groundnut and winged bean along with potato PIs (PI-I, II and III), on the other hand, showed total inhibition of HGP activity at both the pHs except in case of groundnut PIs which inhibited HGP activity up to 84% at pH 10.

Another approach of electrophoretic visualization of inhibition of HGP isoforms by host and non-host plant PIs indicated that major HGPs were insensitive to chickpea and pigeonpea PIs (Fig. 5.1, lanes 2 and 3). Among the non-host PIs, winged bean PIs effectively inhibited all the HGP isoforms (Fig. 5.1, lane 5) whereas groundnut PIs and potato PI-II inhibited

Table 5.1. Inhibition potential of host and non-host plant PIs against gut proteinase activity of *H. armigera*. Activity assays were performed at pH 7.8 and pH 10.0. Double concentration of gut extract was required to obtain equivalent units of BA*p*NAase activity at pH 7.8 than at pH 10.0. Five different concentrations of inhibitor extract were used to assess the potential of inhibitor for inhibiting HGP activity. Maximum possible inhibition of HGP due to respective PIs is given. The values are average of three replicates ± SE

Plant PIs	Maximum inhibition of HGP activity (%)	
	pH 7.8	pH 10.0
Host plants		
Chickpea	45± 3	33± 2
Cicer echinospermum	38± 2	33± 2
Pigeonpea	55± 5	48± 3
Cajanus scaraboides	00	00
Cotton	38± 9	40± 1
Non-host plants		
Groundnut	100	84± 2
Winged bean	100	100
Potato PI-I	100	100
Potato PI-II	100	100
Potato PI-III	100	100

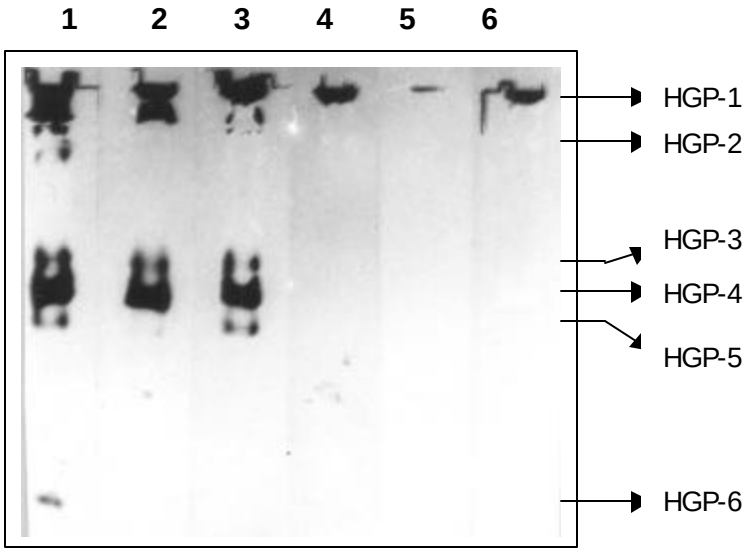

Figure 5.1. Inhibition of HGP isoforms by inhibitors of host and non-host plants. HGP isoforms after electrophoresis were incubated with host and non-host PIs and then visualized by the gel-X-ray film contact print technique. Lane 1,Control; lanes 2-6, HGP-resolved strips incubated in PIs of chickpea, pigeonpea, groundnut, winged bean and potato PI-II, respectively.

all isoforms and partially inhibited HGP-1 (Fig. 5.1, lanes 4 and 6). Based on the data in Table 5.1 and Figure 5.1, it can be concluded that non-host PIs are able to inhibit total proteinase activity and almost all the isoforms of HGP effectively as compared to the host plant PIs, which are poor inhibitors of HGP.

To further check the potential of non-host PIs, their inhibitory activity against HGP from larvae fed on different hosts was checked (Table 5.2). Groundnut PIs showed 50, 70 and 70 % inhibition of HGP from larvae fed on pigeonpea, okra and cotton, respectively. Winged bean PIs and potato PI-II showed total inhibition of HGP of larvae fed on all the three host plants.

5.3.2 In vitro stability of PIs to HGP

The *in vitro* stability of host and non-host plant PIs against HGP were evaluated by enzyme assays after incubation with 0.02 BA*p*NAase units of HGP for 30 min and 3 h (Table 5.3). *In vitro* stability of the host and non-host PIs against HGP was reflected by the extent of inhibition after HGP treatment for 30 min or 3 h. Interestingly, chickpea and pigeonpea PIs after proteolysis by HGP for 3 h showed a modest increase in inhibition of HGP. The non-host PIs, on the other hand, showed total inhibition of HGP even after incubation for 3 h.

Figure 5.2 reveals the stability profiles of TIs of host and non-host plants after treatment with HGP. Host and non-host PI extracts were treated with HGP for 30 min and 3 h and TIs were visualized using gel-X-ray film contact print technique. Stability profiles indicated that chickpea TIs were degraded by HGP leading to generation of active TI fragment after 30 min.

Table 5.2. Inhibition potential of non-host PIs against HGP of larvae fed on pigeonpea, cotton and okra. Assay was performed at pH 10.0 with casein as substrate as described in 'Materials and Methods'. Maximum possible inhibition due to PIs is given. The values are average of three replicates ± SE

Plant PIs	Maximum inhibition (%) of HGP from larvae fed on:		
	Pigeonpea	Cotton	Okra
Groundnut	50± 5	70± 2	70± 2
Winged bean	100	100	100
Potato PI-II	100	100	100

Table 5.3. In vitro stability of host and non-host plant PIs against HGP. Inhibitors were pre-incubated with HGP for 30 min and 3 h at 37^{o}C and then assayed for their inhibitory activity towards HGP as described in 'Materials and Methods'. Each value is the average of three replicates ± SE.

Plant PIs	Inhibition of HGP activity (%)	
	30 min	3 h
Host plants		
Chickpea	33±1.5	47±1.4
Pigeonpea	48±1.0	55±1.3
Non-host plants		
Groundnut	84±2.0	100±0.0
Winged bean	100±0.0	100±0.0
Potato PI-II	100±0.0	100±0.0

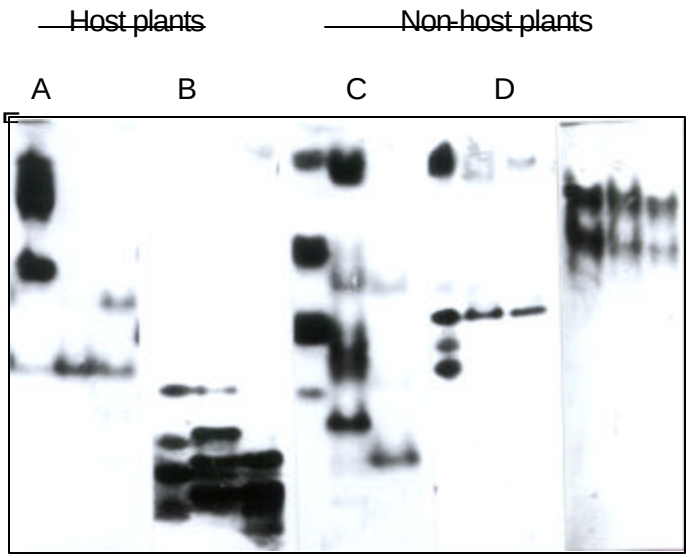

Figure 5.2. Stability of host and non-host plant TIs towards HGP. Seed extracts were incubated with HGP for 30 min and 3 h at 37°C. TI bands were visualized using the X-ray film contact print technique. Lane 1, Control (without pre-incubation with HGP); lane 2, Incubated with HGP for 30 min and lane 3, for 3 h. A, chickpea; B, pigeonpea; C, groundnut; D, winged bean; and E, potato PI-II.

Incubation for 3 h led to the generation of one more TI fragment (Fig. 5.2A). *Cicer echinospermum* TIs were also proteolysed by HGP, similar to chickpea TIs (results not shown). In pigeonpea, among the fast moving four TI bands, first two bands were not stable to HGP on 3 h incubation; while other two remained stable even after 3 h incubation to HGP (Fig. 5.2B). *Cajanus scaraboides* TIs were degraded after 30 min incubation with HGP leading to the formation of four stable TI activity fragments (results not shown). In the non-host group of plants, TI isoforms of groundnut upon 30 min incubation with HGP showed six fragments having TI activity out of which only three remained stable with increasing time (Fig. 5.2C). In case of winged bean, one TI was resistant to proteolysis by HGP, however, slow moving TIs showed partial degradation and fast moving bands disappeared after 3 h incubation (Fig. 5.2D). Native form of potato PI-II was stable to proteolysis by HGP; however, there was partial proteolysis as evident by decreased intensity of bands on incubation for 30 min and 3 h (Fig. 5.2E). The above results suggested that most of the native TI isoforms were susceptible to proteolysis by HGP except potato PI-II and a winged bean TI to a certain extent.

5.3.3 Trypsin and HGP isoinhibitors in host and non-host plants

Figure 5.3 shows electrophoretic profiles of TIs and HGPIs in seed extracts of host and non-host plants. Several bands of TI activity were present in both the host and non-host plants. Chickpea and *Cicer echinospermum* exhibited three TI bands (Fig. 5.3, lanes 1 and 2). In pigeonpea, five fast-migrating TIs were detected (Fig. 5.3, lane 3) while in *Cajanus scaraboides* four TI bands were observed (Fig. 5.3, lane 4). HGPI

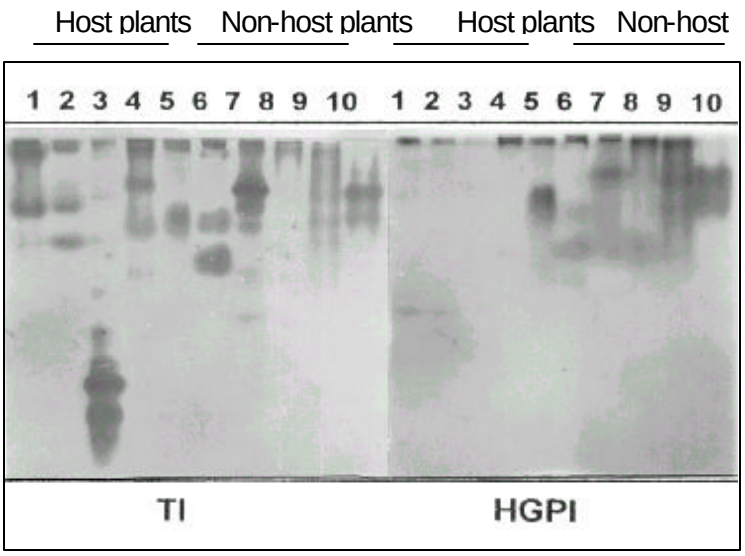

Figure 5.3. TI and HGPI profiles of host and non-host plants. TI and HGPI bands were visualized as described in 'Materials and Methods'. Equal TI units were loaded on both the gels. Lane 1, Chickpea; lane 2, *Cicer echinospermum*; lane 3, pigeonpea; lane 4, *Cajanus scaraboides*; lane 5, cotton; lane 6, groundnut; lane 7, winged bean; lane 8, potato PI-I; lane 9, PI-II and lane 10, PI-III.

bands were absent in host group comprising chickpea, *Cicer echinospermum*, pigeonpea and *Cajanus scaraboides* (Fig. 5.3, lanes 1-4) while cotton TI appeared as HGPI band (Fig. 5.3, lane 5). In the non-host group, winged bean showed six TI bands out of which 3 bands possessed HGPI activity (Fig. 5.3, lane 6). The fast moving TI bands of winged bean did not have inhibitory activity against HGP. Groundnut seed extract revealed 3 bands having both TI and HGPI activity (Fig. 5.3, lane 7). Potato inhibitors viz., PI-I, -II and –III resolved into several TI and HGPI activity bands. Potato PI-I showed a slow moving band active against trypsin and HGP while PI-II showed a smear with two more bands active against trypsin and HGP (Fig. 5.3, lanes 8 and 9). PI-III exhibited two activity bands against trypsin which showed higher intensity against HGP (Fig. 5.3, lane 10). The results indicated that no potential inhibitor(s) of HGP were detected in chickpea, pigeonpea and their wild relatives. In cotton the observed 40% inhibition of HGP in enzyme assay corresponded to a single inhibitor on the activity gel. However, in case of chickpea and pigeonpea, HGP inhibitory activity might be distributed among the several iso-inhibitors or might be too weak to be visualized clearly as HGPI bands. To our knowledge, this is the first study where specific insect PIs have been detected using in-gel assay.

