3.0 Summary

This chapter includes the outcome of the investigations carried out. Where ever possible, the obtained results have been adequately discussed in the light of available literature. The results are broadly presented in three parts, (i) isolation, purification and partial characterization of α-AI from seeds of Amaranthus paniculatus, commonly known as ‘Rajgira’, (ii) activity of obtained α-AI against enzyme α amylase from different sources and (iii) possible role of the α-AI in the pest management against two economically important pests.

******
3.0 Results and Discussion:

3.1 Screening of various members of family Amaranthaceae for the presence of α- Amylase inhibitor activity:

Plant family Amaranthaceae has the most species rich lineage comprising 180 genera and approximately 2500 species. Six plants namely, *Amaranthus paniculatus*, *Achyranthus aspera*, *Celosia arentaeae*, *Amaranthus tricolor*, *Amaranthus spinosa* and *Alternanthera sessilis*, belonging to family Amaranthaceae, were short listed and α amylase inhibitor (α-AI) activity in different parts (seeds, leaves, stems and roots) of them was determined both qualitatively and quantitatively. Qualitative detection of α-AI activity was done by using starch agar plate method and the zones of starch hydrolysis of varying diameters were observed in the extracts of all the plants screened (Figure 3.1). Of the various parts of members of family Amaranthaceae screened, seeds of *Amaranthus paniculatus* showed lowest starch hydrolysis (highest inhibition) on the starch agar plate (Figure 3.1).

3.2 Alpha amylase inhibitor Assay:

The α-AI activity in extracts of different parts of the short listed plants was also determined quantitatively. Of the six plants, the α-AI activity was found to be more in different parts of *A. paniculatus* except in stem. Seeds of *A. paniculatus* showed highest (76.25) relative inhibitor activity followed by leaves (67.50), roots (63.50) and stem (61.50). When compared with the activity from different parts of the other members investigated, it was observed that i) seeds of *A. paniculatus* showed the highest α amylase inhibitory activity, followed by seeds of *Achyranthus aspera* and leaves of *A. paniculatus* and *A. aspera* in decreasing order. The results corroborate well with the results of qualitative tests performed above. The names of the plants screened, plant parts used and α -amylase inhibitory activity in them are shown in Table 3.1.
Figure 3.1: Qualitative screening of α-AI from different parts of Rajgira plant,
- I = Seeds, II = Leaf, III = Stem, IV = Root and V = No inhibitor.
Table 3.1: Quantitative determination of $\alpha$-amylase inhibitor from different parts of six members of family Amaranthaceae.

<table>
<thead>
<tr>
<th>Sr.No</th>
<th>Name of the plants</th>
<th>$\alpha$-AI activity (Relative units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Seed</td>
</tr>
<tr>
<td>1.</td>
<td><em>Amaranthus paniculatus</em></td>
<td>76.25</td>
</tr>
<tr>
<td>2.</td>
<td><em>Achyranthus aspera</em></td>
<td>70.00</td>
</tr>
<tr>
<td>3.</td>
<td><em>Celosia argentea</em></td>
<td>67.50</td>
</tr>
<tr>
<td>4.</td>
<td><em>Amranthus tricolor</em></td>
<td>ND</td>
</tr>
<tr>
<td>5.</td>
<td><em>Amranthus spinosa</em></td>
<td>ND</td>
</tr>
<tr>
<td>6.</td>
<td><em>Alternanthera sessilis</em></td>
<td>ND</td>
</tr>
</tbody>
</table>

ND= Not determined
Many edible plant seeds contain substances that inhibit enzymes, especially hydrolases. Most of these compounds are proteins by nature, which specifically inhibit enzymes by forming complexes that block the active site or alter enzyme conformation, ultimately reducing the catalytic function (Kokiladevi et al., 2005). Substances present in the seeds of *Phaeolus vulgaris* suggested to play a role in insect resistance include heteropoly saccharides, lectins, and protease- and amylase inhibitors (Applebaum et al., 1969). It has also been suggested that the various forms of resistance are mainly due to the presence of genes of the lectin family (Chrispeels and Raikhel, 1991). Alpha-amylase inhibitors have been studied in several common beans (Marshall and Lauda, 1975) maize (Blanco-Labra and Iturbe-Chinas, 1981), sorghum (Kutty and Pattabiraman, 1986), wheat (Warchalewski, 1977), and barley (Mundy and Rogers, 1986). Results observed in the present study are totally in accordance with earlier reports.

### 3.3 Purification of α-AI from seeds of A. paniculatus:

Of the various parts used as source of α-AI, the activity was found to be the highest in the seeds of *A. paniculatus*, commonly called as ‘Rajgira’. Accordingly, these seeds were used for the isolation, purification and characterization of α-AI in present study. The α-AI from ‘Rajgira’ seeds was purified by a four steps published but suitably modified procedure and a typical purification profile is given in Table 3.2.

