International Journal of Entomology Research
www.entomologyjournals.com
ISSN: 2455-4758
Received: 13-03-2024, Accepted: 05-04-2024, Published: 27-04-2024
Volume 9, Issue 4, 2024, Page No. 162-167

Plant protease inhibitors for the control of agricultural pests and its potential for Mosquito control: A review

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Abstract
Mosquito borne diseases are increasing at an alarming rate all around the world. The extensive use of chemical pesticides affects many non-target organisms eventually altering the natural ecological balance. The continuous use of synthetic pesticides also results in pest resistance and health hazards. Thus, it is inevitable to develop better eco-friendly pest control methods. The idea of using Plant Protease Inhibitors (PPIs) as an alternative method of pest control gained momentum recently. Plant protease inhibitors are one of the natural defense strategies of plants and augmenting the same is proved to be effective against insect pests. But the potential application of PPIs for the control of mosquito larvae is not exploited to a great extent. Pests are known to resist the effects of PPIs by different mechanisms such as production of proteases insensitive to PPIs or over expression of existing proteases that are sensitive to PPIs. In this review we discuss about the gut protease of insects, including the larval gut proteases of mosquitoes, the impact of plant protease inhibitors on insect pests and potential use of PPIs in mosquito control. Also, the recent methods emerging for improving the toxicity of PPIs are discussed.

Keywords: Plant protease inhibitors, insect pests, mosquito control, protease inhibitors

Introduction

Insect midgut houses several enzymes including proteases, which plays an important role in digestion. The gut proteases liberate crucial amino acids needed for the insect growth and development by hydrolyzing the peptide bond in the proteins obtained from its feed. When the natural process of digestion is hampered by any means, the insect undergoes malnutrition, leading to stunted growth. One of the natural agents for controlling insect population by targeting gut proteases are Plant Protease Inhibitors (PPIs) (Napoleão *et al*. 2019) [17]. PPIs are produced by the plants as deterrents of phytophagous insects. Understanding more about the protease which dominates the midgut of the target insect and directing a suitable protease inhibitor might be an efficient way of controlling insect population.

There has been great interest in botanical pesticides like plant extracts, oils, protease inhibitors etc., in recent years. Most of the biopesticides are comparatively safer as it is less toxic and degrade faster, thereby reducing the risk of accumulating pesticidal residue in food sources. This makes them a suitable candidate for integrated pest management programs. Thus, biopesticides seems to be a promising candidate in pest management, while posing less threat to human beings and environment.

The ability of mosquitoes to carry and spread diseases to humans affects millions of lives every year. The causative agents of malaria, dengue and filariasis are spread by mosquito genera *Anopheles, Aedes, and Culex* respectively. They also spread a variety of zoonotic arbovirus such as Yellow Fever Virus (YFV), West Nile Virus, and Japanese Encephalitis Virus, Dengue Virus (DENV), and Chikungunya Virus (CHIKV) (Huang *et al*. 2019) [11]. *Aedes aegypti* mosquitoes transmit diseases like Dengue, Chikungunya, Zika and Yellow fever. There is no doubt that Dengue is definitely a major public health concern in the tropical and sub-tropical countries, as it is one of the fastest spreading mosquito-borne disease with about 100-400 million cases per year. To prevent the transmission of mosquito borne diseases and to maintain a healthy life-style, mosquito control is essential.

Vector control against mosquitoes has emerged by the first quarter of 20th century. The strategy was simple, focusing on source reduction, use of larvivorous fish, petroleum oils, and some botanical materials. The mosquito fish, *Gambusia affinis*, a native of South Eastern United States, was primarily used as a biological agent for mosquito control. But, the end of 20th century marked the application synthetic pesticides. The advent of synthetic pesticides like DDT, have brought new definitions to insect control. The use of DDT to eradicate malaria was highly successful. Along with DDT, other synthetic compounds like organochlorine insecticides, pyrethroids, and organophosphates were also used for pest control (Aktar *et al*. 2009) [1].

However, the extensive use of synthetic pesticides has increased pest resistance. Knockdown resistance is the most common type of resistance towards DDT and pyrethroids, which was first identified in houseflies. Target-site insensitivity and metabolic resistance confers resistance to pest insects towards DDT and pyrethroids (Riveron *et al*. 2014) [24]. Studies have also revealed that the use of synthetic pesticides leads to local extinction of aquatic taxa. In short, the deleterious impact of synthetic compounds on non-target organisms, the development of resistance in insects as well as spread of new diseases have paved way to find an alternate way for chemical pesticides.