5.3.4 Effect of host and non-host plant PIs on growth and development of H. armigera larvae

To estimate *in vivo* effects of host and non-host plant PIs on development of *H. armigera* larvae, feeding trials were conducted with appropriate controls. Typical development of larvae reared on a diet-containing host

PIs (chickpea PIs as representative of host group) and on a diet-containing non-host PIs (groundnut PIs as representative of non-host group) is shown in Figure 5.4. About 3 to 4-fold reduction in weight gain in the larvae fed with non-host PIs was observed. Food in-take was drastically reduced in the larvae showing growth retardation. Furthermore, pupation was also delayed for more than 10 days in larvae showing stunted growth.

On the basis of weight gain of larvae on the 10th day, the larval population was distributed into three groups: (A) stunted growth (0.02-0.2 g); (B) intermediate growth (0.21-0.4 g) and (C) normal growth (0.4-0.6 g). More than 65% larvae fed on potato PI-II or PIs of winged bean or of groundnut showed stunted growth (Fig. 5.5A). A small percentage of larvae showed intermediate growth when fed on host or non-host PIs (Fig. 5.5B). As seen in Figure 5.5C, 77 and 81% of larvae showed normal growth when grown on chickpea and pigeonpea PI-containing diet, respectively. We also observed that the instar stage of *H. armigera* larvae was very critical for assessing the potential of dietary inhibitors. From the above results, it can be suggested that inhibitor concentration of non-host PIs used in the diet was sufficient to inhibit growth of early second instar larvae.

5.3.5 Alteration and in vivo inhibition of gut proteinases in H. armigera larvae reared on PIs

To understand *in vivo* effectiveness of non-host PIs, *H. armigera* larvae fed on control diet or on PIs of chickpea, groundnut, winged bean or potato PI-II were dissected after 8 days and the midgut proteinase activity was

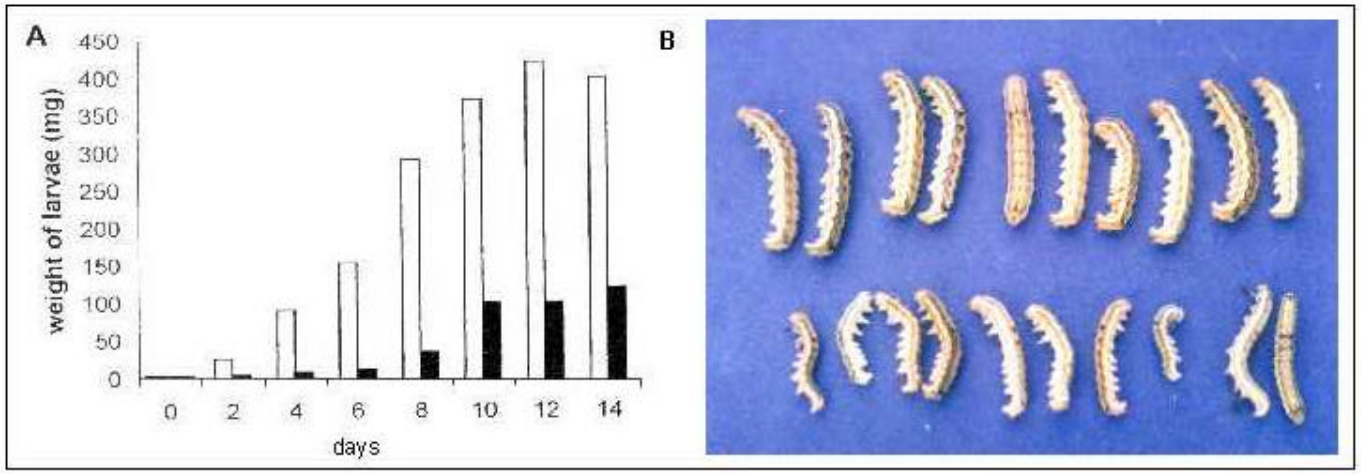

Figure 5.4. Development of *H. armigera* reared on artificial diet containing host (chickpea) and non-host (groundnut) plant PIs. A. Weight of larvae grown on diet containing chickpea PIs (□) and groundnut PIs (■). Weights of larvae were critically measured on every alternate day. B. Photograph of larvae grown on diet containing chickpea PIs showing normal (upper row) and grown on diet containing groundnut PIs showing stunted growth (lower row).

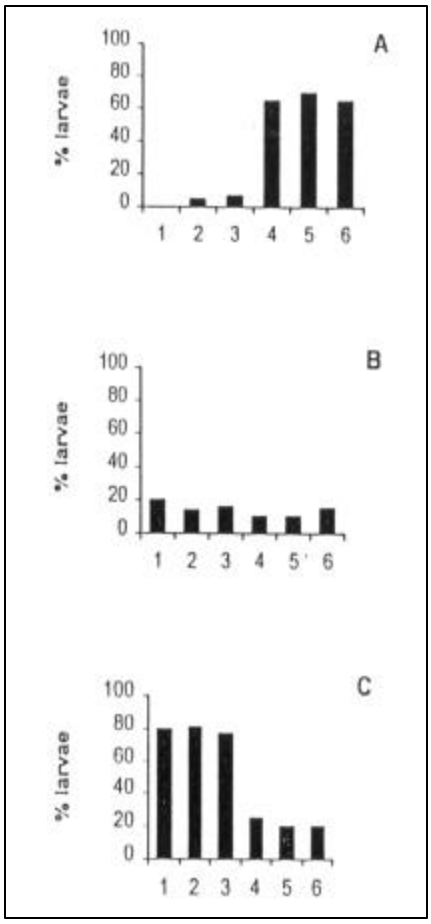

Figure 5.5. *In vivo* effects of host and non-host plant PIs on development of larval *H. armigera. H. armigera* larvae of early second instar were reared on artificial diet supplemented with equal TI units of host and non-host seed extracts as described in 'Materials and Methods'. Weights of larvae were recorded every alternate day. The larvae were classified in to three groups based on their weights. A. Stunted (0.02-0.2 g); B. intermediate (0.21-0.4 g) and C. normal (0.41 g and above) growth of larvae fed on control diet; 1, and diet containing PIs of chickpea, 2; pigeonpea, 3; groundnut, 4; winged bean, 5 and potato PI-II, 6.

estimated (Table 5.4). Proteinase activity of control larvae was considered 100% and accordingly proteinase activity of PI-fed larvae was calculated. The larvae fed on chickpea PIs showed 91% caseinolytic and 72% azocaseinolytic activities whereas in non-host PI-fed larvae the caseinolytic and azocaseinolytic activities were in the range of 35 to 37% and 29 to 30%, respectively. The BApNAase activity which measures trypsin-like proteinases was found to be the lowest (19%) in winged bean PI-fed larvae, 22% in groundnut PI-fed, 42% in potato PI-II fed and 89% in chickpea PI-fed larvae. Very low GLUPHEPAase activity was observed in the guts of control and PI-fed larvae. Larvae reared on non-host PIs showed significant decrease in estimable proteinase activity suggesting that native inhibitors or their fragments were active in the larval gut.

To assess the induction of inhibitor-insensitive proteinase activity, the inhibition potential of maximum amounts (concentration greater than that required to inhibit total proteinase activity of control HGP) of winged bean PIs and potato PI-II required to inhibit the gut extracts of *H. armigera* larvae fed on the same PIs and vice versa were determined (Table 5.5). The larvae fed on winged bean showed 27% inhibitor-insensitive activity while those fed on potato PI-II showed only 5% inhibitor-insensitive activity. Interestingly, winged bean PIs were able to inhibit as high as 96% of the HGP activity induced by potato PI-II while, potato PI-II was able to inhibit only 47% of the HGP activity induced by winged bean PIs.

In order to see if the complement of gut proteinases of *H. armigera* changed following PI ingestion, gut extracts were separated on non-reducing SDS-PA gels to dissociate the enzyme-inhibitor complexes

Table 5.4. Gut proteinase activity of *H. armigera* larvae reared on host and non-host plant PIs. Larvae fed on control diet and PI-containing diet were dissected after 8 days, and proteinase activity was estimated using different substrates as described in 'Materials and Methods'. Parenthesis values are percent proteinase activity of control.

HGPs	Estimable proteinase activity per 10 guts			
	Caseinase	Azocaseinase		BA*p*NAase
	GLUPHEPHAase			
Control	4.65 (100)	4.82 (100)	14.14 (100)	0.013 (100)
Host plants				
Chickpea	4.23 (90.9)	3.48 (72.2)	12.61 (89.2)	0.013 (100)
Non-host plants				
Groundnut	1.66 (35.7)	1.47 (30.5)	3.12 (22.1)	0.000 (0.00)
Winged bean	1.74 (37.4)	1.42 (29.5)	2.70 (19.1)	0.003 (23.1)
Potato PI-II	1.72 (37.0)	1.45 (30.1)	6.01(42.5)	0.005 (38.5)

Table 5.5. Inhibition of gut proteinase activity of *H. armigera* larvae reared on winged bean PIs or potato PI-II. Inhibition potential of winged bean PIs and potato PI-II was assessed against gut proteinases of *H. armigera* reared on the winged bean PIs or on the potato PI-II. Inhibitory activity was estimated by taking various concentrations of inhibitor to obtain maximum inhibition of proteinase activity. Activities were estimated using azocasein as substrate as described in 'Materials and Methods'. The values are average of three replicates ± SE

HGP from larvae reared on	Inhibition of HGP activity (%) by	
	Winged bean PIs	*Potato PI-II*
Control	100	100
Winged bean PIs	73± 3	47± 1
Potato PI-II	96± 1	95± 1

formed *in vivo*. The gut proteinases of larvae fed on control diet and diet-containing plant PIs showed significant differences in the expression of individual proteinases (Fig. 5.6). Gut proteinases of larvae reared on chickpea PIs showed over expression of HGP-1 and decreased expression of HGP-3 and -6 (lane 2). Four isoproteinases viz. HGP-2, -4, -5 and -6 could be detected in the guts of larvae reared on groundnut PIs of which HGP-4 and -6 were highly expressed (lane 3). Interestingly, isoproteinases of larvae fed on winged bean PIs showed an apparently similar profile to that of the control (lane 4). In case of larvae fed on potato PI-II, HGP-2 and -4 were overexpressed, however, HGP-5 and -6 showed a trend similar to larvae fed on control diet (lane 5). HGP-7 was detected as a minor band only in the control and not in the gut extracts of larvae fed on either host or non-host plant PIs (lane 1). The above results indicate that there are significant quantitative and qualitative changes in the gut proteinases in response to dietary PIs.

3.6 Analysis of fecal matter

To study the putative hyperproduction of gut proteinases in response to dietary PIs, the fecal matter from larvae fed on artificial diet with or without PIs was analyzed for proteinase activity (Table 5.6). The fecal matter of larvae fed on artificial diet showed a very high total and trypsin-like proteinase activity compared to that detected in larvae fed on natural diet (section 4.3.5). Surprisingly the fecal matter of larvae fed on groundnut PIs showed higher proteinase activity. This suggests the hyperproduction of proteinases in response to ingested PIs, which are excreted out.