Crude protein extract of *A. paniculatus* seeds was precipitated using ammonium sulphate at different levels of saturation. Greater α-amylase inhibitory activity (85%) was observed in the fraction precipitating between 80 and 100% ammonium sulphate saturation. This fraction was re-suspended in phosphate buffer (pH 7.0) and subsequently applied to sephadex G50 column (2.5 x 10 cm). The elution profile of
### Table 3.2: Purification profile of α-AI from *A. paniculatus* (Rajgira) seeds

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Steps</th>
<th>Volume (ml)</th>
<th>Yield (%)</th>
<th>Total protein (mg)</th>
<th>α-AI Units</th>
<th>Specific activity (units/mg protein)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Seeds crude extract</td>
<td>187</td>
<td>100</td>
<td>748</td>
<td>10397</td>
<td>13.9</td>
<td>1</td>
</tr>
<tr>
<td>2.</td>
<td>Ammonium sulphate precipitation (80-100 %)</td>
<td>25</td>
<td>28</td>
<td>211</td>
<td>5865</td>
<td>27.8</td>
<td>2</td>
</tr>
<tr>
<td>4.</td>
<td>CM Cellulose</td>
<td>10</td>
<td>2.0</td>
<td>15</td>
<td>3367</td>
<td>224.5</td>
<td>16.15</td>
</tr>
</tbody>
</table>
A. paniculatus showed a major peak followed by a minor peak (Figure 3.2A). The fractions of major peak (fractions 10-16; 25 ml) showing inhibitory activity were pooled and subjected to purification in a column of CM-cellulose equilibrated with the same buffer at room temperature. Bound protein was recovered from the column by elution with a gradient (0.1 - 0.5 M) of sodium chloride in 10 mM acetate buffer (pH 7.0) in fraction of 10 ml each. Inhibitor was eluted at approximately 0.2 M sodium chloride concentration as peaks, both of protein and inhibitory activity were obtained at it. The α-AI activity in different fractions collected during the chromatography was found to be almost proportional to the protein content, measured at 280 nm, in them. The fractions containing inhibitor were combined and dialyzed against water, and this was used for characterization of the inhibitor. The elution profile of the inhibitor protein through CM-Cellulose column is represented in (Figure 3.2B). The four stage process resulted in slightly more than 16 fold purification of the α-AI from seeds of A. paniculatus. Examination of samples of the inhibitor prepared in this way, by electrophoresis both on native and denatured polyacrylamide gels showed a single protein band, indicating that the inhibitor is homogeneous and a monomer (Figure 3.3 and 3.5A). Seven point five (7.5) to eighteen point five (18.5) fold purification has been reported by different procedures and different sources (Ho and Whitakar, 1993; Kokiladevi et al., 2005; Hivrale et al., 2011) and our results are in agreement with these reports.

3.4 Characterization of purified α-AI:

3.4.1 Determination of molecular weight of isolated α-AI:

The purified protein from the step-4 of the purification procedure was loaded on a 15% SDS-PAGE along with low molecular weight markers. The purified protein, after silver and coomasie brilliant blue (CBB) staining, showed a single band matching with 14.3 kDa protein band of the marker (Figure 3.3).
Figure 3.2: Elution profile of α-AI from column of (A) sephadex G-50 and (B) CM cellulose.
Figure 3.3: Molecular weight determination of α-AI isolated and purified from seeds *A. paniculatus* (Rajgira). Purified protein (20 μg) was separated on 15% SDS-PAGE and developed by coomassie brilliant blue and silver staining. Lane A: silver staining, Lane B: coomassie blue staining, Lane M: molecular weight marker.
The molecular weight of isolated α-AI was also determined by gel filtration technique. The purified α-AI along with four different proteins of known molecular weight, Bovine serum albumin (66000 Da); Pepsin (35000 Da); Papain (23406 Da) and lysozyme (14300 Da), were eluted through a column of sephadex G-50. The molecular weight of the purified α-AI was calculated to be 14.3 kDa by plotting log molecular weight of known proteins against ratio of elution volume of protein peak and void volume as shown in (Figure 3.4). The α-AI purified from P. vulgaris and other sources has been shown to be an oligomer with three unidentical subunits (Mirkov et al., 1995; Janarathanam et al., 1999; Gupta et al., 2012). Similarly, a heat labile α-AI from white kidney bean was reported to be composed of three subunits (Yamaguchi, 1993; Wato et al., 2000). However, Suzuki and Ishimoto (1999) reported four subunit and Sawada et al (2001) obtained a dimer from P. vulgaris. Our results are in agreement with earlier studies in rye (Iulek et al., 2000) and P. vulgaris (Yang et al., 2008), wherein the α-AI was found to be a monomer as revealed by a single band in SDS-PAGE (Figure 3.3).

Carbohydrate content in the whole α-AI protein was found to be 3.5%. The presence of carbohydrate moiety on the proteinaceous inhibitor molecule was further confirmed by glycoprotein staining which indicated presence of carbohydrate in the α-AI protein (Figure 3.5B). Majority of inhibitors obtained from different sources have been reported to be glycoprotein with carbohydrate content of upto 10% (Marshall et al., 1975; Sawada et al., 2001 and Yang et al., 2008). It is speculated that the carbohydrate moiety in the protein is responsible for inhibitory action on α-amylases by combining at the substrate binding site of the enzyme. Moreover, when the nature of inhibition is non-competitive, it is probable that protein-protein interactions may also be involved in maintaining the stability of enzyme-inhibitor complex.
Figure 3.4: Determination of molecular weight of α-AI by gel filtration chromatography.
Figure 3.5: A) Native PAGE of purified α-AI from Rajgira seed and (B) detection of glycoprotein nature of inhibitor on 15% SDS-PAGE.
The nature of carbohydrate moiety being present in α-AI glycoprotein and effect of its removal on the inhibitory action have not been investigated in majority of cases (Marshall et al., 1975; Gupta et al., 2012).

