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Research Article

SCREENING OF PLANT SEEDS FOR PROTEASE INHIBITOR AGAINST LARVAL GUT PROTEASES OF *SPODOPTERA MAURITIA* (BOISD.) (LEPIDOPTERA: NOCTUIDAE)

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Abstract: Plant protease inhibitors (PPIs) are widely distributed in plants and among other roles, it protects plants from insect attack. Plant protease inhibitors inhibit insect gut protease activity thereby hampering protein digestion. In this study, we screened plant seeds to identify PPIs against larval gut proteases of *Spodoptera mauritia* (Boisd.). Seeds were homogenized in bicarbonate buffer pH 9.0 (1ml/g tissue) and was centrifuged at 10,000 x g for 10 minutes at 4ºC. The supernatant was used for protease inhibition assay using azocasein as substrate. Out of 30 different seeds screened, 10 showed greater than 40% inhibition and the highest percentage of inhibition was showed by *Areca triandra* (73.3±0.04%), followed by *Abelmoschus manihot* (72.57±1.3%), *Mallotus tetracocus* (53±1.7%), *Mucuna pruriens*, (47.30±3.46%). *Crotalaria pallida* (46.56±0.94%), *Nephelium lappacum* (45.66±1.28%), *Persea Americana* (44.38±0.09%), *Ipomoea cairica* (42.53±1.20%), *Ricinus communis* (41.77±3.28%) and *Thunbergia alata* (40.82±0.05%) respectively. This is the first report of presence of PPIs from *Areca triandra*, *Abelmoschus manihot, Mallotus tetracocus, Ipomoea cairica* and *Thunbergia alata*. Though there are reports of the presence of trypsin/ cysteine protease inhibitor from other plants reported here, in this study we showed for the first time that the seed extracts from these plants inhibited larval gut proteases of *S. mauritia*. Proteinase K treatment revealed that the inhibitor in *Abelmoschus manihot* is proteinacious in nature while the inhibitor in *Areca triandra* may be a non-proteinacious inhibitor. Identification and characterization of new PPIs against gut proteases of insects will be helpful in designing better insect control strategies.

Keywords: Plant protease inhibitors, *Spodoptera mauritia*, *Areca triandra* (Roxb), *Abelmoschus manihot* (L)

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Introduction

Pests are the harmful species whose population size or density goes beyond the damage threshold level either throughout the year or during specific season. The insect pest management is crucial for sustained production in economically important crops. *Spodoptera mauritia* (Boisduval) (Noctuidae: Lepidoptera), popularly known as paddy swarming caterpillar or paddy army worm is a sporadic pest which causes serious losses to rice crops. Large swarms of larvae appear suddenly and destroy whole fields of paddy and then march on to the next field. *Spodoptera mauritia* is widely distributed in the East and Southern Asia, Indian subcontinent and in the Australian Region. They usually occur on paddy in India from July to September. They have the ability to migrate to alternate host plant, *Ischaemum aristatum* during off season, at the end of which they make a full scale comeback on the nursery stages of paddy. This ability makes further complication on the status of the pest. For the management of *S. mauritia* conventional pesticides are being used. They are readily available, rapid acting and highly reliable, but poses great threat to man and environment. So alternatives like insect growth regulators, and genetically modified crops are also tried. Toxins from the bacteria, Bacillus thuringiensis (Bt. toxin) which provide protection against a great variety of insect pests were used for the production of the genetically modified crops. Of the different toxins being investigated protease inhibitors from plants (PPI's) are widely accepted as these proteins are naturally present in plants. Proteases are protein hydrolyzing enzymes that specifically breaks the peptide bond present in the proteins, and they are indispensable for the maintenance of normal body function and survival of the living organisms. They are present in almost all forms of life from lower to higher organisms and they are generally encoded by 2% of the genes [1].