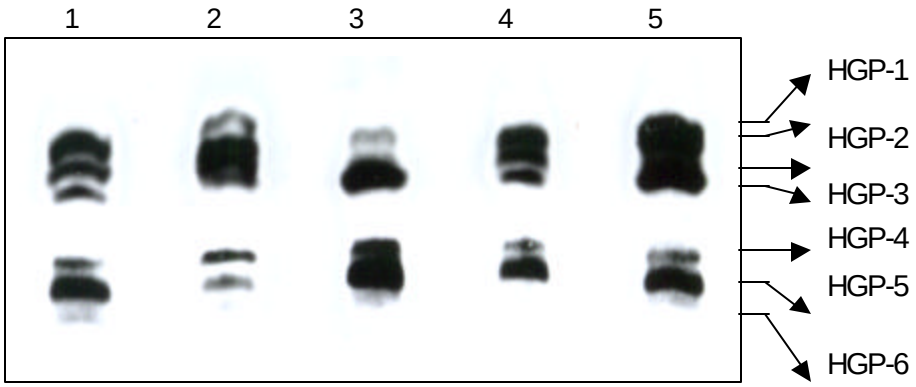

Figure 5.6. Isoproteinase profiles of *H. armigera* larvae fed on diet containing chickpea and non-host PIs. Equal amounts of HGP extract of insects fed on control (lane 1), and on PIs of chickpea (lane 2), groundnut (lane 3) winged bean (lane 4) and potato PI-II (lane 5) were loaded on SDS-PA gel and visualized as described in 'Materials and Methods'.

Table 5.6. Proteinase activity in the fecal matter of larvae fed on PIs. The values are average of three replicates ± SD

Fecal matter from larvae fed on:	Proteinase activity (U/g)	
	Azocaseinase	BApNAase
Control diet	114.3± 2.8	108.2± 1.4
Groundnut PIs	125.0± 2.82	128.4± 6.22

5.4. Discussion

It is an apparent paradox that insects feed on plants in spite of the fact that PIs are ubiquitous, especially in case of legumes. Insect pests adapt to host plant PIs by synthesizing proteinases which are insensitive to inhibitors (Jongsma et al 1995; Broadway 1995; 1997) and/or which have capacity to degrade them (Michaud 1997; Giri et al 1998; Girard et al 1998a). In a stabilized host-pest complex, insects seem to have evolved and adapted to overcome the effect of PIs of their host plants (Bolter and Jongsma 1995; Jongsma et al 1996b; Broadway 1995; 1996; 1997). It is, therefore, necessary to study non-host plant PIs as potential sources to overcome the host inhibitor-insensitive proteinases of insect-pests. The present work deals with evaluation of non-host PIs to establish their potential against HGP through a series of *in vitro* and *in vivo* experiments. For the first time, specific inhibitors of insect gut proteinases have been identified and it has been unequivocally demonstrated that all the TIs do not necessarily possess HGPI activity. The HGPIs when fed to *H. armigera* larvae resulted in antibiosis.

5.4.1 Non-host plant PIs are potent inhibitors of HGP and retard growth of H. armigera larvae

The present study has demonstrated for the first time the presence of inhibitors of insect gut proteinases in the non-host plants. The non-host plant PIs completely inhibited HGP in enzyme assay, and all the HGP isoforms in electrophoretic assay (Table 5.1 and Fig. 5.1). Interestingly, only the non-host plants exhibited presence of PI bands having inhibitory activity against HGP, which were absent in the host plants except cotton

(Fig. 5.3). Earlier and current reports on PIs have dealt with the identification and characterization of specific PIs such as inhibitors of trypsin, chymotrypsin or subtilisin (serine proteinases) and papain (cysteine proteinases). However, it is necessary to identify and evaluate PIs having specific inhibitory activity against insect gut proteinases. In this study we have for the first time visualized the specific HGPIs in non-host plants. Both host and non-host plants showed the presence of TIs but only the non-host plants showed the presence of HGPIs. Our data revealed the dichotomy between the TIs and HGPIs as all the trypsin PIs from the non-host plants could not inhibit HGP, although most of the HGP activity is trypsin-like (Johnston et al 1991; Harsulkar et al 1998). The TIs, which did not appear in HGPI profiles, probably were either ineffective against HGP and/or were degraded by HGP.

Feeding studies showed that PIs of winged bean, groundnut and potato inhibited the growth of nearly 70% of the early second instar larvae, however, did not result in any mortality. Thus the ingested PIs exerted physiological stress on the larvae as evident by retarded growth; however, there was absence of strong selection pressure as there was no mortality. Further there was a significant loss of fertility in addition to severe inhibition of larval growth of *H. armigera* fed on winged bean PIs (Prof. G. P. Gupta, IARI, New Delhi, personal communication).

5.4.2 Stability of PIs

Plant defense proteins can be potentially recognized as substrates by insect gut proteinases (Michaud 1997; Giri et al 1998; Girard et al 1998a). Insects derive dual benefit by digestion of PIs in restoration of the gut

proteinase activity and in availability of valuable sulfur-rich amino acids. Stability of the PIs in the proteolytic environment of the gut is thus an important criterion for selecting candidate PIs. In the present study, upon limited proteolysis by HGP, TIs generated active fragment(s), some of which remained stable up to 3 h (food retention time in the larvae) (Fig. 5.2). Christeller and Shaw (1989) reported that TIs incubated with purified grass grub trypsin retained their activity after limited proteolysis. Chickpea and pigeonpea PIs showed increased inhibition of HGP with prolonged incubation in enzyme assays. This might be a result of formation of modified inhibitor fragments exhibiting higher affinity towards HGP. During incubation of purified chickpea TI with HGP, an increase in HGP inhibition from 33% to 47% was observed (result not shown). Belew and Eaker (1976) also observed that inhibitory activity of trypsin-modified inhibitor towards trypsin increased with the time of exposure which was a common property of TIs. The inhibitory activity which resided in the reactive site of the TI was found to be abolished on removal of the newly-formed carboxy-terminal Lys or Arg from the trypsin-modified inhibitor (Belew and Eaker 1976). Thus, retention of activity rather than integrity of PI proteins is a major factor in assessing their potential utility for insect resistance. In this study, although native forms of few PIs of non-host plants were susceptible to partial proteolysis by HGP, the inhibition potential was not altered as they showed total inhibition of HGP in the enzyme assays (Table 5.3). This indicated their stability against the gut proteinases. It would be of further interest to identify specific fragment(s) possessing HGPI activity from host and non-host plant PIs.

5.4.3 Alteration of H. armigera gut proteinases in response to ingestion of PIs

In the present study, larvae fed on non-host PIs showed a decrease in estimable proteinase activity (Table 5.4). Wu et al (1997) reported a 13% decrease in the total proteinase activity in *H. armigera* larvae fed on transgenically expressed giant taro PI. Bown et al (1997) also reported decrease in overall levels of proteinases and in the levels of mRNAs encoding trypsin-like proteinases of *H. armigera* fed on soybean TI suggesting that the decrease in this activity was at the transcriptional level. The decreased mRNA levels might, however, reflect down-regulation of a particular proteinase which might be compensated by up-regulation of the other proteinases.

Interestingly, a high level of proteinase activity was found in the fecal matter of larvae fed on artificial diet with or without PIs (Table 5.6). This suggested hyperproduction of proteinases in response to ingested PIs. Broadway and Duffey (1986a; b) proposed that hyperproduction of proteinases was the mechanism which led to inhibition of growth by ingested PIs. However, in this study, the larvae fed on artificial diet (not supplemented with non-host PIs) showed normal (uninhibited) growth in contrast to the inhibition of growth of larvae fed on non-host PIs. Also the larvae fed on non-host PIs showed a decrease in the estimable gut proteinase activity. However this decrease was not reflected in electrophoretic profiles (Table 5.4 and Fig. 5.6). These observations taken together strongly suggested the presence of active inhibitors complexed with gut proteinases, which underwent dissociation during SDS-PAGE.

More intense bands actually indicated overexpression of certain proteinases. However, as evident from significant growth retardation, the larvae were suffering from the loss of proteinase activity because of the dietary non-host PIs (Table 5.4 and Fig. 5.5). Recently, Broadway (1997) speculated that insects might possess specific mechanisms for regulation of individual proteinases controlled by a monitor peptide. The latter might be responsible for induction of inhibitor-sensitive and –insensitive proteinases depending upon the nature of the ingested PI.

5.4.4 Winged bean PIs in combination with potato PI-II are ideal for H. armigera resistance

In this study, significant PI-insensitive activity in the larvae fed on winged bean PIs was observed which was 27% insensitive to winged bean PIs and 53% insensitive to potato PI-II (Table 5.5). It is known that potato PI-II is active against a wide range of serine proteinases (Whitworth et al 1998). In this study, however, it was not able to inhibit 53% of the HGP activity of the larvae reared on winged bean PIs (Table 5.5). On the contrary, winged bean PIs inhibited nearly all of the gut proteinase activity of the larvae reared on potato PI-II. This could be attributed to differences in the winged bean PIs and potato PI-II with respect to inhibition of HGP or else to the synthesis of alternative proteinases in response to two different PIs.

The current transgenic research on PIs is mainly focused on expression of a single PI gene in the target plant under the control of universal promoter (reviewed by Schuler et al 1998; Jouanin et al 1998). However, recently several workers have proposed the use of multiple PIs to inhibit a full spectrum of gut proteinases (Jongsma and Bolter 1997; Michaud 1997;

Girard et al 1998a, b). Combinations of PIs targeted to different proteinases have been known to act synergistically (Jongsma and Bolter 1997). Based on the above results, a strategy is proposed to use a combination involving successive expression of winged bean PIs and potato PI-II in a transgenic crop to counteract *H. armigera* infestation. This involves expression of potato PI-II under the control of a 'stay-green' promoter and of winged bean PI under seed-specific promoter.

H. armigera larvae of the first and second instar feed on leaves and flowers and later shift to developing seeds. The rationale in the proposed strategy is to express potato PI-II in vegetative parts so that the growth of early instar larvae will be delayed. When these larvae eventually shift to developing seeds, they would encounter the expressed winged bean PI that has the capacity to inhibit potato PI-II induced proteinases thus forcing the insect to alter the mid-gut proteinase composition at least twice. Such a temporal expression of a suitable combination of inhibitor genes would significantly delay the growth and generation advance of *H. armigera* in the field. Chickpea seed development takes upto two months to form mature seeds. If the larval development is delayed by 10-20 days, it will reduce at least one life cycle with a consequent drastic decrease in the larval population, which grows exponentially with advancing generation. This will result in a significant reduction in yield losses. In feeding studies, the larval growth remained stunted for a long time without any resultant mortality. PIs of the kind or the combination thereof, reported herein are ideal in that they do not target the total elimination of insect pests but merely inhibit larval growth thereby reducing the crop damage. Thus, tandem use of potato PI-

II and winged bean PIs to develop transgenic crop plants will lead to sustainable resistance against *H. armigera*.

CHAPTER 6

Discussion

Proteinase inhibitors as a component of a holistic approach to tackle the problem of insect pests of crops

6.1 A holistic approach to tackle the problem of insect pests on crops

Considering the drawbacks of the use of chemical pesticides, the concept of integrated pest management practices gained significance to reduce the dependence on chemical pesticides and lead to sustainable agriculture. Integrated pest management essentially involves the use of resistant cultivars along with good cultural practices which reduce the emphasis on chemical pesticides (Soon 1997; Thomas 1999). The aim of any sustainable pest management program should not be elimination of the insect pest but to achieve reduction in pest density and thus restrict losses in yield. It is, therefore, essential to consider an approach which is holistic in nature and takes into consideration its impact on the ecosystem's flora and fauna. More specifically, the following three aspects deserve a mention: (1) Study of tritrophic interactions (plant, pest and natural enemies) and their population dynamics. Studies have shown that partial resistance and partially effective biocontrol can be combined to give synergistic reduction in pest density (Thomas 1999), (2) Ecosystem management to take into account the effects of soil, weed, cropping and water practices on the ecosystem fauna. For example, instead of intensive monoculture, diversity of plants along the margins which act as refuges for natural enemies of the insect herbivores will help in herbivore control (Lewis et al 1997), (3) Study of the array of plant defense mechanisms. On attack by insect herbivores, plants release volatile chemicals which attract their predators (e.g. parasitoid wasps) and contain the herbivore attack (Pare and Tumlinson 1999).