Proteases

Proteases, or peptidases are enzymes that are very vital for almost all forms of life. Peptidases or peptide hydrolases are enzymes acting on peptide bond which include the endopeptidases (proteinases) as well as exopeptidases. The terminal amino acid residue in a polypeptide is cleaved by exopeptidases, while an internal amino acid residue is cleaved by endopeptidases. Proteases break peptide bond,

called scissile bond, between amino acids. There are different types of peptidases, where serine, cysteine, aspartic, threonine, glutamic or metallo groups play vital role in catalysis. Among them, the serine, threonine and cysteine peptidases are catalytically distinct from the glutamic, aspartic and metallopeptidases (Rawlings and Salvesen 2013).

In serine proteases, the active sites are made up of three amino acids namely serine, histidine and aspartate which are collectively called as the catalytic triad that works on charge-relay network (Laskar *et al*. 2012) [14]. The active site of aspartate protease is formed by two aspartate residues situated in its catalytic site. Aspartic proteases are single chain enzymes with a molecular weight of approximately 35000 Da. Each of the N-terminal and C- terminal domain of aspartate proteases provide one Aspartic acid (Dunn 2013) [8], whereas the activity of cysteine proteases relies upon a catalytic trad consisting of cysteine, histidine and asparagine. The sulfhydryl group of the cysteine proteases serves as the nucleophile which attacks the peptide bond of the proteins. The fundamental functions of cysteine protease are catabolism and protein processing. To catalyze the addition of water molecule to a peptide bond, metalloproteases use protein-bound metal ions, and coordinated water molecules. In most metalloproteases the metal found in catalytic site is zinc and most frequently use either disulphide bridges or calcium ions for the stabilization of enzyme structure. Threonine peptidases have an active site nucleophile and a basic residue which can act as an acid in the catalytic mechanism. Threonine protease uses a catalytic charge relay system to activate its secondary hydroxyl nucleophile for catalysis. Prokaryotes, fungi, plants and animals require proteases for metabolism. Proteases primarily play an important role in insect digestion. In insect digestion a single protease is not solely responsible for digestion, instead a combination of serine proteases has been identified (Christeller *et al*. 1989) [2]. In insects, proteases also detoxify protein toxins consumed as a part of plant and pathogen feeding. Proteases found in insect gut are sensitive to plant protease inhibitors so that they can be targeted for pest control.

In *Aedes aegypti* serine proteases is responsible for the catabolism of food thereby obtaining nutrients. Previous studies reported that the predominant protease found in the gut of *Culex pipiens* mosquito larvae are serine proteases (Amrutha *et al*. 2018) [2]. Also, Smrithy et. al isolated and characterized soya bean trypsin inhibitor-binding protease from the gut of A*edes albopictus* larvae (Smrithy and Kannan Vadakkadath meethal 2020) [30]. Volz et. al., observed the involvement of serine protease cascades in the initiation of immune responses in *Anopheles gambiae* (Volz *et al*. 2005) [33]. *Aedes albopictus* larvae immensely exhibit trypsin-like serine proteases throughout their larval stages, where a differential rate in expression of proteolytic activity was shown in each larval stage (Sajna *et al*. 2019) [26]. Proteases found in insect gut are very essential for the digestion of blood meal. Other than digestion, proteases can also influence virus infectivity. In light of that, Brackney et.al conducted experiments to find out the relationship of serine proteases of *Aedes aegypti* with Dengue virus – 2 infectivity. They found that some isoforms of trypsin can even limit the infectivity in *Aedes aegypti* (Brackney *et al*. 2008) [4]. From these studies it is evident that proteases play different roles in mosquitoes and it is vital for the growth and development of mosquito.

Toxicity of plant protease inhibitors to insects

Protease Inhibitors (PIs) block the activity of the proteases and acts upon group of proteases that have similar mechanism of action. Above 16000 protease inhibitors, which are divided into 67 families are reported (Rawlings *et al*. 2018) [21]. Most commonly, protease inhibitors are either proteins or peptides. Protease inhibitors are widely distributed in tubers and plant seeds. Also, plants are known to produce protease inhibitors as a result of attack of plants by insects or pathogens. The protease inhibitors hamper the normal digestion of insects, by blocking the action of digestive proteases thereby leading to a deficiency in amino acids which in turn slows the growth and development. Other than blocking the action of a protease, some protease inhibitor plays an important role in regulating growth factor activities, apoptosis, protection against microbes and tumour suppression, resistance to the growth of fungi and boosting immunity. There are four prominent classes of protease inhibitors based on the active amino acid present in the catalytic sites namely, serine, cysteine, aspartate and metallo protease inhibitors.