Proteases also play a critical role in insect physiology and food digestion and they are of great interest as a target for insect pest management [2]. Based on the functional groups present in the active site they are grouped into Serine proteases, Cysteine proteases, metallo proteases and aspartic proteases [3, 4]. Major Coleopteran and Hemipteran gut proteases are belongs to the cysteine proteases, whereas in Orthopterans, Dipterans and Lepidopterans are mainly serine proteases [5]. Protease inhibitors (PIs) are small proteins or peptides that are capable of inhibiting protease and are found in animals, plants and micro-organisms [6] PIs from plants known as Plant protease inhibitors (PPIs) are natural defense proteins which protect plants from insect attack and commonly found in Leguminosea, Solanaceae, and Gramineae families [7]. As early as 1947, Mickel and Standish observed that certain insect larvae were unable to develop normally on soybean product and later Lipke *et al* showed that it is due to the presence of trypsin inhibitors from soybean products which were lethal to the larvae of flour beetle, *Tribolium confusum* [8, 9] Following these early studies, many plant species have been reported to contain PPIs in different tissues like leaves, flowers, seeds and tubers as their defensive tools against pests attack [10-12]. Based on the kind of protease they inhibit, PPIs are classified primarily as serine, cysteine, aspartic or metallo protease inhibitors and among them serine protease inhibitors are well characterized [13, 14]. Based on the homology in their primary structure, active site, the enzyme on which they act and their distribution in the plant kingdom, serine PPIs are categorized into 8 families, which include Bowman-Birk, Kunitz, Potato I, Potato II, Cucurbit, Cereal super- family, Ragi AI and Thaumatin-PR like families [15]. Over the last few decades, a large number of PPIs were isolated and purified from different tissues like seeds, leaves, fruits and tubers of plants of several families and many of them have anti-nutritional

effects against several larval gut proteases of Lepidopteran pests [16-20]. Plant protease inhibitors are capable of interfering with digestive proteases of phytophagous insects resulting in the amino acid deficiency leading to retardation the insect growth, and development [21]. The gene coding for PPIs can be cloned and expressed in host plant to reduce pest attack. This will help to reduce the extensive use of chemical pesticides and thereby its harmful effects on environment and human health. Beyond the defensive role in the pest control, PIs are involved in many biological processes such as blood coagulation, platelet aggregation, immune regulation and anti-carcinogenesis [22, 23]. Modulation of protease activity using synthetic peptidomimetic inhibitors find application in many diseases including cancer, hypertension, cardiovascular diseases *etc*. [24]. In this study we screened different plant seeds to identify extracts containing protease inhibitors against the larval gut proteases of *S. mauritia*.

Materials and Methods

Chemicals

Azocasein was obtained from Sigma Aldrich, St. Louis, USA and Proteinase K from Qiagen, USA. All other chemicals were of Analytical grade.

Collection and rearing of *Spodoptera mauritia* larva

The adult moths of the insect were attracted to light during night and were collected using sweeping net. These moths were then transferred to glass beakers and fed with a dilute solution of honey (10%). They were allowed to mate and lay eggs. Larvae hatched out after 3-4 days. The larvae were reared in glass beakers at initial stages. They were fed with fresh, tender leaves of the grass *Ischaemum aristatum* collected from paddy fields. The larvae were maintained at room temperature with a RH 90 ± 3% and 12:12 dark, photoperiod regime. They were transferred to large plastic troughs as they grew in size. During summer days the cloth covering the troughs was wetted frequently. The pupae were kept separately in beakers for adult emergence.

Preparation of *Spodoptera mauritia* gut extract

Fifth instar larvae were anesthetized to dissect out the mid gut and it was stored at -20°C until use. The gut was homogenized in 0.1M bicarbonate buffer, pH 9.0 (1ml/g of tissue) and were centrifuged at 10,000 x g at 4°C for 10 minutes. The soluble protein recovered from the supernatant was stored as aliquots at -20°C until use.

Collection of plants and preparation of the seed extract

Seeds were collected from Kozhikode, Kannur and Malappuram district of Kerala, India. They were washed and soaked in bicarbonate buffer, pH 9.0 (1ml/g tissue) and homogenized. The homogenates were centrifuged at 10,000 x g for 10 minutes at 4°C. The supernatant containing soluble proteins was used for protease inhibition assay.