3.4.2 Stability studies of isolated α-AI:

The assay for α-AI activity was standardized and it was observed that the best α-AI activity is seen at pH of 6.9, temperature 37°C and incubation period, with α-amylase, of 10 min. To determine the effect of pH and temperature on the stability of α-AI protein, it was incubated at different pH and temperatures. After incubation for 10 min, the α-AI activity was assayed at the optimum conditions of different parameters mentioned above (pH 6.9, temp 37°C and incubation period of 10 min). The α-AI protein was found to be fairly stable over a wide pH range of 4–9 (Figure 3.6A) and temperature range of 25–50°C (Figure 3.6B). Many α-AIs have been shown to be stable over a wide pH and temperature range (Sasikiran et al., 2004; Hivrale et al., 2011) and many others heat labile (Grant et al., 1995 and Gupta et al., 2012) The relatively good temperature, pH stability and low molecular weight indicated that the isolated α-AI may be a member of knottin like α-AI class (Franco et al., 2002). One possible reason of relatively high stability of α-AI protein could be presence of multiple disulphide bond in it (Hivrale et al., 2011).

3.4.3 Determination of nature of inhibition of α-amylase by purified α-AI:

For this purpose, α-amylase activity was determined both in presence and absence of the isolated inhibitor at different substrate concentrations of the enzyme. The obtained rates of reaction were plotted against the substrate concentration used. Both the direct as well as double reciprocal plots yielded the classical noncompetitive inhibitor profile (Figure 3.7). In the double reciprocal plot, the km of the enzyme, α-amylase, both in presence and absence of inhibitor remained unchanged at 370 µM.
Figure 3.6: Stability profile of purified α-AI protein as a function of A) pH and B) temperature.
Figure 3.7: A. Double reciprocal plot and B. Direct plot of the non-competitive inhibitory activity of the purified α-AI protein from Rajgira seeds against α-amylase C. Chinensis.
whereas, the Vmax in presence of inhibitor declined to 100 from 294.1 µmol min$^{-1}$ in its absence. The Ki was calculated to be 2.06 µg. The inhibition pattern of α-AI from different sources has been found to be non-competitive type primarily (Marshall and Lauda, 1975; Frels and Rupnow, 1985; Gupta et al., 2012). However the nature of inhibition by α-AI from Yam bean was found to be uncompetitive type (Sharma and Pattabiraman, 1982).

3.4.4 Effect of chemical Modifiers on α-amylase inhibition potential of isolated inhibitor molecule:

To gain some insight into the role of amino acids/groups in the inhibitory activity of the isolated protein, the effect of four different chemical modifiers of amino acids on the biological activity of isolated α-AI against α-amylase, obtained from C. chinensis, was studied. In present investigation, ninhydrin, 2-mercaptoethanol, cyclohexane dione (CHD) and 5,5'-dithiobis 2-nitrobenzoic acid (DTNB) were used as modifying agents. Under normal conditions (without any chemical modifier), the isolated α-AI, at 200 µg concentration, could inhibit 72% activity of α-amylase isolated from C. chinensis. The data of the effect of these modifiers on activity of α-AI isolated from seeds of A. paniculatus is presented in Table 3.3. The α-AI was pretreated for 10-60 minutes (at an interval of 5 minutes) with each of the chemical agents.

All the chemical modifier except DTNB, in initial phase of incubation i.e. up to 25 minutes, could slightly reduce the inhibition potential. However, the reduction was not significant (~2-4%). After 25 minutes the α-AI regained its potential to inhibit α-amylase and at 60 minutes incubation the inhibition potential was found to be almost same as that of untreated inhibitor. Ninhydrin as well as CHD are reported to be responsible for causing rapid loss of action of inhibitor which require arginyl group for activity (Chaplin, 1976). The disulphide bridges if any, did not
Table 3.3: The effect of chemical modifiers on the activity α-AI isolated from seeds of *A. paniculatus*.

<table>
<thead>
<tr>
<th>Time interval (min)</th>
<th>Inhibition (% of Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ninhydrin</td>
</tr>
<tr>
<td>10</td>
<td>68.56 ± 1.3</td>
</tr>
<tr>
<td>15</td>
<td>68.71 ± 0.9</td>
</tr>
<tr>
<td>20</td>
<td>69.13 ± 2.1</td>
</tr>
<tr>
<td>25</td>
<td>69.89 ± 1.8</td>
</tr>
<tr>
<td>30</td>
<td>70.12 ± 2.4</td>
</tr>
<tr>
<td>35</td>
<td>70.56 ± 1.6</td>
</tr>
<tr>
<td>40</td>
<td>71.24 ± 1.1</td>
</tr>
<tr>
<td>45</td>
<td>71.80 ± 3.1</td>
</tr>
<tr>
<td>50</td>
<td>72.38 ± 1.5</td>
</tr>
<tr>
<td>55</td>
<td>72.79 ± 1.9</td>
</tr>
<tr>
<td>60</td>
<td>72.86 ± 2.2</td>
</tr>
</tbody>
</table>
appear to play a role in activity since 2-mercaptoethanol did not alter the inhibitor potential. Since DTNB had no effect on the inhibition activity of α-AI studied, it appeared that free sulphydryl group also are not essential for its biological activity (Nagaraj and Pattabiraman, 1985).

