2.0 Summary:

This chapter deals with the materials used for experimentation and methods, which have been followed to solve the designed problem. Method of isolation, purification and characterization of α-AI from seeds of A. paniculatus have been described inadequate details. The procedure followed for rearing of the target pests and methods to determine the α-AI activity against insect amylases, both in vitro and in vivo have been discussed. The methods followed for determining the effect on various developmental parameter of insect as well as nutritional parameters of grain legumes as a function of treatment with purified α-AI have been described. The procedure of determining the effect of α-AI on hydrolysis of starch by amylase using scanning electron microscopy has also been provided. For all the procedures/methods/protocols used in the present study, relevant references have been duly acknowledged.

**********
2.1 Materials

2.1.1 Chemicals and Glassware:

All chemicals, media for microbes and reagents used were analytical (AR) grade, mainly procured from M/s. Himedia, Mumbai, India. Broad range protein molecular weightmarkers (3.5-205 kDa) were purchased from Gennie (Bangalore, India) and Sigma Chemicals Co., St. Louis, M.O, USA. Glassware used for all the experiments were of Borosil make.

2.1.2 Grain legumes:

Nine different grain legumes (local varieties) in North Maharashtra; *Cicer arietinum* L. (Chana), *Vigna radiata* L. (Green gram), *Vigna mungo* L. (Black gram), *Vigna unguiculata* L. (Cowpea), *Vigna aconitifolia* J. (Mathbean) *Cajanus cajan* L. (Pigean pea), *Lens culinaris* M. (Lentil), *Pisum satium* L. (Green pea), and *Glycine max* L. (Soybean) were purchased from local farmers of this region. These seeds were stored in airtight tin jars until required for experiments.

2.1.3 Test insects:

*Helicoverpa armigera* (Lepidoptera) larvae were collected from chickpea (*Cicer arietinum* L.) fields near university campus in Jalgaon. They were identified, authenticated and maintained under laboratory conditions on an artificial diet as per protocol provided by Central Institute for Cotton Research (CICR), Nagpur (India) and 3rd instars of subsequent generations were used for feeding assays. The composition of artificial diet for rearing the larvae is given in Table 2.1. The mixture was autoclaved for 15 min at 121°C and 15 psi pressure, allowed to cool and approximately 1 g cakes were casted in pet jars.
Table 2.1: The composition of artificial diet for rearing the larvae of *H. armigera*.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat germ</td>
<td>80 g</td>
</tr>
<tr>
<td>Chickpea flour</td>
<td>30 g</td>
</tr>
<tr>
<td>Sorbic acid</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Sucrose</td>
<td>40 g</td>
</tr>
<tr>
<td>Casein</td>
<td>40 g</td>
</tr>
<tr>
<td>Dried yeast</td>
<td>20 g</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>10 mL</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>4 g</td>
</tr>
<tr>
<td>Multivitamin tablet</td>
<td>1 g</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>4 mL</td>
</tr>
<tr>
<td>Antimold solution agar</td>
<td>24 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>
Similarly, a culture of *Callosobruchus chinensis* L. (Coleoptera) was obtained from a colony initiated and maintained on green gram (*Vigna radiata* L.) at a constant temperature of 30±2°C, relative humidity 70±5% at 16:8 h light: dark photoperiod from Biopesticide Research Laboratory, School of Life Sciences, North Maharashtra University, Jalgaon. It was reared and maintained on green gram as per protocol standardized in the laboratory (Salunke, 2006).

2.1.4 Plant Materials:

Seeds of *Amaranthus paniculatus* linn; (Rajgira), *Achyranthus aspera*, *Celosia argentea*, *Amaranthus tricolor*, *Amaranthus spinosa* and *Alternanthera sessilis* were bought from Nandurbar during the summer season (March-June in India) (Photoplate 2.1). The plants and plant materials were identified by an expert taxonomist. Plant materials were dried in shade for 20 days and ground to fine powder before extraction.