Protease assay and Protease Inhibition assay

The protease assay was done by incubating, 5µl of the gut extract with 0.015 µg/µl azocasein as substrate in a total volume of 20.5µl, at 37°C for 30 minutes. The reaction was stopped by adding 80 µl 5% TCA. After centrifugation, 50 µl supernatant was mixed with 150 µl of 0.5M NaOH. The absorbance was measured at 440nm using a Microplate reader (SYNERGY HTX, Bio Tek). In protease inhibition assay 10 µl of the seed extract was pre-incubated with the 5 µl of the gut extract and assay done as described in protease assay. All assays were done in duplicate and the experiments were repeated three times.

Proteinase K treatment of plant extracts

Seed extracts with higher inhibition was tested to assess whether the inhibitor is proteinacious in nature or not. Proteinacious nature of the inhibitor was assessed by overnight incubation of the plant extract (90µl) with Proteinase K (10µl) at 56°C followed by the inactivation of the proteinase K by heating the mixture at 75°C for 15minutes. A buffer control and inhibitor alone control were also kept without proteinase K for incubation. Centrifuged 10000 x g for 1 minute and the supernatant was used for protease inhibition assay. Inhibitor control without any incubation was also done. All assays were done in duplicate and the experiments were repeated three times.

Statistical analysis

Statistical analysis was done by using SPSS software, version 16.

Results and Discussion

Among the thirty plant extracts screened, ten of them are having greater than 40% inhibition against the gut protease activity of *S. mauritia* [Table-I]. Of these the highest percentage of inhibition is shown by *Areca triandra* (Roxb.), (73.33±0.04%) followed by *Abelmoschus manihot* (L) (72.57±1.3 %). No protease inhibitor was reported from these two plants.

Table-1 *List of plants screened for protease inhibition against larval gut proteases of S. mauritia and their percentage inhibition*.

SN	Name of plant	% inhibition (Mean ± SE)
1	*Areca triandra* (Roxb.)	73.33 ±0.04
2	*Abelmoschus manihot* (L.)	72.57 ±1.30
3	*Mallotus tetracoccus* (Roxb.)	53.40 ±1.77
4	*Mucuna pruriens* L.	47.30±3.46
5	*Crotallaria pallida* L.	46.56±0.94
6	*Nephelium lappaceum* L.	45.66 ±1.28
7	*Persea americana* Mill.	44.38±0.09
8	*Ipomoea cairica* L.	42.53±1.20
9	*Ricinus communis* L.	41.77±3.28
10	*Thunbergia alata* Boj. Ex Sims	40.82±0.05
11	*Croton tiglium* L.	39.01±0.48
12	*Passiflora foetida* L.	37.43±1.36
13	*Crotalaria retusa* L.	34.41±2.14
14	*Careya arborea* Roxb.	33.34±0.54
15	*Zanthoxylum rhetsa* (Roxb).DC	26.92±0.53
16	*Sterculia guttata* (Roxb.)	28.99±3.90
17	*Lagerstroemia speciosa* L.	29.10±0.02
18	*Debregeasia longifolia* (Burm.f.) Wedd.	26.79±2.16
19	*Meremia umbellate* (L).Hallier f.	19.01±0.08
20	*Colubrina travancorica* Bedd	16.06±1.22
21	*Senna tora* (L)	15.47±3.37
22	*Clerodendrum infortunatum*	15.32±0.22
23	*Geophila repens* L.	15.97±0.31
24	*Annona squamosal* L.	14.43±2.02
25	*Rubus ellipticus*	13.00±0.02
26	*Fioria vitifolia* (L) Mattei	12.62±1.83
27	*Aristolochic indica* L.	12.53±0.32
28	*Flacourtia jangomas* (Lour.) Raeusch.	12.27±2.81
29	*Anethum graveolens* (L)	10.51±1.45
30	*Rauvolfia serpentina* (L.) Benth. ex Kurz.	10.54±3.40