**3.4.5 Protease inhibitory activity of isolated α-Amylase Inhibitor:**

The isolated α-AI in present study was also tested for its protease inhibitory action to check for its bi-functional nature (α-Amylase and Protease inhibitory potential). For this study, four different proteases viz. i) Trypsin; ii) Chymotrypsin; iii) Collagenase (type I) and iv) Papain were used. The isolated α-AI was incubated with all these proteases at the conditions previously optimized for the inhibitor molecule. The protease inhibition data of α-AI under investigation is presented in Table 3.4. The data suggest that the present protein is not bifunctional as it could not inhibit any of the proteases significantly. The highest inhibition (9.63 %) was observed in case of trypsin, whereas, it could not inhibit Collagenase (type I) at all (Table 3.4).

The characteristics of partly characterized α-AI from seeds of *A. paniculatus* are shown at a glance in (Table 3.5).

**3.5 Inhibitory activity against α-amylases from different sources:**

For the purpose of screening of presence of α-AI in different parts of short listed plants, enzyme diastase was used. The activity of isolated, purified and partly characterized α-AI from seeds of *A. paniculatus* was determined against α-amylases obtained from different sources viz; human saliva, *Bacillus* species, larvae of pulse beetle and *H. armigera* (Figure 3.8). Alpha-AI of *Bacillus* species was found to be rapidly inhibited even at very low concentration of the inhibitor protein followed by insect gut amylases, *H. armigera* and *C. chinensis* in increasing order. At the
Table 3.4: Evaluation of protease inhibitory activity of isolated $\alpha$-Amylase inhibitor

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Protease enzyme</th>
<th>Activity (μmole casein hydrolyzed/min)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Trypsin</td>
<td>463.4</td>
<td>9.63</td>
</tr>
<tr>
<td></td>
<td>Trypsin + $\alpha$-Al</td>
<td>418.8</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Chymotrypsin</td>
<td>333.92</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>Chymotrypsin + $\alpha$-Al</td>
<td>331.25</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Callagenase</td>
<td>455.35</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Callagenase + $\alpha$-Al</td>
<td>457.14</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Papain</td>
<td>295.53</td>
<td>1.81</td>
</tr>
<tr>
<td></td>
<td>Papain + $\alpha$-Al</td>
<td>290.17</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.5 Characteristics of isolated α-AI from seeds of *A. paniculatus*.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td>14.3 kDa (SDS-PAGE &amp; Gel permeation chromatography)</td>
</tr>
<tr>
<td>Carbohydrate content</td>
<td>3.5%</td>
</tr>
<tr>
<td>pH stability</td>
<td>Yes (Range 4-9)</td>
</tr>
<tr>
<td>Temperature stability</td>
<td>Yes (Range 25-50°C)</td>
</tr>
<tr>
<td>Nature of inhibition against α-amylase</td>
<td>Noncompetitive</td>
</tr>
<tr>
<td>Amino acids/ Functional groups</td>
<td>Arginyl,-SH &amp; -S-S not required for action</td>
</tr>
<tr>
<td>Protease inhibitory activity</td>
<td>No protease inhibitory activity (Not bifunctional in nature)</td>
</tr>
</tbody>
</table>
Figure 3.8: Alpha amylase inhibition activity of isolated inhibitor protein against α-amylases obtained from i) Bacillus species (BS); ii) Helicoverpa armigera (HGA); iii) Callosobruchus chinensis (CC) and iv) Human saliva (HS)
maximum concentration of the inhibitor studied (200 µg), α-amylase from Bacillus 
species was inhibited to the tune of almost 90% whereas, human salivary amylase was 
found to be the least inhibited showing only 30% inhibition at that concentration. The 
insect gut amylases were inhibited to the tune of approximately 70% under identical set 
of conditions. These finding were corroborated by starch gel detection method wherein 
bands of varying colour intensity of starch iodine complex were visible due to inhibition 
of different α-amylases (Figure 3.9). The ability of α-AI to inhibit α-amylases from 
different sources can be selectively utilized. For example, α-AI which inhibit human 
salivary amylase can be utilized in prevention and therapy of obesity, and as a template 
for drug design targets in diabetic treatment. Many isolated α-AIs from different 
sources have shown this characteristic (Yoshikawa et al., 1999; Iulek et al., 2000; 
Heidari et al., 2005; Hivrale et al., 2011; Gupta et al., 2012). On the contrary, no or less 
activity against human salivary α-amylase is a desirable characteristic in α-AIs to be 
utilized in pest management strategies. Many proteinaceous α-AIs from chick pea, 
kidney bean, maize, wheat, and millet seeds show this characteristic (Khan, 2011).