2.2 Methods:

2.2.1 Isolation of alpha amylase inhibitor (α-AI):

Isolation of alpha amylase inhibitor from plant parts (root, leaf, stem and seeds) of *Amaranthus paniculatus*, *Achyranthus aspera*, *Celosia argentea*, *Amaranthus tricolor*, *Amaranthus spinosa* and *Alternanthera sessilis* was done as per the method of Kokiladevi et al (2005). Finely ground plant material (200 mg) was extracted in an extraction buffer (1% 2-marcaptoethanol, 0.1% Triton X-100, 2 mM phenylmethyl sulphonyl fluoride (PMSF), 2 mM tris HCl and 500 mM NaCl) by homogenization followed by incubation at 4°C for 24 h. It was then centrifuged at 15,000 rpm for 15 min at 4°C. The supernatant was collected and stored frozen in aliquots. The protein content in the extract was quantified following the method of Lowry et al (1951).
Photoplate 2.1: Plant screened for the isolation of α-amylase inhibitor A. *Amaranthus paniculatus*; B. *Achyranthus aspera*; C. *Celosia argentea*; D. *Amaranthus tricolor*; E. *Amaranthus spinosa* and *Alternanthera sessilis*
2.2.2 Starch-Agar plate method for qualitative detection of α-AI:

Qualitative detection of α-AI in the crude extracts of different parts of plants under study was done by the method described by Fossum and Whitaker (1974) with slight modifications. The petri plate containing 1.5% agar, 0.1% starch, 20 mM phosphate buffer (pH 6.9) and 0.5 mM NaCl was used. Alpha-amylase and inhibitor (200 µg each) were pre-incubated for 10 minute at room temperature and loaded in wells of 5 mm diameter made in the starch agar plate. The plate was covered with a tight fitting glass plate and kept at 37°C for 18 h. It was then flooded with lugol solution (1 g Iodine, 2 g KI dissolved in 100 mL of distilled water) and the excess solution poured off. The presence of α-amylase was indicated by clear lysis zones around the wells as a result of hydrolysis of starch whereas, presence of inhibitor was indicated by no hydrolysis of the starch.

2.2.3 Alpha amylase inhibitor Assay:

The α-AI was assayed by quantifying the reducing sugar (maltose equivalent) liberated under the assay conditions. The enzyme inhibitory activity is expressed as the decrease in units of maltose liberated. A modified dinitrosalicyclic acid (DNS) method of Bernfeld, (1955) was followed to estimate the maltose content. Alpha-AI extract equivalent to 200 µg protein was pre-incubated with 200 µg of alpha amylase for 10 min and 1 mL (0.1%) starch solution was added before further incubating at 37°C for 10 min. The reaction was stopped by adding 1 mL (DNS) reagent (1 g DNS, 200 mg crystalline phenol, and 50 mg sodium sulphite dissolved in 1% NaOH [w/v] and prepared fresh), then the contents were heated in a boiling water bath for 5 min. One mL potassium sodium tartarate (40%, [w/v]) solution was added while the contents were still warm and volume in each tube was made up to 10 mL. A blank was set without α-AI extract, another one without amylase enzyme(s) which were replaced by
equal quantities of extraction buffer. The absorbance was measured at 530 nm in a UV-
Visible spectrophotometer (Shimadzu, Model-UV-1601, Japan). The reducing sugar
released from starch was estimated as maltose equivalent from standard graph. The
same assay was followed using α-amylase obtained from different sources like human
saliva, *Bacillus* species, and guts of insect *C. chinensis* and *H. armigera*. One unit of α-
amylase is defined as the amount which causes release of 1 µmole maltose/min. One
unit of α-AI is defined as the amounts which inhibits 1 unit of α-amylase.

### 2.2.4 Purification of Alpha amylase inhibitor from seeds of *A. paniculatus* seeds:

The α-AI from seeds of *A. paniculatus* was isolated and purified as per the
procedure described by Kokiladevi at al., (2005) with slight modifications. The steps
involved in purification are briefly described below:

**(A) Step-I: Crude extract of α-AI from Rajgira seeds**

Finely ground seed flour of *Amaranthus paniculatus* (25 g) was extracted in the
200 mL extraction buffer (10 m M Tris HCl [pH 7.5], 500 mM NaCl, 1% tritonX-100,
2 mM phenylmethylsulphonyl fluoride, (PMSF) by homogenization followed by
incubation at 4°C for 24 h. It was centrifuged at 15,000 rpm for 15 min at 4°C. The
supernatant was collected and stored frozen in aliquots. The protein content was
quantified following the method of Lowry et al (1951) and inhibitory activity in it was
measurement by a modified dinitrosalicylic acid (DNS) method of Bernfeld (1955).