Seeds of *Mallotus tetracocus* (Roxb.), showed an inhibition of 53.4±1.7%, and no protease inhibitor was reported from this plant. Antioxidant properties against DPPH (2, 2-diphenyl-1-picrylhydrazyl radical) were reported from the bark of *Mallotus philippinensis* [25]. *Mucuna pruriens* L., *Crotallaria pallida* L., *Nephelium lappaceum* L., *Persea americana* (Mill) and *Ricinus communis* L. showed percentage inhibition of 47.30±3.46, 46.56±0.94, 45.66±1.28, 44.38±0.09 and 41.77±3.2 respectively against gut proteases of *S. mauritia*. Although trypsin/cysteine protease inhibitors were reported from these plants, no protease inhibition against gut proteases of *S. mauritia* is reported. Sane *et al*., isolated single trypsin inhibitor from the seed extract of *Mucuna pruriens* [26]. A 32.5 kDa trypsin inhibitor CpTi was purified from the seeds of *Crotallaria pallida* and the inhibitor showed the inhibitory activity against the digestive enzymes of insect pest [27]. Fang and Ng isolated a trypsin inhibitor (NLTI) of 22. 5 kDa from the seeds of *Nephelium lappaceum* L. [28]. The complete amino acid sequence of an 11.3kDa cysteine protease inhibitor was reported from the fruit of *Persea americana* (Mill) by Kimura *et al*., [29]. Further purification and characterization is needed to check whether the protease inhibitor from the seed extract reported in this study is similar to the protease inhibitor reported from the fruit. Soomro *et al*., identified protease inhibitor in the crude extract of *Ricinus communis* [30]. *Ipomoea cairica* L. and *Thunbergia alata* showed inhibition of 42.53±1.20 and 40.82±0.05 percentage respectively towards gut protease activity of *S. mauritia*. A trypsin inhibitor (SPTI) was reported from the root of Sweet potato, Ipomoea batatas by Hou and Lin, [31]. Obey and Swamy studied and showed the antibacterial activities of ethanolic extract of *Thunbergia alata* leaves against some selected microorganisms [32]. No protease inhibitor was reported from the seed extract of *Ipomoea cairica* L. and *Thunbergia alata*.

Proteinase K treatment of the selected plant extracts

Plant extract with high percentage of inhibition was treated with proteinase K to

Table-2 *Effect of Proteinase K treatment on inhibition by plant seed extracts on S. mauritia gut protease inhibition*

SN	Plant used	Proteinase K Control % inhibition (Mean ± SE)	Proteinase K Test % inhibition (Mean ± SE)	Proteinacious nature of the inhibitor
1	*Areca triandra*	65.56 ± 2.30	64.51±1.80	-
2	*Abelmoschus manihot*	61.01 ± 1.20	20.02 ± 0.42	+