Osborne and Mendel in 1917, provided the first report of protease inhibitor from plants as they observed that raw soy bean does not aid in the growth of rats unless they are cooked for 3 hours (Osborne *et al*. 1917) [19]. It was found that moderate heating of raw soybean increased its nutritional quality and subsequent researches provided additional evidence to the works of Hayward and his co-workers. In 1947, Michael and Standish observed that the larvae of *Tribolium castenium* failed to complete their development in soy flour (Mikel and Standish 1947) [16]. Following these observations many works have been done on protease inhibitors and later Kunitz isolated a crystalline form of trypsin inhibitor from raw soy bean. The effect of protease inhibitor on pest development was also proven by *in vivo* studies. For instance, it has been found that the inclusion of soy bean trypsin inhibitor in the diet of Sunn pest larvae, showed a decrease in weight and developmental retardation (Lawrence and Koundal 2002) [15]. In a study it was also found that a trypsin inhibitor from *Plathymenia foliolosa* had a toxic effect against Mediterranean flour moth, *Anagasta kuehniella* (Silveira Ramos *et al*. 2009) [29]. Clitocypin, a cysteine protease inhibitor from *Clitocybe nebularis*, was found to have insecticidal activity against younger larvae of the pest in its natural or recombinant form. It was also observed that, there is a positive correlation between the amount of protease inhibitor and pest resistance. For example, *Vigna cultivar* has a high amount of protease inhibitor which confer resistance to its main pest *callosobruchus maculates*. Another study revealed that in some pests, a defensive strategy towards protease inhibitor is seen where the organism is induced to produce insensitive trypsin (Oliveira *et al*., 2013) [18]. *In vivo* studies using a combination of two protease inhibitors in artificial diet has exhibited a synergetic effect on *Tribolium castanum.* In vitro studies using trypsin inhibitor purified from winged bean has shown that it delayed the development of melon fruit fly larvae in larval as well as pupal stages, which is a serious agricultural pest (Kaur and Sohal 2019) [13]. It is also evident that protease inhibitor has

deleterious effects on molting of insects. For instance; in vitro studies of an aspartic protease inhibitor on *Helicoverpa armigera* resulted in deformed larvae and pupa. It is also known that protease inhibitor not only retard the growth and development of insects but also reduces the fecundity. For example, a protease inhibitor isolated from the seeds of Bitter gourd directly affects the fecundity of *Helicoverpa armigera* and *Spodoptera litura* (Sajitha et. al 2020) [25] due to the inhibition of its gut proteases. Remya et. al purified and characterized a new protease inhibitor from the seeds of *Spatholobus parviflorus* which inhibits the larval gut proteases of *Spodoptera mauritia*.