**(B) Step-II: Precipitation with ammonium sulphate:**

After quantification, 187 mL crude extract was subjected to ammonium
sulphate precipitation (80-100% saturation) under cold conditions for 2 h. The resulting
precipitate after centrifugation at 5000Xg for 5 min, was then dissolved in 25 mL
phosphate buffer (20 mM, pH 6.9) and transferred in dialysis bags and dialyzed
overnight against the same buffer.
(C) **Step-III: Size exclusion chromatography:**

The partially purified protein, after dialysis was fractionated by size exclusion chromatography on sephadex G-50 column (26 X 1.2 cm, L x dia). The resin was allowed to swell, by soaking overnight in sufficient quantity of phosphate buffer (20 mM, pH 6.9), packed in the glass column and wash thoroughly by same buffer. The partially purified protein, from previous step was loaded on column and eluted with 20 mM phosphate buffer (pH 6.9) at 4°C. Fraction (2 mL each) were collected and those showing high optical density at 280 nm were pooled and dialyzed against 10mMTris-HCl buffer (pH 6.9) overnight.

(D) **Step –IV: Ion exchange chromatography:**

The pooled and dialyzed fractions from the previous step were loaded on a column (10X2.5 cm) of CM cellulose equilibrated with 10 mM Tris-HCl buffer (pH 6.9). Bound protein was recovered from the column by elution with a gradient (0-0.5M) of sodium chloride in 10 mM Tris-HCl buffer (pH 6.9). Fraction (10 mL each) were collected and analyzed for protein (ΔA 280 nm) and inhibitor activities. The fraction(s) showing activity were pooled, dialyzed against water and stored frozen at -20 °C in small aliquots (Marshall and Lounda, 1975).

### 2.2.5 SDS-PAGE analysis:

For this purpose, the following reagents were prepared-

**Acrylamide- bis-acrylamide solution (30%):** It was prepared by dissolving 30 g acrylamide and 0.8 g bis-acrylamide in 100 mL distilled water, filtered, stored in an amber coloured bottle and used as stock.

**Resolving gel buffer:** It was prepared by dissolving 22.7 g Tris-HCl (1.87 M, pH 8.8) in 100 ml deionized H₂O.
Stacking gel buffer: It was prepared by dissolving 7.26 g Tris-HCl (0.6 M, pH 6.8) in 100 ml deionized H₂O.

Ammonium persulfate: A small amount of 10% (w/v) stock solution was prepared in deionized H₂O freshly before use.

Tris-glycine electrophoresis buffer: This buffer contains 25 mM Tris base and 250 mM glycine (electrophoresis grade). The pH is adjusted to 8.3 by adding 0.1 N HCl followed by addition of 0.1% SDS

Sodium dodecyl sulfate (SDS): A 10% (w/v) stock solution was prepared in deionized H₂O.

SDS gel loading buffer (1X): 50 mMTris-Cl (pH 6.8), 10% SDS, 0.1% bromophenol blue, 10% (v/v) glycerol was used for the sample loading buffer.

Gel stainer: 1.6 g coomassie brilliant blue (CBB) was dissolved in a mixture of 200 mL CH₃OH + 50 mL glacial acetic acid, diluted to 500 mL by deionized H₂O and filtered through Whatman filter paper No. 1.

Gel destainer: It was prepared by mixing 300 mL CH₃OH + 100 mL glacial acetic acid and diluted to 1 L by deionized H₂O.

Stacking gel: It was poured by mixing 6.8 mL deionized H₂O + 1.7 mL 30% stock acrylamide mix + 1.25 mL tris buffer (0.6 M, pH 6.8) + 0.1 mL 10% Ammonium persulfate + 0.1 mL 10% SDS + 0.01 mL N. N. N’, N’ tetramethylethlenediamine (TEMED) in the given sequence.