check whether the inhibitor is a protein or not. Two plant extract (*Abelmoschus manihot* and *Areca triandra*) showing higher percentage of inhibition against larval gut proteases of *S. mauritia* were selected for Proteinase K treatment. Proteinase K (PK) treatment of *Areca triandra* extract, showed an inhibition of 64.51±1.8 % compared to untreated control 65.56±2.3% [Table-2]. From the data it is clear that the inhibitor is not a protein, but it may be a small molecule. Abhilash and Kannan reported a protease inhibitor from Areca catechu seed extract [33]. Proteinase K treatment of *Abelmoschus manihot* seed extract showed an inhibition of 20.02 ±0.42 % whereas untreated control showed 61.0±1.20% inhibition towards gut enzyme (Table 2). This result indicates that the major inhibitor is a protein. Trypsin inhibitors have been reported from another species, *Abelmoschus moschatus* seeds by Dokka *et al*. [34]. Further purification and characterization of the *A. manihot* seeds is necessary to find out whether the inhibitor/s is similar to the one reported from *A. moschatus* seeds or a novel one. Further purification and characterization of the inhibitor from these plants are ongoing in our laboratory. Plant protease inhibitor may be exploited for pest control and is a better choice as these are naturally occurring and the adverse effect on health and environment will be minimal. Plant protease inhibitors being naturally present in plants, augmenting their activity by over expressing it in transgenic plants is less likely to produce undesirable effects compared to plants expressing toxin genes like Bt. toxin. Genetically modified (GM) plants with genes encoding PPIs are a modern attractive and alternative to conventional pesticides. The Cowpea Trypsin inhibitor, CpTi was the first PPI gene to be successfully transferred to tobacco plant and transgenic tobacco plant containing CpTi showed significant resistance against tobacco hornworm (*Manduca sexta*) [35]. Growth of *Manduca sexta*, tobacco hornworm, larvae feeding on transgenic tobacco leaves expressing tomato or potato inhibitor II, a powerful inhibitor of both trypsin and chymotrypsin, was significantly retarded, compared to growth of larvae fed on untransformed leaves [36]. Sane *et al*., studied the efficacy of transgenic tobacco plants expressing CpTi gene against larval development of *Spodoptera litura* under laboratory condition and it was observed that 50% reduction in biomass of *S. litura* larvae fed on transgenic leaves expressing CpTi gene [37]. A major storage protein, Sporamin, and a Kunitz type trypsin inhibitor was isolated and characterized from the tuberous root of sweet potato [38, 39]. Transgenic tobacco and cauliflower expressing sporamin gene, SpTI-1 is found to confer resistance to beet cyst nematode (*Heterodera schachtii* Schm.) [40]. Also transgenic tobacco plant expressing rice cysteine proteinase inhibitor induces resistance against two potyviruses, tobacco etch virus (TEV) and potato virus Y (PVY) [41]. Duan *et al*, introduced potato proteinase inhibitor II gene (pin2) into several Japonica rice and produced a large number of GM rice plants. Bioassay showed that these transgenic rice plants have increased resistance to pink stem borer (*Sesamia inferens*), a major rice pest [42]. Transgenic tomato plant expressing two PPIs, potato serine proteinase inhibitor (PI-II) and carboxypeptidase inhibitor (PCI) results in increased resistance to *Heliothis obsoleta* and *Liriomyza trifolii* larvae [43]. Thus identifying and characterizing better protease inhibitors from plants will be helpful in formulating better insect control strategies. Plant protease inhibitors are ideal for control of insect pests as they are part of natural defense mechanism employed by the plants and adverse effects on other organisms will be less likely than that of synthetic pesticides.

Conclusion

Out of the different plant seeds screened in this study, the highest inhibition towards the gut proteases of *S. mauritia* was shown by *Areca triandra* (73.3±0.04%) followed by *Abelmoschus manihot* (72.57±1.3%). The inhibitor present in the seed extract of *Areca triandra* is non proteinacious and that from *Abelmoschus manihot* is mainly proteinacious in nature. The other plants having greater than 40 % inhibition are *Mallotus tetracocus* (53±1.7%), *Mucuna pruriens*, (47.30±3.46%). *Crotalaria pallida* (46.56±0.94%), *Nephelium lappacum* (45.66±1.28%), *Persea Americana* (44.38±0.09%), *Ipomoea cairica* (42.53±1.20%), *Ricinus communis* (41.77±3.28%) and *Thunbergia alata* (40.82±0.05%) respectively. This is the first report of presence of PIs from the seeds of *Areca triandra* (Roxb), *Abelmoschus manihot* (L), *Mallotus tetracocus* (Roxb.), *Ipomoea cairica* and *Thunbergia alata*. To our knowledge this is the first report of protease inhibitor from the seeds of *Areca triandra* (Roxb), *Abelmoschus manihot* (L), *Mallotus tetracocus* (Roxb.), *Mucuna pruriens* L., *Crotallaria pallida* L., *Nephelium lappaceum* L. *Persea americana* Mill., *Ipomoea cairica, Ricinus communis* and *Thunbergia alata* against larval gut proteases of *S. mauritia*. Further studies on the purification and characterization of inhibitors reported in this study will be helpful in formulating better insect control strategies.

Application of research: Genetically modified plants containing protease inhibitors for pest control.

Research Category: Crop pest management.

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