The above management practices should be integrated for effective pest control. During the last decade, it has been advocated to replace chemical pesticides with biologically active molecules or 'biopesticides' (notably Bt). However, the use of such therapeutics like chemical pesticides and Bt should form a 'backdrop' to management practices and not be a frontline strategy (Lewis et al 1997). Based on available literature and the work embodied in this thesis, proteinase inhibitors (PIs) are good candidates to be integrated in a sustainable pest management strategy. PIs do not have therapeutic effect as Bt or chemical pesticides thus minimizing the possibility of development of resistance in insect pests. PIs can be used synergistically along with sublethal doses of Bt to give significant reduction in pest density.

6.2 Basis of selection of PIs for effective inhibition of gut proteinases and growth of insect pests

The success of PI-based strategy depends upon the selection of appropriate PIs and their proper expression. An ideal effective PI should have the following characteristics: (1) It should have specific activity against insect gut proteinases, (2) It should have activity against broad spectrum of gut proteinases, (3) It should remain stable to proteolysis during the food retention time in the larval gut. Chronic ingestion of stable PIs leads to an *in vivo* inhibition of gut proteinase activity. The PI should be able to significantly inhibit gut proteolytic activity, only then will it result in retarded growth of the insect pest. Ideally it should adversely affect the development of the insect (fertility and fecundity). Our study has demonstrated the dichotomy between trypsin inhibitors (Tis) and *Helicoverpa armigera* gut

proteinase inhibitors (HGPIs) in the host and non-host plants and presence of HGPIs only in the non-host plants which are stable to HGP and inhibit the growth of *H. armigera* upon ingestion. Also the winged bean PIs inhibit HGP of larvae fed on different host plants pointing to their broad-range activity thus fulfilling most of the characteristics of an ideal effective PI.

Insect pests have adapted to the host plant PIs by synthesis of inhibitor-insensitive and / or inhibitor-degrading proteinases (Jongsma et al 1995; Michaud 1997). Thus the non-host plants represent one of the best sources for selecting out potential effective PIs. Additionally, the use of PIs from sources other than plants has also been considered and exploited (Gruden et al 1998; Schuler et al 1998). For example, Gruden et al (1998) screened twenty-one PIs of different structural types for their ability to inhibit the induced gut proteolytic activity of Colorado potato beetles (*Leptinotarsa decemlineata*) and found that the thyroglobulin type-1 domain-like inhibitors, equistatin and MHC class II-associated p41 invariant fragment strongly inhibited the induced proteolytic activity. Also larval growth was inhibited strongly on ingestion of equistatin-coated leaves. In another approach, PIs isolated from the gut of the insect pest *Manduca sexta* were found to offer protection against insect attack (Schuler et al 1998).

In addition to screening for effective PIs from natural sources, it is now possible to create variants of the PI molecules *in vitro* using the techniques of directed molecular evolution (Jongsma et al 1996b). The variants of PIs thus created using *in vitro* mutagenesis techniques can be efficiently screened by the technique of phage display (Jongsma et al 1996b; Koiwa

et al 1998). The latter makes use of gene fusions with the coat protein of the M13 phage to generate a library which 'displays' the variants of the PI. This library is screened with the appropriate proteinase to select out the PI with the highest binding affinity. For example, Koiwa et al (1998) selected two variants of soyacystatins with increased affinity using the phage display technique.

The efficacy of the candidate PI can be increased by the use of protein engineering to modify selected bases and achieve higher inhibitory activity. Urwin et al (1995) built a model of oryzacystatin I-papain depending upon sequence alignments and the stefinB-papain structural model, carried out deletions of amino acids using site-directed mutagenesis and obtained deletion mutants of oryzacystatin I having greater inhibitory activity.

Importantly, feeding trials with the selected PIs and further investigation of adaptive response of particular insect species are necessary before their use in transforming the target plant. Thus selection of proper PI(s) still remains a challenge to obtain sustainable resistance against the insect.

6.3 Combinatorial use of PIs and their targeted expression for sustainable resistance

From the discussion so far, it is apparent that no single PI can confer exploitable resistance in target plants and hence using PIs in combination is advisable to attain total inhibition of gut proteinases. A proper combination of PIs targeted to inhibit the total spectrum of insect gut proteinases improves each other's stability and thus efficiently impairs digestion of dietary proteins in the insect gut. Simultaneous inclusion of potato carboxypeptidase inhibitor with cystatins was found to prevent

degradation and to restore activity of otherwise susceptible cystatins in the guts of *Diabrotica* larvae, which eventually retarded the larval growth (Orr et al 1994). It is necessary to ensure that combination of PIs should include PIs complementary to each other and targeted to different mechanistic classes of proteinases. Oppert et al (1993) have shown that dietary mixture of serine and cysteine PIs exhibited synergistic toxicity towards the red flour beetle *Tribolium castaneum*. The use of a combination of serine PI along with peptidase inhibitor or cysteine PI against three Coleopteran pests has been proposed (Girard et al 1998a; b; Bonade-Bottino et al 1999).

Use of PIs with defense proteins other than PIs has also been advocated to gain protection from unwanted proteolysis of defense proteins (Michaud, 1997). Combination of PIs with Bt (MacIntosh et al 1990; Zhang et al 2000), amylase inhibitors (Giri and Kachole 1998) and lectins (Boulter et al 1990) has shown potentiation of activity against the target insect pest.

It is also essential to ensure proper expression of such a combination of PIs. A major constraint of the current strategy using a single PI under universal promoter is that a very high level of expression is necessary for effective protection of the plant from the insect, otherwise the insect adapts to the single inhibitor and overgrows (deLeo et al 1998). This constitutive high level of expression in all tissues (under universal promoter) may put a physiological burden on the plant. Moreover, most of the insects preferentially feed on a specific tissue or a group of tissues and almost never on all of the plant organs. For example, nematodes feed on roots whereas most of the lepidopteran pests feed on either foliar parts or on

seeds and in very few cases on both. *H. armigera* moth lays eggs on leaves of the host plant and the newly hatched larvae feed on foliage and floral tissues. The larvae then shift on to the developing seeds for the large deposits of proteins and nutrients. Considering this feeding behavior of insect pests, targeted expression of PIs will be more appropriate and effective. Specifically the differential temporal expression will ensure exposure of the insect to different PIs in succession, forcing the insect to alter its mid-gut composition more than once leading to an additional physiological stress. Based on our results on non-host PIs, we have proposed a strategy to use a combination of PIs involving tissue specific expression of potato PI-II and winged bean PIs in a transgenic crop to counteract *H. armigera* infestation. The strategy proposes to express potato PI-II in foliage and winged bean PIs in developing seeds so as to counteract *H. armigera* infestation.

Another new approach of expression of transgenes via chloroplast transformation has been advocated (Daniell 1999, Kota et al 1999). Genetic engineering through chloroplast genome presents several advantages like: (1) extremely high level of expression of the transgenic protein in foliage, (2) absence of position effect, an artefact of nuclear transformation, (3) compartmentalization of the transgene within chloroplast, (4) prevention of 'gene pollution' among other non-transgenic plants (via pollen) as chloroplasts are maternally inherited and (5) tissue-specific expression in foliage with absence of expression in fruits and pollen which will ensure the containment of harmful effects on beneficial insects like pollinators. The expression of PIs in foliage via chloroplast

transformation will lead to effective resistance against the lower instars of *H. armigera* which are foliar feeders which in turn will protect the crop from damage.

6.4 Possible adverse effects of the transgenic use of PIs for insect control

Although PIs represent the plants' own defense response and do not put high selection pressure on insects, there are a few reports about their adverse effects on non-target insects such as beneficial insects and predators. For example, Malone et al (1998) studied the effects of several serine PIs (mixed with nectar) on honey bees and found that high concentration of PIs affected the gut proteolytic activity and survival of bees; however, low doses did not affect growth. Walker et al (1998) studied effects of cysteine PIs on ladybirds (predators of sucking pests aphids) and found that ingestion inhibited the gut proteolytic activity; however, effects on growth were not studied. In a significant result, Ashouri et al (1998) showed the adverse effects of oryzacystatin I on the fitness and survival of the stinkbug which is a predator of the important pest Colorado potato beetle. Considering these results, many such studies need to be done in view of the potential transgenic use of PIs for insect resistance.

In nature, plants have been known to contain very high levels of (serine) PIs which do not inhibit the endogenous proteinases or affect the physiology of the plant. Nevertheless, the possible effects of the transgenically expressed PIs on plant physiology should be considered.

In addition it is essential to conduct toxicity trials of PIs which are expressed in tissues meant for human consumption (e.g. chickpea seeds). Ideally PIs which get degraded on germination and cooking should be used so as to eliminate their anti-nutritional property.

In summary, there is an urgent need to study and use plants' natural defense mechanisms like PIs to control the problem of insect infestation. The success of transgenic PI-based strategies depends upon (i) identification of potent inhibitors of insect gut proteinases, (ii) detailed *in vitro* and *in vivo* studies to check their efficacy to inhibit larval growth, (iii) study of the response of the insect to the ingested PIs, (iv) use of PIs in combinations and more importantly, (v) differential tissue-specific high-level expression targeting exposure of the insect to two different PIs in succession giving it a minimal chance to overcome the effect of PIs. Devising a strategy having such merits will retard insect growth, delay generation advance and arrest the exponential growth of insect population in the field thus minimizing the damage to crop yields.

Summary

Chickpea is the most important legume in India providing high quality protein in a predominantly vegetarian diet. Productivity of chickpea is severely restricted due to the two fungal diseases namely wilt and blight caused by *Fusarium oxysporum* and *Ascochyta rabei*, respectively. However, the polyphagous insect pest *Helicoverpa armigera* (Hübner) represents the most important biotic stress to chickpea. It feeds on foliage, flowers and particularly on developing seeds leading to severe losses in crop yields. Incorporating genes of proteinase inhibitors (PIs) for resistance against insect pests is a promising strategy as the PIs represent the plants' own defense (Ryan 1990). In addition, use of PIs does not lead to high selection pressure as compared to the 'wipe out' approach executed by other pest control measures including chemical pesticides and Bt toxin thus minimizing the possibility of developing resistance in insect population against PIs.

In my laboratory, research on PIs was initiated to explore their potential in conferring resistance to chickpea against *H. armigera*. Earlier work to study the chickpea-*H. armigera* interactions showed that although chickpea produced differentially expressed trypsin inhibitors (TIs) in developing seeds, they were degraded by *H. armigera* gut proteinases (HGP). I was fascinated by these results and decided to carry out these studies further. Essentially I decided to study the potentially effective PIs in *Cicer* germplasm and other non-host plants and also the complement of gut proteinases in *H. armigera*. The main findings of my thesis are summarized below:

Diversity of proteinase inhibitors in *Cicer* species and in moisture stress-induced chickpea at various stages of seed development

- ***TI / HGPI content in developing seeds of chickpea:*** Developing seeds of eight chickpea cultivars (12 to 60 d after flowering) showed a significant variation in TI and HGPI content. The electrophoretic patterns showed variation in TI bands during early stages of seed development.
- ***Localization of TIs in seed organs:*** TI and HGPI activities were highly localized in embryo-axis as compared to cotyledons in immature and mature seeds of chickpea.
- ***Effect of moisture stress:*** Moisture stress, as effected under rainfed conditions, resulted in reduced PI levels in the chickpea cultivars.
- ***PIs in wild relatives of chickpea:*** Wild relatives of chickpea revealed variability in terms of number and intensity of TI bands. However, none of the wild *Cicer* species showed more than 35% inhibition of HGP suggesting that a large proportion of HGP was insensitive to PIs from *Cicer.*

These results represent the biochemical basis for adaptation of *H. armigera* to PIs of *Cicer* species.