Mosquitocidal effect of plant protease inhibitors

It was found that, *Adenanthera pavonina* (ApTI), a Kunitz type non-competitive inhibitor, has delayed the developmental stages of *Aedes aegypti* larvae. The larvae were treated with protease inhibitor and it was observed that they were vulnerable to concentrations above 0.06mg/ml. A concentration hike of ApTI also leads to a decline in their survival rate. The *Cassia leiandra* trypsin inhibitor (ClTI) is a kunitz-type trypsin inhibitor purified from its seeds. A 50% reduction in the midgut activity of *Aedes aegypti* larvae was observed when the larvae were treated with ClTI at a concentration of 4.65 x 10^{-6} M. In a long-term exposure of 10 days, it was observed that the mortality rate was 44% and also delayed the process of larval development (Dias *et al*. 2017) [7]. There are many reports on the in vitro studies, highlighting the effect of protease inhibitor on mosquito larvae. For instance, Silvia *et al*., demonstrated the inhibition of larval midgut protease of *Aedes aegypti* by a trypsin inhibitor from *Ricinus communis*, in vitro (Silva *et al*. 2015) [28]. Shamsi *et al*., observed that on administration of *Allium sativum* Protease Inhibitor (ASPI), impaired the developmental stages of *Aedes aegypti* larvae and also showed a dose dependent acute toxicity. It was also observed that a trypsin inhibitor from *Moringa oleifera* flowers has significantly hampered the hatching rates of *Aedes aegypti* larvae when *Aedes aegypti* eggs were incubated with flower extract at a concentration of 8.5-17mg/mL. The *Moringa oleifera* Trypsin Inhibitor promoted mortality of the *Aedes aegypti* larvae giving an LC_{50} of 0.3mg/mL. A low molecular weight trypsin inhibitor isolated from *Leucaena leucocephala* seeds reduced in vitro midgut proteolytic activity of *Aedes aegypti* larvae by 70%. It was demonstrated that plant extract containing trypsin inhibitor is toxic to the lrvae of *Aedes aegypti* (Aparna *et al*. 2021) [3]. The long-term exposure of protease inhibitor purified from *Lonchocarpus sericeus* seeds as well as *Enterolobium contortisiliquum* seeds (Tabosa *et al*. 2020) [32] are known to have caused inhibition of midgut proteases and high mortality in *Aedes aegypti* larvae. In a study, it was also revealed that a cysteine protease inhibitor has access to the food vacuole of *Plasmodium falciparum* (which is present in the midgut of the mosquito) and can block digestion of haemoglobin in gametocytes. It also leads to the reduction of early gametocytes due to shrinkage as well as oocyte production, when it is treated with a protease inhibitor, but no reports on cessation of malaria transmission is reported (Czesny *et al*. 2009) [6]. Protease inhibitor can be used to reduce the hike in mosquito midgut infection due to Dengue virus mediated recruitment of a protease. For example, a study demonstrated that a Kazal type inhibitor can effectively inhibit plasmin so as to reduce the enhancement of mosquito mid gut infection caused by Dengue virus (Ramesh *et al*. 2019) [20].

Recent advancement of plant protease inhibitors in insect pest management

The application of plant protease inhibitors seems to be a promising tool for pest control. Different strategies are adopted by the researchers in recent years to develop resistance to plants against pests. Some of them are as follows:

a. **Gene pyramiding:** It is the combination of different genes from multiple parents into a single genotype so as to enhance the trait performance. In recent years PIs are used in gene pyramiding or gene packaging for conferring enhanced resistance to insect pests. Careful selection and combination of two genes can produce synergetic effect which is much efficient when compared to the performance of a single gene (Goulet *et al*. 2008) [9]

b. **Molecular phage display:** Phage display includes random mutations in different sites in the sequence of a protease inhibitor, and a large collection of its variants are produced. Genes can be manipulated for all coding frames, so that each modified protein varies in specificity, binding and other properties. For instance; selection of a variant of mustard trypsin inhibitor by phage display has shown to have highest affinity towards bovine chymotrypsin among the recombinant chymotrypsin inhibitor of Mustard Trypsin Inhibitor (MTI-2) family. Another study revealed that variants of a protease inhibitor, identified by phage display has shown to be good candidates in mosquito control by hampering the digestion in the gut (Soares *et al*. 2013) [31].

c. **RNAi mediated gene silencing:** A novel strategy for managing insect pests is micro-RNA mediated gene silencing. In order to inhibit the gut protease of the target pest, synthetic RNAi could be prepared which mimic the role a protease inhibitor. For instance, studies show that there is an increased larval mortality of *Helicoverpa armigera*, when synthetic RNAi that mimics a protease inhibitor was orally fed (Jayachandran *et al*. 2013) [12]. RNAi mediated sequence specific gene silencing could effectively manage pest by unaffecting other related species.

d. **Transgenic plants expressing protease inhibitors:** Identifying and sequencing of the gene encoding for a PPI, or a combination of genes encoding two or more PPI, makes it facile to develop a variety of transgenic plants. It was observed that many insects belonging to Lepidoptera, Orthoptera and Coleoptera showed incomplete development in their larval stage (Hilder *et al*. 1987) [10] when fed on such transgenic plants. Among them, Lepidopteran pests (Fig 1) are the major pests of agricultural crops and transgenic crops expressing PPIs against the gut proteases of such Lepidopteran pests will be a more eco-friendly alternative for conventional synthetic pesticides.