3.6 Scanning Electron Microscopic analysis inhibition of starch 
hydrolysis by α-AI:

Scanning electron microscopy images of soluble starch granules that had been 
treated with two different amylases, prepared after homogenizing mid gut and entire 
larvae of H. armigera and C. chinensis, respectively and that of granules treated with 
enzymes in presence of isolated inhibitor were obtained to determine the effect on 
pattern of hydrolysis. The images of starch granules treated with enzymes in presence 
and absence of inhibitor were taken after 10 minutes of incubation. Treatment of starch 
granules with enzymes obtained from both the sources resulted in similar patterns of 
granule degradation (Figure 3.10). From the images it was clear that when
Figure 3.9: In gel detection of purified α-AI from seeds of *A. paniculatus* (Rajgira) against different α-amylases. Purified α-AI (20 µg) was loaded on 7% native starch polyacrylamide gel containing 0.1% soluble starch and allowed to interact with amylases from different sources, lane 1) *Bacillus species* (BS); lane 2) *Helicoverpa armigera* (HGA); lane 3) *Callosobruchus chinensis* (CC) and lane 4) Human saliva (HS).
Figure 3.10: Scanning Electron Microscopy (SEM) of starch granule after \textit{in vitro} digestion I) larval amylase (\textit{C. chinensis}) II) larval amylase (\textit{C. chinensis}) with inhibitor III) larval amylase (\textit{H. armigera}) and IV) larval amylase (\textit{H. armigera}) with inhibitor.
treated with amylases from both the sources, surface part of the granule is hydrolyzed (Figure 3.10 I and 3.10 III). The pattern and extent of hydrolysis of the surface of granule is more or less similar suggesting the similar mechanism of hydrolysis even though the amylases are obtained from two different sources. It should be noted here that the topology of degradation of starch granules observed is as a result of 10 minutes incubation period which was optimized for determining the activity in in vitro. The granules digested in presence of isolated inhibitor were observed to be protected from degradation which can be seen clearly in Figure 3.10 II and 3.10 IV. SEM images of interaction of starch obtained from maize, potato and wheat with amylases of Tenebrio molitor and Zabrotes subfasciatus in vivo and invitro showed similar pattern of degradation but magnitude of degradation varied as a function of starch source and incubation time with amylases (Meireles et al., 2008). Our results are in agreement with these studies in terms of degradation pattern of amylases obtained from two different sources. Similar results were obtained for bacterial and plant α-amylases (Helbert et al., 1996; Gallant et al., 1997; Sarikaya et al., 2000 and Smith et al., 2005). The same pattern of granule degradation was also observed during in vivo and in vitro digestion of wheat starch granules by larval Tribolium castaneum amylases (Baker et al., 1992). A comparison of the digestion of starch granules isolated from two legume species V. unguiculata and P. vulgaris also showed that different α-amylases produced the same pattern of granules degradation (Silva et al., 2001a). Though, there are several reports on SEM based analysis of interaction of amylases with starch granules nobody have used SEM images to confirm the action of α-AI. In our opinion, confirmation of inhibitory action of isolated α-AI with the help of SEM is reported for the first time.
3.7 Effect of salts on α-AI activity:

Effect of different salts (NaCl, KCl and Iodine) at varying ionic strengths on α-AI activity of isolated and purified protein was determined with α-amylases from different sources, i) Bacillus species ; ii) Helicoverva armigera ; iii) Callosobruchus chinensis and iv) Human saliva. We observed that there was no effect of any of the salts at any ionic strengths studied on the inhibitory potential of the isolated α-AI with the amylase from different sources used and it remained intact even after treatment with various ionic strengths of NaCl, KCl and Iodine (Figure 3.11). The α-AI activity observed here was exactly the same to that of activity after following all the purification steps. On the contrary, Jane and John (1985) reported that the purified alpha amylase inhibitor loses its activity after dialysis and which could be restored by adding small amounts of NaCl to the medium. Similarly, O’Donnell and McGreeney (1976) also reported that increase in phosphate buffer concentrations from (0.1 to 50mM) did not have any effect on the degree of inhibition of salivary alpha-amylase by inhibitor isolated from wheat, while the addition of NaCl (1 mg) to phosphate buffer (0.1 mM), increased the activity from 68 to 85%. While supporting results, these researchers have claimed that an increase in salt ionic strength might facilitate inhibition by shielding charge groups on the enzyme or inhibitor, which could cause a conformational change or an alkaline shift at the optimum pH. However, our findings do agree with earlier reports and the reason behind this may be the ability α-AI to remain stable at a wide range of pH.
Figure 3.11: Effect of various concentrations of A) NaCl, B) KCl and C) I₂ on α-AI activity of isolated protein on amylases from *C. chinensis*, *H. armigera*, human saliva and *Bascillus spp.*
3.8 Role of α-AI in pest management:

3.8.1 Effect on *C. chinensis* (α-amylase activity):

*C. chinensis* (pulse beetle) is one of the two target pests chosen for evaluating the role of α-AI in its management. *C. chinensis* is an economically important post-harvest primary pest of grain legumes in our country. Of the stages in its life cycle, the larval stage is the most damaging, as it bores deep into grain, consuming the endosperm and making the grain unfit for human as well as animal consumption. Therefore, attempts were made to evaluate the changes in larval amylases of *C. chinensis* as a function of variation in diet. Five freshly emerged pairs (male and female) of *C. chinensis* were added in the jars containing five grams each of green gram, black gram, math bean, cowpea chickpea and pigeon pea. Simultaneously, five pairs of *C. chinensis* were also allowed to feed on five grams of mixture of equal quantities of all above grains.

All the grains used for this experiment were analyzed for total carbohydrate and soluble protein content (Figure 3.12). The legumes green gram, black gram, math bean, cowpea chickpea and pigeon pea contained significant amounts of soluble proteins and total carbohydrates. Total carbohydrate content was the highest in blackgram (58.60 gm%) and the lowest in soybean (20.90 gm%). Among all the grains evaluated the highest protein content was found in soybean (40.20 gm%) while chickpea showed the lowest protein content (18.97 gm%).

The presence of larval amylase was detected after 15 days of feeding on various grains and mixture of them qualitatively using PAGE. PAGE profiles revealed that same type of amylases are expressed in all the larvae feeding on different grains or mixture of them albeit in different quantities (as evident by the varying intensities of bands on gel). Interestingly, an additional fast moving amylase isoform (low
Figure 3.12: Total carbohydrate and soluble protein content of grains used for feeding assays. The graph represents values of carbohydrates and proteins (gm/100gm grains). Results are an average of three independent experiments conducted in duplicate. Error bars represent mean ± SD.
molecular weight) was observed in *C. chinensis* larvae feeding on green gram and mixture of all the grains (Figure 3.13A).