Resolving gel: It was poured by mixing 11.5 mL deionized H₂O + 25.0 mL 30% stock acrylamide mix + 12.5 mL tris buffer (1.5 M, pH 8.8) + 0.5 mL 10% Ammonium persulfate + 0.5 mL 10% SDS + 0.02 mL N. N. N’, N’ tetramethylethlenediamine (TEMED) in the given sequence.
For molecular weight determination of the isolated \( \alpha \)-AI, the purified protein and molecular weight marker were resolved in 15\% polyacrylamide gel in the discontinuous buffer system as described by Laemmli (1970). Alpha –AI, (20 \( \mu \)l/\( \mu \)g protein/ml) and markers were loaded using gel loading buffer. It was allowed to run at 50 V for almost 2.5 h. After electrophoresis the gel was stained both with silver nitrate and CBB-250 and molecular weight of the purified inhibitor \( \alpha \)-AIwas estimated by comparing its band with the molecular weight marker.

### 2.2.6 Determination of molecular weight of \( \alpha \)-AI by gel filtration:

The molecular weight of the \( \alpha \)-AI was also estimated by gel filtration on a sephadex G-50 column (26 X 1.2cm, L x dia). The column was pre-equilibrated with physiological saline (0.85 \% NaCl). The column was calibrated using the following proteins:

- Bovine serum albumin (\( \text{Mr} = 66,000 \) Da)
- Pepsin (\( \text{Mr} = 35,000 \) Da)
- Papain (\( \text{Mr} = 23,406 \) Da)
- Lysozyme (\( \text{Mr} = 14,300 \) Da)

A calibration curve (Log MW \( \times 10^4 \) versus \( V_e/V_0 \)) was plotted wherein \( V_e \) = elution volume at which the protein peak is obtained and \( V_0 \) = void volume as determined by using blue dextran.

Purified \( \alpha \)-AI (2 mL, 1.0 mg) was applied on the gel filtration column and fractions of 3 mL each were collected. Each fraction was analyzed for the protein content and for the alpha amylase inhibitory activity. The molecular weight of the \( \alpha \)-AIwas determined by extrapolating the \( V_e/V_0 \) value on the calibration curve.
2.2.7 Determination of carbohydrate content in purified α-AI:

The carbohydrate content in the purified α-AI was determined both qualitatively and quantitatively. The quantitatively estimation of carbohydrate content in α-AI was done by phenol-sulfuric acid method of Dubois et al (1956) whereas, qualitatively it was confirmed by gel protein staining method described by Gander (1984). The protein was loaded and resolved on 12% denaturing polyacrylamide gel. The resolved gel was incubated with fixing solution (25% isopropanol, 10% acetic acid, and 65% water) with gentle shaking. It was transferred to equilibration solution (0.2% thymol blue (w/v) in fixing solution). It was then transferred to staining solution comprising 80% sulphuric acid and 20% ethanol. The reddish-brown band of glycoproteins against light background was visualized and documented.

2.2.8 Studies on stability of α-Amylase inhibitor:

Majority of inhibitor protein are reported to be stable over a wide range of pH and temperature (Fronco et al., 2000). For determining the pH stability of the isolated α-AI, it was incubated (200 µg in 150 µl) with buffers of different pH (100 mM acetate pH 4.0 & pH 5.0, 100 mM citrate phosphate pH 6.1, 100 mM phosphate pH 6.9, 100 mM Tris HCl pH 8.0 & pH 9.0) for 20 h at 4°C. The α-AI was then assayed as described earlier at optimum conditions. Similarly, for temperature stability, the isolated α-AI (200 µg, 150 µl) was pre-incubated in 20 mM phosphate buffer (pH 6.9) in total reaction volume of 1 mL at different temperatures (25, 30, 35, 37, 40, 45, 50, 60, and 70°C) for 10 min. The α-AI activity was then assayed at optimum conditions.

2.2.9 Kinetic Studies on α-AI:

The α-amylase activity at different starch concentration was determined in presence and absence of α-AI. For this purpose C. chinensis larval amylase was used. The assay was done in 50 mM phosphate buffer (pH 6.9). Alpha-amylase (200 µg) was
incubated with α-AI (200 µg) in phosphate buffer (pH 6.9, 10 mM) at 37°C for 45 min in a total reaction volume of 0.9 mL before initiating the reaction with different concentrations of the substrate (0.1% starch solution). Direct and double reciprocal plots of α-amylase activity and starch concentration were drawn to determine the Km and Vmax of the enzyme in presence and absence of inhibitor. Inhibitor constant (Ki) for inhibition of α-amylase by the isolated α-AI was calculated by the following formula and Km & Vmax were obtained from the double reciprocal plots

\[ V_{\text{max}}(i) = \frac{1}{V_{\text{max}}} \left(1 + \frac{I}{K_i}\right) \]

Where:

- \( V_{\text{max}}(i) \) = maximum velocity of the inhibited enzyme reaction.
- \( V_{\text{max}} \) = maximum velocity of the uninhibited enzyme reaction.
- \( I \) = concentration of the inhibitor.
- \( K_i \) = inhibitory constant.