Complex nature of *Helicoverpa armigera* gut proteinases is responsible for its adaptation to chickpea and other host plants

- *HGP activity of larvae fed on chickpea and other host plants:* ***H. armigera* fed on chickpea showed more than 2.5 to 3-fold gut proteinase activity than those fed on the other host plants. HGP**

composition revealed the predominance of serine proteinase activity, however, the larvae fed on pigeonpea revealed presence of metalloproteinases and low levels of aspartic and cysteine proteinases as well.

- *HGP activity during larval development:* **Gut proteinase activity increased during larval development with the highest activity seen in the fifth instar larvae which, however, declined sharply in the sixth instar. Over 90% of HGP activity of the fifth instar larvae was of the serine proteinase type, however, the second instar larvae showed the presence of proteinases of other mechanistic classes as evident by inhibition studies.**
- *Analysis of fecal matter of larvae fed on different diets:* **Analysis of fecal matter of larvae showed a significant increase in proteinase activity when fed on artificial diet than larvae fed on natural diet.**

Successive use of non-host plant proteinase inhibitors offers a potential solution for effective inhibition of *Helicoverpa armigera*

- ***Dichotomy of TIs and HGPIs in host and non-host plants:*** TI activity bands were detected in all the host and non-host plants but HGPI activity bands were present only in non-host plants excepting cotton in the host plant group.
- ***Non-host plant PIs are effective inhibitors of HGP and larval growth:*** Enzyme assays and electrophoretic analysis of interaction of HGP with PIs revealed that non-host PIs inhibited HGP activity efficiently whereas host PIs were ineffective. *H. armigera* larvae reared

on a diet containing non-host PIs showed growth retardation, reduction in total and trypsin-like proteinase activity and production of inhibitor-insensitive proteinases.

- ***Alteration of gut proteinase activity in response to ingested PIs:*** Electrophoretic analysis of PI-induced HGP showed differential regulation of proteinase isoforms. Interestingly, HGP activity induced in response to dietary potato PI-II was inhibited by winged bean PIs.

Based on these results, strategy involving optimized combination of potato PI-II and winged bean PIs identified in the present study and their successive use has been proposed which has a significant potential in developing *H. armigera*-resistant transgenic plants.

List of publications

1. **Patankar AG**, Harsulkar AM, Giri AP, Gupta VS, Sainani MN, Ranjekar PK, Deshpande VV, (1999). Diversity in inhibitors of trypsin and *Helicoverpa armigera* gut proteinases in chickpea (*Cicer arietinum*) and its wild relatives. *Theoretical and Applied Genetics* **99:** 719-726

2. Harsulkar AM, Giri AP, **Patankar AG**, Gupta VS, Sainani MN, Ranjekar PK, Deshpande VV (1999) Successive use of non-host plant proteinase inhibitors required for effective inhibition of *Helicoverpa armigera* gut proteinases and larval growth. *Plant Physiology* **121:** 497-504

3. Harsulkar AM, Giri AP, Gupta VS, Sainani MN, Deshpande VV, **Patankar AG**, Ranjekar PK (1998) Characterization of *Helicoverpa armigera* gut proteinases and the interaction with proteinase inhibitors using gel X-ray film contact print technique. *Electrophoresis* **19:** 1397-1402

4. Sant VJ, **Patankar AG**, Gupta VS, Sarode ND, Mhase CB, Sainani MN, Deshmukh RB, Ranjekar P K (1999) Potential of DNA markers in detecting divergence and in analyzing heterosis in Indian elite chickpea cultivars. *Theoretical and Applied Genetics* **98:** 1217-1225

5. Giri AP, Harsulkar AM, **Patankar AG**, Gupta VS, Sainani MN, Deshpande VV, Ranjekar PK (1998) Association of induction of protease and chitinase in chickpea roots with resistance to *Fusarium oxysporum* f. sp. *ciceri*. *Plant Pathology* **47:** 693-699

6. **Patankar AG**, Giri AP, Harsulkar AM, Sainani MN, Deshpande VV, Ranjekar PK, Gupta VS, 2000. Complexity in specificities and expression of *Helicoverpa armigera* gut proteinases explains polyphagous nature of the insect pest. Revised and communicated to *Insect Biochemistry and Molecular Biology*

References

Alstad DN, Andow DA, 1995. Managing the co-evolution of insect resistance to transgenic plants. Science 268: 1394-1396

Ambekar SS, Patil SC, Giri AP, Kachole MS, 1996. Proteinaceous inhibitors of trypsin and amylase in developing and germinating seeds of pigeonpea (*Cajanus cajan* L. Millsp). J Sci Food Agric 72: 57-62

Anon 1993. Annu Report of Research Work on Pulses 1992-93 (Submitted to the Research Review Committee, Department of Entomology, Mahatma Phule Krishi Vidyapeeth, Rahuri, India)

Anon 1994. Annu Report of Research Work on Pulses 1993-94 (Submitted to the Research Review Committee, Department of Entomology, Mahatma Phule Krishi Vidyapeeth, Rahuri, India)

Anon 1995. Annu Report of Research Work on Pulses 1994-95 (Submitted to the Research Review Committee, Department of Entomology, Mahatma Phule Krishi Vidyapeeth, Rahuri, India)

Armes NJ, Jadhav DR, DeSouza KR, 1996. A survey of insecticide resistance in *Helicoverpa armigera* in the Indian subcontinent. Bulletin Entomol Res 86: 499-514

Ashouri A, Overney S, Michaud D, Cloutier C, 1998. Fitness and feeding are affected in the two-spotted stinkbug, *Perillus bioculatus*, by the cysteine proteinase inhibitor, oryzacystatin I. Arch Insect Biochem Physiol 38: 74-83

Barton KA, Whiteley HR, Yang NS, 1987. *Bacillus thuringiensis* delta-endotoxin expressed in transgenic *Nicotiana tabacum* provides resistance to lepidopteran insects. Plant Physiol 85: 1103-1109

Belew M, Eaker D, 1976. The trypsin and chymotrypsin inhibitors in chickpea (*Cicer arietinum* L.). Identification of the trypsin reactive site, partial amino acid sequence and further physico-chemical properties of the major inhibitor. Eur J Biochem 62: 499-508

Belew M, Porath J, 1970. Extracellular proteinase from *Penicillium notatum* In Methods in Enzymology. vol. 19 (G.E. Perlmann and L. Lorand, eds), pp. 576-581

Belew M, Porath J, Sundberg L, 1975. The trypsin and chymotrypsin inhibitors in chickpeas (*Cicer arietinum* L.). Purification and properties of the inhibitors. Eur J Biochem 60: 247-258

Bolter CJ, 1993. Methyl jasmonate induces papain inhibitors in tomato leaves. Plant Physiol 103: 1347-1353

Bolter CJ, Jongsma MA, 1995. Colorado potato beetles (*Leptinotarsa decemlineata*) adapt to proteinase inhibitors induced in potato leaves by methyl jasmonate. J Insect Physiol 41: 1071-1078

Bolter CJ, Latoszek-Green M, 1997. Effect of chronic ingestion of the cysteine proteinase inhibitor, E-64, on Colorado potato beetle gut proteinases. Entomol Experi Appli 82: 295-303

Bonade-Bottino M, Lerin J, Zaccomer B, Jouanin L, 1999. Physiological adaptation explains the insensitivity of *Baris coerulescens* to transgenic oilseed rape expressing oryzacystatin I. Insect Biochem Mol Biol 29: 131-138

Borchers R, Ackerson CW, 1947. Arch Biochem 13: 291-293

Botella MA, Xu Y, Prabha TN, Zhao Y, Narasimhan ML, Wilson KA, Nielsen SS, Bressan RA, Hasegawa PM, 1996. Differential expression of soybean cysteine proteinase inhibitor genes during development and in response to wounding and methyl jasmonate. Plant Physiol 112: 1201-1210

Boulter D, 1993. Insect pest control by copying nature using genetically engineered crops. Phytochem 34: 1453-1466

Boulter D, Edwards GA, Gatehouse AMR, Gatehouse JA, Hilder VA, 1990. Additive protective effects of different plant-derived insect resistance genes in transgenic tobacco plants. Crop Protect 9: 351-354

Bown DP, Wilkinson HS, Gatehouse JA, 1997. Differentially regulated inhibitor-sensitive and -insensitive protease genes from the phytophagous insect pest, *Helicoverpa armigera*, are members of complex multigene families. Insect Biochem Mol Biol 27: 625-638

Bown DP, Wilkinson HS, Gatehouse JA, 1998. Midgut carboxypeptidase from *Helicoverpa armigera* (Lepidoptera: Noctuidae) larvae: enzyme characterization, cDNA cloning and expression. Insect Biochem Mol Biol 28: 739-749.

Bradford MM, 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254

Brar DS, Khush GS, 1997. Alien introgression in rice. Plant Mol Biol 35: 35-47

Broadway RM, 1995. Are insects resistant to plant proteinase inhibitors? J Insect Physiol 41: 107-116

Broadway RM, 1996. Dietary proteinase inhibitors alter complement of midgut proteases. Arch Insect Biochem Physiol 32: 39-53

Broadway RM, 1997. Dietary regulation of serine proteinases that are resistant to serine proteinase inhibitors. J Insect Physiol 43: 855-874

Broadway RM, Duffey SS, 1986a. The effect of dietary protein on the growth and digestive physiology of larval *Heliothis zea* and *Spodoptera exigua*. J Insect Physiol 32: 673-680

Broadway RM, Duffey SS, 1986b. Plant proteinase inhibitors: mechanism of action and effect on the growth and digestive physiology of larval *Heliothis zea* and *Spodoptera exigua*. J Insect Physiol 32: 827-833

Broadway RM, Duffey SS, 1988. The effect of plant protein quality on insect digestive physiology and toxicity of plant proteinase inhibitors. J Insect Physiol 34: 1111-1117

Broadway RM, Villani MG, 1995. Does host range influence susceptibility of herbivorous insects to non-host plant proteinase inhibitors? Entomol Experi Appli 76: 303-312.

Brock RM, Forsberg CW, Buchanan-Smith JG, 1982. Proteolytic activity of rumen microorganisms and effect of proteinase inhibitors. Appl Environ Microbiol 44: 561-569

Brousseau R, Masson L, Hegedus D, 1999. Insecticidal transgenic plants: are they irresistible? Review. AgBiotechNet July, ABN022

Brzin J, Kidric M, 1995. Proteinases and their inhibitors in plants: Role in normal growth and in response to various stress conditions. Biotech Genet Engineer Rev 13: 421-467

Burgess EPJ, Main CA, Stevens PS, Christeller JT, Gatehouse AMR, Laing WA, 1994. Effects of protease inhibitor concentration and combinations on the survival, growth and gut enzyme activities of the black field cricket, *Telleogryllus commodus*. J Insect Physiol 40: 803-811

Casaretto JA, Corcuera LJ, 1995. Plant proteinase inhibitors: A defensive response against insects (Review). Biol Res 28: 239-249

Casaretto JA, Corcuera LJ, 1998. Proteinase inhibitor accumulation in aphid-infested barley leaves. Phytochem 49: 2279-2286

Charity JA, Anderson MA, Bittisnich DJ, Whitecross M, Higgins TJV, 1999. Transgenic tobacco and peas expressing a proteinase inhibitor from *Nicotiana alata* have increased insect resistance. Mol Breed 5: 357-365

Christeller JT, Shaw BD, 1989. The interaction of a range of serine proteinase inhibitors with bovine trypsin and *Costelytra zealandica* trypsin. Insect Biochem 19: 233-241

Christeller JT, Shaw BD, Gardiner SE, Dymock J, 1989. Partial purification and chracterization of the major midgut proteases of grass grub larvae (*Costelytra zealandica*, Coleoptera: Scarabaeidae). Insect Biochem 19: 221-231

Christeller JT, Laing WA, Markwick NP, Burgess EPJ, 1992. Midgut protease activities in 12 phytophagous lepidopteran larvae: dietary and protease inhibitor interactions. Insect Biochem Mol Biol 22: 735-746.