At the end of month, larval amylases of *C. chinensis* were isolated by crushing 50 larvae feeding on different grains and mixture as well. The protein content of all the larval homogenates was determined and equated to same concentration (20 mg/ml) that was required for evaluation of inhibition potency of isolated α-AI. The effect of purified inhibitor on digestive amylases of *C. chinensis* fed on six different gains and their mixture was determined by pre-incubating the two for 10 min followed by analysis by PAGE. The results depicted that isolated α-AI could efficiently and significantly inhibit the amylase that was expressed as a function of type of diet. The amylase isoform that was detected in the larvae feeding on green gram and mixture of grains was also inhibited and amylase activity for this was not detected in-gel. This isoform was already expressed in a very small quantity and that is why it may be completely inhibited (Figure 3.13 B).

### 3.8.2 Effect on *C. chinensis* (growth and development):

Having established the inhibitory activity of α-AI isolated and purified from Rajgira seeds against larval amylases of *C. chinensis*, we studied its effect on growth and development of pulse beetle (*C. chinensis*) under laboratory conditions.

Response of *C. chinensis* upon feeding with purified α-AI at 200 mg/gm dose was evaluated by reconstituting pellets out of flour of i) green gram; ii) black gram; iii) chickpea; iv) pigeon pea and v) soybean. Numbers of eggs laid on seeds and on the walls of jar were recorded. Number of adults emerged; their development time and weights of both male and female insects were recorded after 30 days. Similarly effect of α-AI feeding on first generation was also monitored. In this, parameters like
Figure 3.13: A) Larval amylase expression profile of *C. chinensis* feeding on I) Greengram; II) Blackgram; III) Mixture of Grains; IV) Moth bean; V) Cowpea; VI) Pigeon pea and VII) Chickpea. B) Inhibition of amylases of *C. chinensis* feeding on various grains by Rajgira α-AI
longevity (days), fecundity (eggs/female), number of adults emerged and developmental time (Days) were recorded.

In case of grains reconstituted out of flour of green gram and fortified with partially purified $\alpha$-AI (PP$\alpha$-AI) and purified $\alpha$-AI (P$\alpha$-AI), It was observed that the P$\alpha$-AI and P$\alpha$-AI reduced the total eggs laying by 20 and 25%, respectively over the control. They also retarded the adult emergence by one third and one fourth of control, respectively. The developmental time of the insects was also slightly increased as compared to the control. The weight of male insect was found to be reduced by nearly 23% and in case of female insect the weight reduction was to the tune of around 21% after feeding upon P$\alpha$-AI and P$\alpha$-AI proteins containing reconstituted grains (Table 3.6 A). Effect of P$\alpha$-AI and P$\alpha$-AI proteins on F1 generation of C. chinensis with respect to longevity, fecundity, adult emergence and development time was also recorded (Table 3.6 B). It was observed that longevity of F1 insects was reduced by 46% and 67% as a result of feeding on P$\alpha$-AI and P$\alpha$-AI proteins, respectively. Egg laying capacity of F1 females was found to be reduced drastically as a result of treatment with P$\alpha$-AI and P$\alpha$-AI proteins (87 and 95%, respectively). Adult emergence was also reduced by 90% in case of P$\alpha$-AI and P$\alpha$-AI didn’t allow any insect to emerge. Development time of insects was also observed to be increased by 10%. Effect of $\alpha$-AI proteins on the efficacy of C. chinensis in terms of ability to lay eggs and damage the grains in reconstituted grains of green gram is shown in (Figure 3.14).

Data presented in Table No. 3.7 A & B show the effect of P$\alpha$-AI and P$\alpha$-AI proteins supplemented through reconstituted grains made out of flour of Chickpea. Briefly, 38 and 50% reduction in number of total eggs laid, 70 & 88% reduction in adult emergence and 11% reduction in development time was noted for both P$\alpha$-AI
Table 3.6: Effect of feeding of PPα-AI and Pa-AI through reconstitute green gram grain on A) development of *C. chinensis* and B) its first generation

**A)**

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of eggs laid on seeds (Mean ± SD)</th>
<th>No. of eggs on Jar wall (Mean ± SD)</th>
<th>Total No. of eggs (Mean ± SD)</th>
<th>No. of adults emerged (Mean ± SD)</th>
<th>Development time (Day)</th>
<th>Weight (mg) Male</th>
<th>Weight (mg) Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control untreated</td>
<td>96 ± 6.8</td>
<td>8.3 ± 0.1</td>
<td>104 ± 6.9</td>
<td>16 ± 0.8</td>
<td>29 ± 0.0</td>
<td>2.6 ± 0.05</td>
<td>3.8 ± 0.05</td>
</tr>
<tr>
<td>Partially purified α-Al</td>
<td>77 ± 3.5</td>
<td>5.3 ± 1.2</td>
<td>83 ± 4.5</td>
<td>5.6 ± 0.6</td>
<td>30 ± 0.0</td>
<td>2.1 ± 0.13</td>
<td>3.2 ± 0.05</td>
</tr>
<tr>
<td>Purified α-Al</td>
<td>75 ± 3.1</td>
<td>4 ± 0.5</td>
<td>79 ± 3.5</td>
<td>4 ± 0.0</td>
<td>31 ± 0.0</td>
<td>2.0 ± 0.06</td>
<td>3.0 ± 0.03</td>
</tr>
</tbody>
</table>