### 2.2.10 Chemical modification studies:

For having some insight into the role of specific amino acid(s) in inhibitory activity, isolated α-AI was incubated with chemical(s)/compound(s) which specifically modify an amino acid/group/bond (Nagaraj and Pattabiraman, 1985). A list of amino acid/group/bond modifying chemicals/compounds is given in Table 2.2. The inhibitor protein (200 µg) was incubated separately with the chemical modifier (500 µg CHD, 200 µg ninhydrin, 500 µg DTNB and 1% 2 mercaptoethanol) in 20 mM phosphate buffer (pH 6.9) in a reaction volume of 1 mL. After 10 minutes, the mixture was dialyzed against distilled water. The α-AI activity was then assayed as described earlier.
Table 2.2: A list of amino acid/group/bond modifying chemicals/compounds.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Amino acid/group / bond</th>
<th>Modifying compound</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Sulphydryl group</td>
<td>5,5'-dithiobis 2-nitrobenzoic acid (DTNB)</td>
<td>Ellman (1559)</td>
</tr>
<tr>
<td>3</td>
<td>Disulphide bond</td>
<td>2-marcaptoethanol</td>
<td>Nagaraj and Pattabiraman (1985)</td>
</tr>
</tbody>
</table>
2.2.11 Protease inhibitory activity of isolated α-AI:

For this purpose, different protease like pepsin, trypsin, chymotrypsin and collagenase (200 µg each) were pre-incubated with purified α-AI (200 µg) in 1.5 ml of appropriate buffer at 37°C for 10 min. This was followed by the addition of casein (1%, 1 ml) to it and the mixture was allowed to stand at 37°C for 10 min. The reaction was terminated by addition of TCA (5%). The tubes were kept for 30 min at room temperature and filtered through Whatmann filter paper no.1. To 1 mL of filtrate, 5 mL of 0.4 M sodium carbonate was added which was followed by addition of 0.5 mL folin phenol reagent and incubated for 10 min at 37°C. Absorbance of the filtrate was measured at 660 nm in a UV-Visible spectrophotometer (Shimadzu, Japan) against a suitable control. The activities of protease in the absence of α-AI were also measured under similar conditions and the two were compared.

2.2.12 Detection of α-AI by Starch PAGE:

The purified α-AI was resolved electrophoretically on 7% native starch polyacrylamide gel containing 0.1% soluble starch. After electrophoresis, the native gel was equilibrated with 20 mM phosphate buffer (pH 6.9) containing 1.5 mM NaCl for 5-10 min. After equilibration the gel was placed in solution of different amylases (Bacillus species, C. chinensis, H. armigera and Human saliva) for 30 min at room temperature. After incubation the gel was rinsed briefly with deionized H₂O to remove amylase and placed in iodine solution for 2-5 min followed by washing with deionized H₂O. The α-AI on the starch gel was visible as a dark band against a light background which was recorded.
2.2.13 Scanning Electron Microscopy:

Scanning electron microscopy of starch granules subjected to hydrolysis by the insect \(H.\ \text{armigera}\) and \(C.\ \text{chinensis}\) amylases, both in presence and absence of isolated and purified \(\alpha\)-AI, was done to determine their pattern of degradation in vitro. Sample preparation was done as per the procedure of Meireles et al., (2009). The procedure, in brief is given below:

Starch was incubated with insect amylases separately for 10 min after which reaction was terminated by 1 mL 90% ethanol. The mixture was allowed to dehydrate and granules were mounted on specimen stub area. Samples were then coated with gold and observed under Scanning Electron Microscopy (FESEM, Model: 54800, Hitachi, Japan) at 12 kV.

In another case, insect \(\alpha\)-amylase(s) were pre-incubated with isolated \(\alpha\)-AI for 10 min and then added to the starch solution. Rest of procedure was same as described above.