Cloutier C, Fournier M, Jean C, Yelle S, Michaud D, 1999. Growth compensation and faster development of Colorado potato beetle (Coleoptera: Chrysomelidae) feeding on potato foliage expressing oryzacystatin I. Arch Insect Biochem Physiol 40: 69-79.

Cloutier C, Jean C, Fournier M, Yelle S, Michaud D, 2000. Adult Colorado potato beetles, *Leptinotarsa decemlineata* compensate for nutritional stress on oryzacystatin I-transgenic potato plants by hypertrophic behaviour and overproduction of insensitive proteases. Arch Insect Biochem Physiol 44: 69-81

Conconi A, Smerdon MJ, Howe GA, Ryan CA, 1996. The octadecanoid signaling pathway in plants mediates a response to ultraviolet radiation. Nature 383: 826-829

Confalonieri M, Allegro G, Balestrazzi A, Fogher C, Delledonne M, 1998. Regeneration of *Populus nigra* transgenic plants expressing a Kunitz proteinase inhibitor (*Kti*$_3$) gene. Mol Breed 4: 137-145

Cordero MJ, Raventos D, sanSegundo B, 1994. Expression of a maize proteinase inhibitor gene is induced in response to wounding and fungal infection: systemic wound response of a monocot gene. Plant J 6: 141-150

Daniell H, 1999. The next generation of genetically engineered crops for herbicide and insect resistance: containment of gene pollution and resistant insects. Review. AgBiotechNet August ABN024

Davis BJ, 1964. Disc electrophoresis II: methods and application to human serum. Ann NY Acad Sci 121: 404-429

DeLeo F, Bonade-Bottino MA, Ceci LR, Gallerani R, Jouanin L, 1998. Opposite effects on *Spodoptera littoralis* larvae of high expression level of a trypsin proteinase inhibitor in transgenic plants. Plant Physiol 118: 997-1004

Denolf P, Hendrickx K, Van Damme J, Jansens S, Peferoen M, Degheele D, Van Rie J, 1997. Cloning and characterization of *Manduca sexta* and *Plutella xylostella* midgut aminopeptidase N enzymes related to *Bacillus thuringiensis* toxin-binding proteins. Eur J Biochem 248: 748-761.

Doares SH, Syrovets T, Weiler EW, Ryan CA, 1995. Oligogalacturonides and chitosan activate plant defensive genes through the octadecanoid pathway. Proc Natl Acad Sci USA 92: 4095-4098

Domoney C, Welham T, Sidebottom C, Firmin JL, 1995. Multiple isoforms of *Pisum* trypsin inhibitors results from modification of two primary gene products. FEBS Lett 360: 15-20

Duan X, Li X, Xue Q, Abo-el-saad M, Xu D, Wu R, 1996. Transgenic rice plants harbouring an introduced potato inhibitor II gene are insect resistant. Nature Biotechnol 14: 494-498

Duranti M, Gius C, 1997. Legume seeds: protein content and nutritional value. Field Crops Res 53: 31-45

Ehrlich PR, Raven PH, 1964. Butterflies and Plants: A study in evolution. Evolution 18: 586-608

Elzinga RJ, 1978. Fundamentals of Entomology. Prentice Hall of India Pvt Ltd., New Delhi.

Erlanger BF, Kokowesky N, Cohen W, 1964. The preparation and properties of two new chromogenic substrates of trypsin. Arch Biochem Biophys 95: 271-278

Estruch JJ, Carozzi NB, Desai N, Duck NB, Warren GW, Koziel MG, 1997. Transgenic plants: An emerging approach to pest control. Nature Biotechnol 15: 137-141

Farmer EE, Ryan CA, 1990. Interplant communication: airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. Proc Natl Acad Sci USA 87: 7713-7716

Felicioli R, Garzelli B, Vaccari L, Melfi D, Balestreri E, 1997. Activity staining of protein inhibitors of proteases on gelatin containing polyacrylamide gel electrophoresis. Anal Biochem 244:176-178.

Fischhoff DA, Bowdish KS, Perlak FJ, Marrone PG, McCormick SM, Niedermeyer JG, Dean DA, Kusano-Kretzmer K, Mayer EJ, Rochester DE, Rogers SG, Fraley RT, 1987. Insect tolerant transgenic tomato plants. Bio/Technol 5: 807-813

Foissac X, Loc NT, Christou P, Gatehouse AMR, Gatehouse JA, 2000. Resistance to green leafhopper (*Nephotettix virescens*) and brown planthopper (*Nilaparvata lugens*) in transgenic rice expressing snowdrop lectin *Galanthus nivalis* agglutinin; (GNA). J Insect Physiol 46: 573-583

Forcada C, Alcacer E, Garcera MD, Martinez R, 1996. Differences in the midgut proteolytic activity of two *Heliothis virescens* strains, one susceptible and one resistant to *Bacillus thuringiensis* toxins. Arch Insect Biochem Physiol 31: 257-272

Frutos R, Rang C, Royer M, 1999. Managing insect resistance to plants producing *Bacillus thuringiensis* toxins. Crit Rev Biotechnol 19:227-276

Garcia-Olmedo F, Salcedo G, Sanchez-Monge R, Gomez L, Royo J, Carbenero P, 1987. Plant proteinaceous inhibitors of proteinases and α-amylases. Oxford Surveys Plant Mol Cell Biol 4: 275-334

Gatehouse AMR, Boulter D, 1983. Assessment of the antimetabolic effects of trypsin inhibitors from cowpea (*Vigna unguiculata*) and other legumes on development of the bruchid beetle *Callosobruchus maculatus.* J Sci Food Agri 34: 345-350

Gatehouse AMR, Davison GM, Newell CA, Merryweather A, Hamilton WDO, Burgess EPJ, Gilbert RJC, Gatehouse JA, 1997. Transgenic potato plants with enhanced resistance to the tomato moth *Lacanobia oleracea*: growthroom trials. Mol Breed 3: 49-63

Gatehouse JA, Evans IM, Croy RRD, Boulter D, 1986. Differential expression of genes during legume seed development. Phil Trans R Soc Lond B 314: 367-384

Gatehouse LN, Shannon AL, Burgess EP, Christeller JT, 1997. Characterization of major midgut proteinase cDNAs from *Helicoverpa armigera* larvae and changes in gene expression in reponse to four proteinase inhibitors in the diet. Insect Biochem Mol Biol 27: 929-944

Girard C, Jouanin L, 1999. Molecular cloning of cDNAs encoding a range of digestive enzymes from a phytophagous beetle, *Phaedon cochleariae.* Insect Biochem Mol Biol 29: 1129-1142

Girard C, Le Metayer M, Bonade-Bottino M, Pham-Delegue MH, Jouanin L, 1998a. High level of resistance to proteinase inhibitors may be conferred by proteolytic cleavage in beetle larvae. Insect Biochem Mol Biol 28: 229-237

Girard C, Le Metayer M, Zaccomer B, Bartlet E, Williams I, Bonade-Bottino M, Pham-Delegue MH, Jouanin L, 1998b. Growth stimulation of beetle larvae reared on trangenic oilseed rape expressing a cysteine proteinase inhibitor. J Insect Physiol 44: 263-270.

Girard C, Bonade-Bottino M, Pham-Delague M-H, Jouanin L, 1998c. Two strains of cabbage seed weevil (Coleoptera: Curculionidae) exhibit differential susceptibility to a transgenic oilseed rape expressing oryzacystatin. J Insect Physiol 44: 569-577

Giri AP, Harsulkar AM, Deshpande VV, Sainani MN, Gupta VS, Ranjekar PK, 1998. Chickpea defensive proteinase inhibitors can be inactivated by podborer gut proteinases. Plant Physiol 116: 393-401

Giri AP, Kachole MS, 1998. Amylase inhibitors of pigeonpea (*Cajanus cajan* L.) seeds. Phytochemistry 47: 197-202

Goldberg RB, Barker SJ, Perez-Grau L, 1989. Regulation of plant gene expression during embryogenesis. Cell 56: 149-160

Gould F, 1998. Sustainability of transgenic insecticidal cultivars: Integrating pest genetics and ecology. Annu Rev Entomol 43: 701-726

Gould F, Anderson A, Jones A, Sumerford D, Heckel DG, Lopez J, Micinski S, Leonard RR, Laster M, 1997. Initial frequency of alleles for resistance to *Bacillus thuringiensis* toxins in field populations of *Heliothis virescens*. Proc Natl Acad Sci USA 94: 3519-3523

Graham J, McNicol RJ, Greig K, 1995. Towards genetic based insect resistance in strawberry using the cowpea trypsin inhibitor gene. Ann Appl Biol 127: 163-173

Green TR, Ryan CA, 1972. Wound induced proteinase inhibitor in plant leaves: A possible defence mechanism. Science 175: 776-777.

Gruden K, Strukelj B, Popovic T, Lenarcic B, Bevec T, Brzin J, Kregar I, Herzog-Velikonja J, Stiekema WJ, Bosch D, Jongsma MA, 1998. The cysteine protease activity of Colorado potato beetle (*Leptinotarsa decemlineata* Say) guts, which is insensitive to potato protease inhibitors, is inhibited by thyroglobulin type-I domain inhibitors. Insect Biochem Mol Biol 28: 549-560

Harsulkar AM, Giri AP, Gupta VS, Sainani MN, Deshpande VV, Patankar AG, Ranjekar PK, 1998. Characterization of *Helicoverpa armigera* gut proteinases and their interaction with proteinase inhibitors using gel-X-ray film contact print technique. Electrophoresis 19: 1397-1402

Harsulkar AM, Giri AP, Kothekar VS, 1997. Protease inhibitors of chickpea (*Cicer arietinum* L.) during seed development. J Sci Food Agric 74: 509-512

Hatchett JE, Starks KJ, Webster JA, 1987. Insect and mite pests of wheat. In Wheat and wheat improvement. (Ed. EG Heyne). Am Soc Agron, USA. pp 625-675

Heath RL, McDonald G, Christeller JT, Lee M, Bateman K, West J, van Heeswijck R, Anderson MA, 1997. Proteinase inhibitors from *Nicotiana alata* enhance plant resistance to insect pests. J. Insect Physiol 43: 833-842

Hilder VA, Gatehouse AMR, Boulter D, 1993. Transgenic plants conferring insect tolerance. Proteinase inhibitor approach. In Transgenic plants vol. 1. engineering and utilization. (Eds. SD Kung and R Wu). Academic Press, Inc. California, pp 317-338.