**B)**

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Group</th>
<th>Longevity (Day) (Mean ± SD)</th>
<th>Fecundity (eggs/female) (Mean ± SD)</th>
<th>Adults Emerged (Mean ± SD)</th>
<th>Development Time (Day) (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>13.6±0.6</td>
<td>107±7.0</td>
<td>23.3±1.2</td>
<td>28±0.0</td>
</tr>
<tr>
<td>2</td>
<td>PP-α-Al</td>
<td>11.6±0.3</td>
<td>13.3±0.8</td>
<td>3.6±0.8</td>
<td>31±0.0</td>
</tr>
<tr>
<td>3</td>
<td>P-α-Al</td>
<td>8.6±0.0</td>
<td>5.6±</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The data presented is average of triplicate independent experiments ± SD
Figure 3.14: Pictorial representation of effect of α-AI on grain damage and eggs laying by *C. chinensis* with reconstituted grains of green gram
Table 3.7: Effect of feeding of PPα-AI and Pα-AI through reconstituted Chickpea grain on A) development of *C. chinensis* and B) its first generation

### A)

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of eggs laid on seeds</th>
<th>No. of eggs on Jar wall</th>
<th>Total No. of eggs</th>
<th>No. of adults emerged</th>
<th>Development time (Day)</th>
<th>Weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Male</td>
</tr>
<tr>
<td>Control untreated</td>
<td>99±1.5</td>
<td>3.33±0.8</td>
<td>102.3±0.8</td>
<td>17±0.8</td>
<td>28±0.0</td>
<td>2.3±0.1</td>
</tr>
<tr>
<td>Partially purified α-AI</td>
<td>62±1.2</td>
<td>3.33±0.8</td>
<td>63.6±0.8</td>
<td>5.0±1.1</td>
<td>31±0.0</td>
<td>2.2±0.1</td>
</tr>
<tr>
<td>Purified α-AI</td>
<td>51±0.5</td>
<td>2.1±0.1</td>
<td>51.6±0.3</td>
<td>2.3±0.3</td>
<td>31±0.0</td>
<td>1.9±0.0</td>
</tr>
</tbody>
</table>

### B)

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Group</th>
<th>Longevity (Day)</th>
<th>Fecundity (eggs/female)</th>
<th>Adults Emerged</th>
<th>Development Time (Day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>14±1.0</td>
<td>100±1.5</td>
<td>21.3±1.8</td>
<td>28±0.0</td>
</tr>
<tr>
<td>2</td>
<td>PP-α-Al</td>
<td>7±0.5</td>
<td>10±1.1</td>
<td>4.3±0.3</td>
<td>31±0.0</td>
</tr>
<tr>
<td>3</td>
<td>P-α-Al</td>
<td>6±0.5</td>
<td>3±0.5</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The data presented is average of triplicate independent experiments ± SD
Studies on plant alpha amylase inhibitor and its role in pest management

and Pα-AI proteins (Table 3.7 A). PPα-AI and Pα-AI proteins exposure drastically affected the longevity, fecundity, adult emergence and developmental time in case of F1 generation (Table 3.7 B). Effect of α-AI proteins on the efficacy of C. chinensis in terms of ability to lay eggs and damage the grains in reconstituted grains of chickpea is shown in (Figure 3.15).

Similarly, grains were reconstituted out of flour of pigeon pea which was fortified with PPα-AI and Pα-AI proteins and it also had an impact on C. chinensis development and on its F1 generation. Number of eggs laid was reduced by 26 and 41% as a result of PPα-AI and Pα-AI proteins exposure, respectively. Emergence of adults was significantly reduced by 57 and 78%, development time required for development of an insect was observed to be increased by 7 and 11%. Reduction in the weight of male and female insects was also recorded and it was found that PPα-AI and Pα-AI proteins in pigeon pea flour could reduce weights of male insects to the tune of 23 and 38%, respectively while in case of female insects the reduction was 40 and 54% for PPα-AI and Pα-AI proteins, respectively when added in the reconstituted grains of pigeon pea (Table 3.8 A & B). Effect of α-AI proteins on the efficacy of C. chinensis in terms of ability to lay eggs and damage the grains in reconstituted grains of pigeon pea is shown in Figure 3.16.

When the insects were fed on the grains constituted out of flour of black gram fortified with both PPα-AI and Pα-AI proteins, respectively it was observed that i) number of egg laid reduced by 10 and 51% ii) adult emergence reduced by 60% and 80%, iii) development time increased by 11% because of PPα-AI and Pα-AI proteins and iv) the weight of female insects was observed to be reduced drastically i.e. 29 and 51%, (Table 9 A & B), respectively.
Figure 3.15: Pictorial representation of effect of α-AI on grain damage and eggs laying by *C. chinensis* with reconstituted grains of chick pea
Table 3.8: Effect of feeding of PPa-AI and Pa-AI through reconstituted pigeon pea grain on A) development of *C. chinensis* and B) its first generation