2.2.14 Effect of salt on alpha amylase inhibitor activity:

The effect of salts like NaCl, KCl and I\(_2\) on \(\alpha\)-AI was studied by pre-incubating them (0.2 to 1 mg salt & 200 \(\mu\)g inhibitor protein) for 30 min at 37\(^\circ\)C in sodium phosphate buffer (20 mM, pH 6.9). The reaction mixture was further incubated with 200 \(\mu\)g of alpha amylase for 10 min and 1 mL (0.1%) starch solution was added and kept at 37\(^\circ\)C for 10 min. The reaction was stopped by adding 1 mL (DNS) reagent, then the contents were heated in a boiling water bath for 5 min. A blank was set without \(\alpha\)-AI extract and salts, another one without amylase enzymes which were replaced by equal quantities of extraction buffer in the reaction medium. The absorbance was measured at 530 nm.
2.2.15 Isolation of insect α-amylase(s):

Isolation of alpha amylase from larvae of *C. chinensis* was done as per Bernfeld et al (1955). Infested green gram seeds were dissected and the active larvae (50 no) were homogenized in 1 mL extraction buffer (20 mM sodium phosphate (pH 6.9), 300 mM sodium chloride and 2 mM calcium chloride followed by centrifugation at 15,000 rpm for 30 min at 4°C. The supernatant was collected and stored at -20°C. This was used as enzyme source in further experiments. The concentration of protein in the supernatant was determined by the method of Lowry et al (1951).

Similarly, α-amylase from larvae of *H. armiger* was isolated by surgically removing the midguts in 0.9% NaCl under ice cold conditions. Midguts were homogenized using ice-cold pestle and mortar with 20 mM NaCl and 0.1 mM CaCl₂. The homogenate was centrifuged at 15,000 rpm for 20 min at 4°C. The supernatant was collected and stored at -20°C. This was used as enzyme source in further experiments. This supernatant was also used to estimate the protein content in the supernatant was estimated by the method of Lowery et al (1951).

2.2.16 In gel visualization of insect amylase activity:

The α-amylase activity in crude larval homogenates of *C. chinensis* and *H. armigera* was visualized in gel as described by Kotkar et al (2009). After infestation, grains of green gram, black gram, mix diet, math bean, cowpea, red gram and pigeon pea were dissected and the larval amylase was prepared as described above. It was resolved separately using 10% native PAGE with a (4%) stacking gel. The resolving gel buffer contained 1.5M Tris-Cl (pH 8.8) while stacking gel buffer contained 1.0M Tris-HCl (pH 6.8). The electrode buffer comprised 25 mMTris and 250 mM glycine (pH 8.3). The gels were run using pre-cooled buffer until the blue dye reached the end in a cold room at 4°C, approximately for 3.5 h at a constant voltage of 100 V. For
visualization of amylase activity, the gel was incubated in starch (1%) prepared in 0.02M sodium phosphate buffer (pH 6.9) containing 10mM NaCl for 30 min at 37°C, briefly rinsed in water and amylolytic activity was stopped by transferring the gel to the staining solution (10 mM I<sub>2</sub> in 14mM KI) for 5 minute. Excess I<sub>2</sub> was washed off with water and light band against a dark background indicated presence of amylase isoforms. In-gel inhibition assay were performed by pre-incubating the gel for 30 min in purified α-AI and then in starch (0.1%) for 30 min at 37°C followed by staining solution to visualize the amylase activity bands as described above.

**2.2.17 Determination of invivo α-AI activity:**

**(A) C. Chinensis:**

The evaluation of inhibitory activity of the isolated protein invivo was done by reconstituting the grains after incorporating the isolated α-AI protein. Briefly, isolated inhibitor protein was mixed with finely powdered green gram, black gram, red gram, pigeonpea and soybean @ 200 µg per gram. Water in required quantity, was then added to each test material, mixed thoroughly into dough and cake of 6 mm thickness was prepared. Pellets were cut from this cake with teflon tube (5 mm diameter x 6 mm height) and they were dried in an oven at 55 °C overnight. Before commencing bioassays the dried pellets were held in controlled environment room 25±2°C and 75±5% RH for 24h to equilibrate them to 10±2 moisture content.