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

Hilder VA, Powell KS, Gatehouse AMR, Gatehouse JA, Gatehouse LN, Shi Y, Hamilton WDO, Merryweather A, Newell CA, Timans JC, 1995. Expression of snowdrop lectin in transgenic tobacco plants results in added protection against aphids. Transgen Res 4: 18-25

Hildmann T, Ebneth M, Pena-Cortes H, Sanchez-Serrano J, Willmitzer L, Prat S, 1992. General roles of abscisic and jasmonic acids in gene activation as a result of mechanical wounding. Plant Cell 4: 1157-1170

Huang F, Buschman LL, Higgins RA, McGaughey WH, 1999. Inheritance of resistance to *Bacillus thuringiensis* toxin (Dipel ES) in the European corn borer. Science 284: 965-967

Ishimoto M, Chrispeels MJ, 1996. Protective mechanism of the Mexican bean weevil against high levels of α-amylase inhibitor in the common bean. Plant Physiol 111: 393-401

Ishimoto M, Sato T, Chrispeels MJ, Kitamura K, 1996. Bruchid resistance of transgenic azuki bean expressing seed α-amylase inhibitor of common bean. Entomol Experi Appli 79: 309-315

Jadhav DR, Armes NJ, 1996. Comparative status of insecticide resistance in the *Helicoverpa* and *Heliothis* species (Lepidoptera: Noctuidae) of South India. Bulletin Entomol Res 86, 525-531

Janzen DH, 1980. When is it coevolution? Evolution 34: 611-612

Johnson R, Narvaez J, An G, Ryan CA, 1989. Expression of proteinase inhibitors I and II in transgenic tobacco plants: effects on natural defense against *Manduca sexta*. Proc Natl Acad Sci USA 86: 9871-9875

Johnston KA, Gatehouse JA, Anstee JH, 1993. Effect of soybean protease inhibitors on the growth and development of larval *Helicoverpa armigera*. J Insect Physiol 39: 657-664

Johnston KA, Lee MJ, Brough C, Hilder VA, Gatehouse AMR, Gatehouse JA, 1995. Protease activities in the larval midgut of *Heliothis virescens*: evidence for trypsin and chymotrypsin-like enzymes. Insect Biochem Mol Biol 25: 375-383

Johnston KA, Lee MJ, Gatehouse JA, Anstee JH, 1991. The partial purification and characterization of serine protease activity in midgut of larval *Helicoverpa armigera*. Insect Biochem 21: 389-397

Jongsma MA, Bakker PL, Peters J, Bosch D, Stiekema WJ, 1995. Adaptation of *Spodoptera exigua* larvae to plant proteinase inhibitors by induction of gut proteinase activity insensitive to inhibition. Proc Natl Acad Sci USA 92: 8041-8045

Jongsma MA, Bakker PL, Visser B, Stiekema WJ, 1994. Trypsin inhibitor activity in mature tobacco and tomato plants is mainly induced locally in response to insect attack, wounding and virus infection. Planta 195: 29-35

Jongsma MA, Bolter CJ, 1997. The adaptation of insects to plant protease inhibitors. J Insect Physiol 43: 885-895

Jongsma MA, Peters J, Stiekema WJ, Bosch D, 1996a. Characterization and partial purification of gut proteinases of *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae). Insect Biochem Mol Biol 26: 185-193

Jongsma MA, Stiekema WJ, Bosch D, 1996b. Combating inhibitor-insensitive proteases of insect pests. Trends Biotechnol 14: 331-333

Jouanin L, Bonade-Bottino M, Girard C, Morrot G, Giband M, 1998. Transgenic plants for insect resistance. Plant Sci 131: 1-11

Kazan K, Muehlbauer FJ, 1991. Allozyme variation and phylogeny in annual species of *Cicer* (Leguminosae). Plant Syst Evol 175: 11-21

Keller M, Sneh B, Strizhov N, Prudovsky E, Regev A, Koncz C, Schell J, Zilberstein A, 1996. Digestion of δ-endotoxin by gut proteases may explain reduced sensitivity of advanced instar larvae of *Spodoptera littoralis* to Cry IC. Insect Biochem Mol Biol 26: 365-373

Khush GS, Brar DS, 1991. Genetics of resistance to insects in crop plants. Adv Agron 45: 223-274

Koiwa H, Bressan RA, Hasegawa PM, 1997. Regulation of protease inhibitors and plant defense. Trends Plant Sci 2: 379-384

Koiwa H, Shade RE, Zhu-Salzman K, Subramanian L, Murdock LL, Nielsen SS, Bressan RA, Hasegawa PM, 1998. Phage display selection can differentiate insecticidal activity of soybean cystatins. Plant J 14: 371-379

Kollipara KP, Singh L, Hymowitz T, 1994. Genetic variation of trypsin and chymotrypsin inhibitors in pigeonpea (*Cajanus cajan* (L.) Millsp) and its wild relatives. Theor Appl Genet 88: 986-993

Korth KL, Dixon RA, 1997. Evidence for chewing insect-specific molecular events distinct from a general wound response in leaves. Plant Physiol 11: 1299-1305

Kota M, Daniell H, Varma S, Garczynski SF, Gould F, Moar WJ, 1999. Overexpression of the *Bacillus thuringiensis* (Bt) Cry2Aa2 protein in chloroplasts confers resistance to plants against susceptible and Bt-resistant insects. Proc Natl Acad Sci USA 96: 1840-1845

Kothekar VS, Harsulkar AM, Khandelwal AR, 1996. Low trypsin and chymotrypsin inhibitor mutants in winged bean (*Psophocarpus tetragonolobus* (L.) D.C.). J Sci Food Agric 71: 137-140

Kramer KJ, Muthukrishnan S, 1997. Insect chitinases: molecular biology and potential use as biopesticides. Insect Biochem Mol Biol 27: 887-900

Ladizinsky G, Adler A, 1976. Genetic relationships among the annual species of *Cicer* L. Theor Appl Genet 48: 197-203

Larocque AM, Houseman JG, 1990. Effect of ingested soybean, ovomucoid and corn protease inhibitors on digestive processes of the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Pyralidae). J Insect Physiol 36: 691-697

Laskowski M Jr, Kato I, 1980. Protein inhibitors of proteinases. Annu Rev Biochem 49: 593-626

Laskowski M Jr, Kato I, Kohr WJ, Park SJ, Tashiro M, Whatley HE, 1988. Positive Darwinian selection in evolution of protein inhibitors of serine proteinases. Cold Spring Harbor Symp Quant Biol 52: 545-553

Lee MJ, Anstee JH, 1995. Characterization of midgut exopeptidase activity from larval *Spodoptera littoralis*. Insect Biochem Mol Biol 25: 63-71

Lee SI, Lee SH, Koo JC, Chun HJ, Lim CO, Mun JH, Song YH, Cho MJ, 1999. Soybean Kunitz trypsin inhibitor (SKTI) confers resistance to the brown planthopper (*Nilaparvata lugens* Stal) in transgenic rice. Mol Breed 5: 1-9

Lenz CJ, Kang J, Rice WC, McIntosh AH, Chippendale GM, Schubert KR, 1991. Digestive proteinases of larvae of the corn earworm, *Heliothis zea*: characterization, distribution and dietary relationships. Arch Insect Biochem Physiol 16: 201-212

Leple JC, Bonade-Bottino M, Augustin S, Pilate G, Dumanois Le Tan V, Delplanque A, Cornu D, Jouanin L, 1995. Toxicity to *Chrysomela tremulae* (Coleoptera: Chrysomelidae) of transgenic poplars expressing a cysteine proteinase inhibitor. Mol Breed 1: 319-328

Lewis WJ, van Lenteren JC, Phatak SC, Tumlinson III JH, 1997. A total system approach to sustainable pest management. Proc Natl Acad Sci USA 94: 12243-12248

Liu YB, Tabashnik BE, 1997. Inheritance of resistance to the *Bacillus thuringiensis* toxin Cry1c in the diamondback moth. Appl Environ Microbiol 63: 2218-2223

Liu YB, Tabashnik BE, Dennehy TJ, Patin AL, Bartlett AC, 1999. Development time and resistance to Bt crops. Nature 400: 519

Losey JE, Raynor LS, Carter ME, 1999. Transgenic pollen harms monarch larvae. Nature 399: 214

MacIntosh SC, Kishore GM, Perlak FJ, Marrone PG, Stone TB, Sims SR, Fuchs RL, 1990. Potentiation of *Bacillus thuringiensis* insecticidal activity by serine proteinase inhibitors. J Agric Food Chem 38: 1145-1152

Malone LA, Burgess EPJ, Christeller JT, Gatehouse HS, 1998. *In vivo* responses of honey bee midgut proteases to two protease inhibitors from potato. J Insect Physiol 44: 141-147

Manjunath TM, Bhatnagar VS, Pawar CS, Sithanantham S, 1989. Economic importance of *Heliothis* spp. in India and an assessment of their natural enemies and host plants. In: Proceedings of the workshop on biological control of *Heliothis*: Increasing the effectiveness of natural enemies. (Eds. King EG and Jackson RD) Far Eastern Regional Research Office, USDA, New Delhi pp. 197-227

Marchetti S, Chiaba C, Chiesa F, Bandiera A, Pitotti A, 1998. Isolation and characterization of two trypsins from the larval midgut of *Spodoptera littoralis* (Boisduval). Insect Biochem Mol Biol 28: 449-458.

Markwick NP, Reid SJ, Laing WA, Christeller JT, 1995. Effects of dietary protein and protease inhibitors on codling moth (Lepidoptera: Tortricidae). J Econ Entomol 88: 33-39

Mazumdar-Leighton S, Babu CR, Bennett J, 2000. Identification of novel serine proteinase gene transcripts in the midguts of two tropical insect pests, *Scirpophaga incertulas* (Wk.) and *Helicoverpa armigera* (Hb.). Insect Biochem Mol Biol 30: 57-68

McManus MT, Burgess EPJ, 1995. Effects of the soybean (Kunitz) trypsin inhibitor on growth and digestive proteases of larvae of *Spodoptera litura*. J Insect Physiol 41: 731-738

McManus MT, White DWR, McGregor PG, 1994. Accumulation of a chymotrypsin inhibitor in transgenic tobacco can affect the growth of insect pests. Transgen Res 3: 50-58

Michaud D, 1997. Avoiding protease-mediated resistance in herbivorous pests. Trends Biotechnol 15: 4-6

Michaud D, Bernier-Vadnais N, Overney S, Yelle S, 1995a. Constitutive expression of digestive cysteine proteinase forms during development of the Colorado potato beetle, *Leptinotarsa decemlineata* Say. (Coleoptera: Chrysomelidae). Insect Biochem Mol Biol 25: 1041-1048

Michaud D, Cantin L, Vrain TC, 1995b. Carboxy-terminal truncation of oryzacystatin II by oryzacystatin-insensitive insect digestive proteinases. Arch Biochem Biophys 322: 469-474

Miller EA, Lee MC, Atkinson AH, Anderson MA, 2000. Identification of a novel four-domain member of the proteinase inhibitor II family from the stigmas of *Nicotiana alata*. Plant Mol Biol 42: 329-333

Milne RE, Pang ASD, Kaplan H, 1995. A protein complex from *Choristoneura fumiferana* gut juice involved in the precipitation of δ-endotoxins from *Bacillus thuringiensis* subsp. *sotto*. Insect Biochem Mol Biol 25: 1101-1114

Mishra S, 1998. Baculoviruses as biopesticides. Curr Sci 75: 1015-1022

Morton RL, Schroeder HE, Bateman KS, Chrispeels MJ, Armstrong E, Higgins TJV, 2000. Bean α-amylase inhibitor 1 in transgenic peas (*Pisum sativum*) provides complete protection from pea weevil (*Bruchus pisorum*) under field conditions. Proc Natl Acad Sci USA 97: 3820-3825

Mueller R, Weder JKP, 1989. Isolation and characterization of two trypsin–chymotrypsin inhibitors from lentil seeds (*Lens culinaris* Medik). J Food Biochem 13: 39-63

Murdock LL, Brookhart G, Dunn PE, Foard DE, Kelley S, Kitch L, Shukle RH, Wolfson JL, 1987. Cysteine digestive proteinases in Coleoptera. Comp Biochem Physiol 87B: 783-787

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

Novillo C, Castanera P, Ortego F, 1997. Inhibition of digestive trypsin-like proteases from larvae of several lepidopteran species by the diagnostic cysteine proteinase inhibitor E-64. Insect Biochem Mol Biol 27: 247-254.