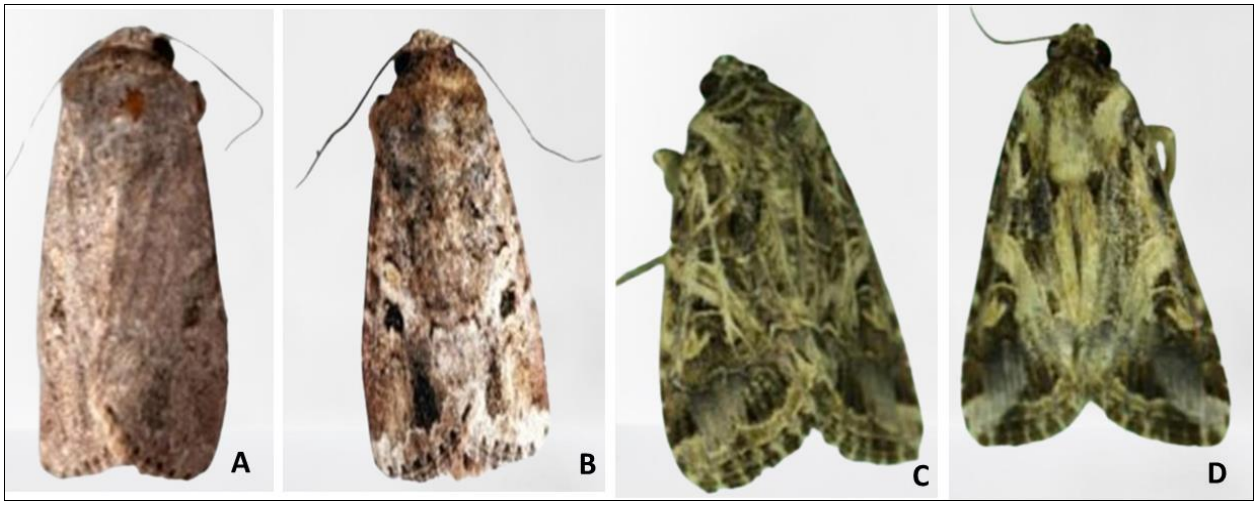

Fig 1: Some Lepidopteran pests of agricultural crops, A: *spodoptera mauritia* adult female B: *Spodoptera mauritia* adult male C: *Spodoptera litura* adult female D: *Spodoptera litura* adult male

Future prospects

The phytophagous insects that are present today and the plants they consume exist in nature as a result of co-evolution. Insect population are diverse in nature and are found in almost every part of the world. Plants contain protease inhibitors which are natural reservoirs of toxins against pests, at the same time; interaction between plants and herbivorous insects is an important aspect of plant productivity in the wild. Though different experiments have been conducted using plant protease inhibitors in the past, no much effective strategies have been described due to the high adaptive capability of plant pests towards its host.

Insect resistance towards a particular PPI largely depends on the abundance of the type of proteases present in their gut. Out of all types of Plant Protease Inhibitors, it is very essential to identify the potential candidate for targeting the gut protease of a desired insect pest. For example, a study suggested that the gut of *Culex pipens* mosquito larvae is dominated by serine proteases among other types of proteases (Amrutha *et al*. 2018) [2]. Advanced techniques like 3D modelling may aid in finding the most potent type of PPI. Also, protease inhibitors from unrelated plants in which the insect least feed should be identified and isolated. In order to resist pest attack on a broad scale, new methods like gene stacking have come into play. Implementing a combination of multiple protease inhibitors imparts extensive range of resistance to plants against insect pests. Also, RNAi mediated gene silencing is a newly emerging field of insect pest control.

Microbial symbiosis in insect gut might contribute to resistance of insect pests against PPI. For instance, reports have shown that a strain of *Bacillus subtillis* isolated from gut of *Helicoverpa armigera* produce chymotrypsin-like proteases (Shinde *et al*. 2012) [27].

Though PPIs have gained interest in the scientific field against insect pests, the effects of animal protease inhibitors are least explored. Knowing much about animal protease inhibitors and the molecular mechanisms by which they inhibit insect growth is also yet to be elucidated.

Conclusions

The use of chemical pesticide has an adverse effect on environment, as most of them also harm non-target organisms. As a biological alternative, the potential of plant protease inhibitors as a pesticide must be explored. As these inhibitors are natural deterrents of insect pests, we could genetically manipulate them, or may use then in combination to procure a more promising result. In recent times, the use of PPI against mosquito larvae is also demonstrated. To combat the exponential growth of mosquitoes in nature, plant protease inhibitors can be directed to stunt the growth in its larval form. However, the molecular mechanism by which insect combat the defence mechanism of plants, including transcription and expression of defence genes are not fully understood. Exploring more genes that impart resistance to insect pests, its manipulation and introduction into transgenic crops might decrease crop damage due to insect pests.

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