Ten pre-conditioned pellets (green gram, red gram, black gram, pigeon pea and soybean)(approximately 2 g) were placed in each plastic jar and incubated along with two pairs of freshly emerged adult of *C. chinensis* for 3 days. After 3 days adults were removed and the jar along with pellets seeded with eggs were left in the same environment until adult emergence.
A pair of insects emerged (F1 progeny) from various treatments in the above set were released on 10 grain seeds in plastic jars. Longevity of adults, fecundity of female, development and emergence of adults (F2 progeny) were studied and recorded.

(B) *H. armigera*:

For determination of effect on *H. armigera* larvae, bioassays were conducted by feeding early 3rd instar larvae on a control and test diet. The test diet contained purified inhibitor protein @ 200 µg/ml. Twenty early third instars larvae were reared on this diet and their rate of pupation and mortality were recorded at 24 h intervals. The assay was started at early 3rd instar stage and continued up to pupation. The larvae surviving after the first day continued to be fed on the same diet on the second and subsequent days. The experiment was done in triplicate and repeated at least three times. After periodic interval, amylase activity from the midgut of larvae feeding on test diet was determined both qualitatively (based on native PAGE) and quantitatively by DNS method (Bernfeld, 1955).

2.2.18 Determination of nutritional indices:

Effect of α-AI mixed diet on different nutritional indices of *H. armigera* larvae were measured on the dry weight basis. The quantity of food ingested was determined in control (without inhibitor) and test groups (with inhibitor). The following formulae were used to calculate the CI (consumption index), AD (approximate digestibility), ECI (efficiency of conversion of ingested food) and ECD (efficiency of conversion of digested food) (Waldbauer, 1968):

\[
CI = \frac{E}{A}
\]

\[
AD = \frac{E - F}{E}
\]

\[
ECI = \frac{P}{E}
\]

\[
ECD = \frac{P}{E - F}
\]
Where, \( A \) = mean dry weight of insect over unit time, \( E \) = dry weight of food consumed, 
\( F \) = dry weight of food consumed, \( F \) = dry weight of feces produced and \( P \) = insect dry weight gain.

### 2.2.19 Differential susceptibility of grain legumes to *C. chinensis*:

The basis of differential susceptibility of grain legumes to *C. chinensis* infestation was evaluated as function of following criteria:

(A) **Physical characteristic of grain legumes:**

Variations in some physical characteristics like colour, shape, size, texture; seed coat thickness (with Vernier caliper) and weight of seeds of different grain legumes were investigated to analyze the differential behavior of *C. chinensis* on these seeds.

(B) **Analysis of nutritional factors in grain legumes:**

**Sample preparation:** The dried grains were finely grounded in grinder to make fine powder and powdered samples were used for estimation of various nutritional constituents in different grain legumes.

(i) **Estimation of reducing sugars:** 100 mg of powdered sample of each grain legume was extracted with 80% ethanol twice (5 mL each time). Supernatant was collected and evaporated by keeping it in water bath at 80°C. 10 mL distilled water was added to dissolve the sugars. 0.1 mL aliquots from each tube were taken in separate test tubes and total reducing sugars were estimated as per Miller, (1959).

(ii) **Estimation of moisture content:** Ground sample were analyzed for moisture (AOAC, 2000). Briefly, 1 g whole grain sample of each pulse was taken in a pre-weighed dish and kept at 105°C for 2 h. Loss in its weight after drying was recorded by taking a final weight and moisture content estimated on the basis of % weight of loss.
(iii) Germination: 10 seeds of each grain legume were soaked in water for 2 h and grains were placed on moist filter papers (Whatmann No. 2, 5 cm diameter) kept in the bottom of a petri plates (5.5 cm diameter X 1.2 cm). Germinated grains were counted and % germination was recorded.

2.2.20 Study of chronology of infestation of insect and microbial pests in grain ecosystem:

Two thousand green gram seeds (around 200 g) were infested with a pair of C.chinensis adults (1 male and 1 female) in plastic jars. The infestation trend of C.chinensis was studied over a period of 1 year by taking monthly observation. The observation included measurement of moisture content of the grains, insect number, microbial count, number of grains damaged and undesirable odour.

Statistical analysis:

Data was expressed as mean ± S.D. Statistical analysis was carried out by repeated measure ANOVA followed by post hoc Dunnett’s multiple comparison tests (GraphPadInStat version 3.00 for Windows Vista TM BASIC). P<0.05 is considered statistically significant.

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