Oppert B, Kramer KJ, Beeman RW, Johnson D, McGaughey WH, 1997. Proteinase-mediated insect resistance to *Bacillus thuringiensis* toxins. J Biol Chem 272: 23473-23476

Oppert B, Kramer KJ, Johnson D, Upton SJ, McGaughey WH, 1996. Luminal proteinases from *Plodia interpunctella* and the hydrolysis of *Bacillus thuringiensis* CryIA(c) protoxin. Insect Biochem Mol Biol 26: 571-583

Oppert B, Morgan TD, Culbertson C, Kramer KJ, 1993. Dietary mixtures of cysteine and serine proteinase inhibitors exhibit synergistic toxicity toward the red flour beetle *Tribolium castaneum*. Comp Biochem Physiol 105B, 379-385.

Orr GL, Strickland JA, Walsh TA, 1994. Inhibition of *Diabrotica* larval growth by a multicystatin from potato tubers. J Insect Physiol 40: 893-900

Overney S, Fawe A, Yelle S, Michaud D, 1997. Diet-related plasticity of the digestive proteolytic system in larvae of the Colorado potato beetle (*Leptinotarsa decemlineata* Say). Arch Insect Biochem Physiol 36: 241-250.

Panda N, Khush GS, 1995. Host plant resistance to insects. Oxon, London. IRRI-CAB Intl.

Pare PW, Tumlinson JH, 1999. Plant volatiles as a defense against insect herbivores. Plant Physiol 121: 325-331

Pautot V, Holzer FM, Walling LL, 1991. Differential expression of tomato proteinase inhibitor I and II genes during bacterial pathogen invasion and wounding. Mol Plant Microbe Interact 4: 284-292

Pearce G, Strydom D, Johnson H, Ryan CA, 1991. A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins. Science 253: 895-898

Peña-Cortés H, Fisahn J, Willmitzer L, 1995. Signals involved in wound-induced proteinase inhibitor II gene expression in potato and tomato plants. Proc Natl Acad Sci USA 92: 4106-4113

Peña-Cortés H, Sanchez-Serrano JJ, Mertens R, Willmitzer L, Prat S, 1989. Abscisic acid is involved in the wound-induced expression of the proteinase inhibitor II gene in potato and tomato. Proc Natl Acad Sci USA 86: 9851-9855

Perlak FJ, Deaton WR, Armstrong TA, Fuchs RI, Sims SR, Greenplate JT, Fischhoff DA, 1990. Insect resistant cotton plants. Bio/Technol 8: 939-943

Perlak FJ, Fuchs RL, Dean DA, McPherson SL, Fischhoff DA, 1991. Modification of the coding sequence enhances plant expression of insect control protein genes. Proc Natl Acad Sci USA 88: 3324-3328

Peterson AM, Barillas-Mury CV, Wells MA, 1994. Sequence of three cDNAs encoding an alkaline midgut trypsin from *Manduca sexta*. Insect Biochem Mol Biol 24: 463-471

Peterson AM, Fernando GJP, Wells MA, 1995. Purification, characterization and cDNA sequence of an alkaline chymotrypsin from the midgut of *Manduca sexta*. Insect Biochem Mol Biol 25: 765-774.

Pichare MM, Kachole MS, 1994. Detection of electrophoretically separated protease inhibitors using X-ray film. J Biochem Biophy Meth 28: 215-224

Pichare MM, Kachole MS, 1996. Protease inhibitors of pigeon-pea (*Cajanus cajan*) and its wild relatives. Physiol Planta 98: 845-851

Purchell JP, Greenplate JT, Simmons RD, 1992. Examination of midgut luminal proteinase activities in six economically important insects. Insect Biochem Mol Biol 22: 41-47

Raina AK, 1997. Development of biorational strategies for the management of *Helicoverpa* spp pest complex. In Proc Intl Conf Ecol Agri: Towards Sust Devt Vol 2 (Eds. Dhaliwal GS, Randhawa NS, Arora R, Dhawan AK) Chandigarh, India pp 81-92

Romeis J, Shanower TG, 1996. Arthropod natural enemies of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) in India. Bioc Sci Technol 6: 481-508

Ryan CA, 1990. Protease inhibitors in plants: Genes for improving defenses against insect and pathogens. Annu Rev Phytopathol 28: 425-449

Sastry MCS, Murray DR, 1987. The contribution of trypsin inhibitors to the nutritional value of chickpea seed protein. J Sci Food Agric 40: 253-261

Saxena D, Flores S, Stotzky G, 1999. Transgenic plants: Insecticidal toxin in root exudates from Bt corn. Nature 402: 480

Saxena MC, Singh KB, 1987. The chickpea. CAB International, Wallingford, UK.

Schaller A, Ryan CA, 1995. Systemi n-a polypeptide defense signal in plants. BioEssays 18: 27-33

Schnepf HE, Whiteley HR, 1981. Cloning and expression of the *Bacillus thuringiensis* crystal protein gene in *Escherichia coli*. Proc Natl Acad Sci USA 78: 2893-2897

Schuler TH, Poppy GM, Kerry BR, Denholm I, 1998. Insect-resistant transgenic plants. Trends Biotechnol 16: 168-175

Shade RE, Schroeder HE, Pueyo JJ, Tabe LM, Murdock LL, Higgins TJV, Chrispeels MJ, 1994. Transgenic pea seeds expressing α-amylase inhibitor of the common bean are resistant to bruchid beetles. Bio/Technol 12: 793-796

Singh KB, 1997. Chickpea (*Cicer arietinum* L). Field Crops Res 53: 161-170

Smirnoff P, Khalef S, Birk Y, Applebaum SW, 1976. A trypsin and chymotrypsin inhibitor from chickpeas (*Cicer arietinum*). Biochem J 157: 745-751

Smirnoff P, Khalef S, Birk Y, Applebaum SW, 1979. Trypsin and chymotrypsin inhibitor from chickpeas-selective chemical modification of the inhibitor and isolation of two isoinhibitors. Intl J Peptide Protein Res 14: 186-192

Sohonie K, Bhandarkar AP, 1954. J Sci Ind Res (India) 13B: 500-503

Soon LG, 1997. Integrated pest management in developing Asia. In Proc Intl Conf Ecol Agri: Towards Sust Devt (Eds. Dhaliwal GS, Randhawa NS, Arora R, Dhawan AK) Chandigarh, India pp 3-16

Stankovic B, Davies E, 1997. Intercellular communication in plants: electrical stimulation of proteinase inhibitor gene expression in tomato. Planta 202: 402-406

Steffens R, Fox FR, Kassel B, 1978. Effect of trypsin inhibitors on growth ad metamorphosis of corn borer larvae *Ostrinia nubilalis* (Hübner). J Agric Food Chem 26: 170-174

Subbarao GV, Johansen C, Slinkard AE, Nagenswar Rao RC, Saxena NP, Chauhan YS 1995. Strategies for improving drought resistance in grain legumes. Crit Rev Plant Sci 14: 469-523

Tamayo MC, Rufat M, Bravo JM, San Segundo B, 2000. Accumulation of a maize proteinase inhibitor in response to wounding and insect feeding, and characterization of its activity towards digestive proteinases of *Spodoptera littoralis* larvae. Planta 211: 62-71

Tayyar RI, Waines JG, 1995. Genetic relationships among annual species of *Cicer* (Fabaceae) using isozyme variation. Theor Appl Genet 92: 245-254

Terra WR, Ferriera C, 1994. Insect digestive enzymes: properties, compartmentalization and function. Comp Biochem Physiol 109B: 1-62

Thomas MB, 1999. Ecological approches and the development of 'truly integrated' pest management. Proc Natl Acad Sci USA 96: 5944-5951

Urwin PE, Atkinson HJ, Waller DA, McPherson MJ, 1995. Engineered oryzacystatin I expressed in transgenic hairy roots confers resistance to *Globodera pallida*. Plant J 8: 121-131

Vaeck M, Reynaerts A, Hofte H, Jansens S, DeBeukeleer M, Dean C, Zabeau M, Van Montagu M, Leemans J, 1987. Transgenic plants protected from insect attack. Nature 327: 33-37

Vain P, Worland B, Clarke MC, Richard G, Beavis M, Liu H, Kohli A, Leech M, Snape J, Christou P, Atkinson H, 1998. Expression of an engineered cysteine proteinase inhibitor (Oryzacystatin IΔD86) for nematode resistance in transgenic plants. Theor Appl Genet 96: 266-271

Valaitis AP, 1995. Gypsy moth midgut proteinases: Purification and characterization of luminal trypsin, elastase and the brush border membrane leucine aminopeptidase. Insect Biochem Mol Biol 25: 139-149

Valaitis AP, Augustin S, Clancy KM, 1999. Purification and characterization of the western spruce budworm larval midgut proteinases and comparison of gut activities of laboratory-reared and field-collected insects. Insect Biochem Mol Biol 29: 405-415

Wadman M, 1999. US groups sue over approval of Bt crops. Nature 397: 636

Walker AJ, Ford L, Majerus MEN, Geoghegan IE, Birch N, Gatehouse JA, Gatehouse AMR, 1998. Characterization of the mid-gut digestive proteinase activity of the two-spot ladybird (*Adalia bipunctata* L.) and its sensitivity to proteinase inhibitors. Insect Biochem Mol Biol 28: 173-180

Walker AJ, Urwin PE, Atkinson HJ, Brain P, Glen DM, Shewry PR, 1999. Transgenic Arabidopsis leaf tissue expressing a modified oryzacystatin shows resistance to the field slug *Deroceras reticulatum* (Muller). Transgen Res 8: 95-103

Welham T, O' Neill M, Johnson S, Wang TL, Domoney CW, 1998. Expression patterns of genes encoding seed trypsin inhibitors in *Pisum sativum*. Plant Sci 131: 13-24

Whitworth ST, Blum MS, Travis J, 1998. Proteolytic enzymes from larvae of the fire ant, *Solenopsis invicta*: isolation and characterization of four serine proteinases. J Biol Chem 273: 14430-14434

Whitworth ST, Kordula T, Travis J, 1999. Molecular cloning of Soli E2: an elastase-like serine proteinase from the imported fire ant (*Solenopsis invicta*). Insect Biochem Mol Biol 29: 249-254

Wildon DC, Thain JF, Minchin PEH, Gub IR, Reily AJ, Skipper YD, Doherty HM, O' Donnell PJ, Bowles DJ, 1992. Electrical signaling and systemic proteinase inhibitor induction in the wounded plant. Nature 360: 62-65

Wu Y, Llewellyn D, Mathews A, Dennis ES, 1997. Adaptation of *Helicoverpa armigera* (Lepidoptera: Noctuidae) to a proteinase inhibitor expressed in transgenic tobacco. Mol Breed 3: 371-380

Xu D, Xue Q, McElroy D, Mawal Y, Hilder VA, Wu R, 1996. Constitutive expression of a cowpea trypsin inhibitor gene, *CpTi*, in transgenic rice plants confers resistance to two major rice insect pests. Mol Breed 2: 167-173

Zhang JH, Wang CZ, Qin JD, 2000. The interactions between soybean trypsin inhibitor and delta-endotoxin of *Bacillus thuringiensis* in *Helicoverpa armigera* larvae. Invertebr Pathol 75: 259-266

Zhao X-F, Wang J-X, Wang Y-C, 1998. Purification and characterization of a cysteine proteinase from eggs of the cotton bollworm, *Helicoverpa armigera*. Insect Biochem Mol Biol 28: 259-264

Zhao Y, Botella MA, Subramanian L, Niu X, Nielsen SS, Bressan RA, Hasegawa PM, 1996. Two wound-inducible soybean cysteine proteinase inhibitors have greater insect digestive proteinase inhibitory activities than a constitutive homolog. Plant Physiol 111: 1299-1306

Zhu Y-C, Baker JE, 2000. Molecular cloning and characterization of a midgut chymotrypsin-like enzyme from the lesser grain borer, *Rhyzopertha dominica*. Arch Insect Biochem Physiol 43: 173-184