### A)

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of eggs laid on seeds</th>
<th>No. of eggs on Jar wall</th>
<th>Total no. of eggs</th>
<th>No. of adults emerged</th>
<th>Develop -ment time (Day)</th>
<th>Weight (mg)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control untreated</td>
<td>89±0.5</td>
<td>7.6±0.8</td>
<td>96.3±0.3</td>
<td>14.6±0.3</td>
<td>28±0.0</td>
<td>2.6±0.0</td>
<td>4.2±0.1</td>
<td></td>
</tr>
<tr>
<td>Partially purified α-AI</td>
<td>63.3±0.3</td>
<td>3.3±0.3</td>
<td>71±0.5</td>
<td>6.3±0.3</td>
<td>30±0.0</td>
<td>2.0±0.1</td>
<td>2.5±0.2</td>
<td></td>
</tr>
<tr>
<td>Purified α-AI</td>
<td>54.3±0.8</td>
<td>2.3±0.3</td>
<td>56.6±0.6</td>
<td>3.3±0.3</td>
<td>31±0.0</td>
<td>1.6±0.0</td>
<td>1.9±0.0</td>
<td></td>
</tr>
</tbody>
</table>

### B)

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Group</th>
<th>Longevity (Day)</th>
<th>Fecundity (eggs/female)</th>
<th>Adults Emerged</th>
<th>Development Time (Day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>15±0.5</td>
<td>103.06±4.2</td>
<td>20.3±0.3</td>
<td>28±0.0</td>
</tr>
<tr>
<td>2</td>
<td>PP-α-AI</td>
<td>8±0.5</td>
<td>13±0.5</td>
<td>2.6±0.3</td>
<td>31±0.0</td>
</tr>
<tr>
<td>3</td>
<td>P-α-AI</td>
<td>5±0.5</td>
<td>5±0.0</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The data presented is average of triplicate independent experiments ± SD
Figure 3.16: Pictorial representation of effect of α-AI on grain damage and eggs laying by *C. chinensis* with reconstituted grains of pigeon pea.
Table 3.9: Effect of feeding of PPa-AI and Pu-AI through reconstituted black gram grain on A) development of *C. chinensis* and B) its first generation.

A)

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of eggs laid on seeds</th>
<th>No. of eggs on Jar wall</th>
<th>Total no. of eggs</th>
<th>No. of adults emerged</th>
<th>Development time (Day)</th>
<th>Weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Male</td>
</tr>
<tr>
<td>Control untreated</td>
<td>105±1.5</td>
<td>5.6±0.8</td>
<td>110.6±0.6</td>
<td>15.6±0.4</td>
<td>28±0.0</td>
<td>2.3±0.0</td>
</tr>
<tr>
<td>Partially purified α-AI</td>
<td>86±0.5</td>
<td>3.6±0.6</td>
<td>89.6±0.8</td>
<td>6.66±0.3</td>
<td>31±0.0</td>
<td>2.0±0.0</td>
</tr>
<tr>
<td>Purified α-AI</td>
<td>51±0.5</td>
<td>3.0±0.5</td>
<td>54±1.0</td>
<td>3.33±0.3</td>
<td>31±0.0</td>
<td>1.8±0.0</td>
</tr>
</tbody>
</table>

B)

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Group</th>
<th>Longevity (Day)</th>
<th>Fecundity (eggs/female)</th>
<th>Adults Emerged</th>
<th>Development Time (Day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>14.3±0.3</td>
<td>103.3±3.3</td>
<td>15.6±0.3</td>
<td>28±0.0</td>
</tr>
<tr>
<td>2</td>
<td>PP-α-AI</td>
<td>7.3±0.3</td>
<td>11±0.5</td>
<td>4.6±0.3</td>
<td>31±0.0</td>
</tr>
<tr>
<td>3</td>
<td>P-α-AI</td>
<td>4.3±0.3</td>
<td>5.6±0.3</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The data presented is average of triplicate independent experiments ± SD
Effect of α-AI proteins on the efficacy of *C. chinensis* in terms of ability to lay eggs and damage the grains in reconstituted grains of black gram is shown in Figure 3.17.

Grains reconstituted out of flour of soybean and PPα-AI and Pα-AI proteins appeared most effective in all the parameters of insect growth and development observed. At the end of one month the total number of egg laid were reduced by 26 and 42% by virtue of presence of PPα-AI and Pα-AI proteins in the reconstituted grains. No further emergence of adults was observed hence the reduction in development time, weight and effect on F1 generation was not detected (Table 3.10 A&B). Effect of α-AI proteins on the efficacy of *C. chinensis* in terms of ability to lay eggs and damage the grains in reconstituted grains of soybean is shown in Figure 3.18.

The purified α-AI from seeds of Rajgira showed remarkable effect on all the developmental parameters including eggs laying capacity of *C. chinensis* in reconstituted grains except in soybean. The effects were also observed in the F1 generation of the surviving insects from the treated sets. Of the 5 grains reconstituted, soybean was found to be resistant to the infestation by itself. There was only marginal difference in egg laying capacity when purified α-AI was incorporated in the grain flour as compared to control and there was no adult emergence in all sets including control. Of the remaining four, the effect of incorporation of purified α-AI on the developmental parameters and egg laying capacity of *C. chinensis* was observed to be variable with the maximum being in green gram and the minimum in black gram under identical set of conditions and same concentration of the inhibitor. It should be pointed out here that grains vary in their biochemical composition, endogenous inhibitor concentration and physical characteristics. Table 3.11 shows the biochemical composition and Table 3.12 presents the physical characteristics of the grains under
Figure 3.17: Pictorial representation of effect of α-AI on grain damage and eggs laying by *C. chinensis* with reconstituted grains of black gram
Table 3.10: Effect of feeding of PPα-AI and Pa-AI through reconstituted soybean grain on A) development of *C. chinensis* and B) its first generation.

A)

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of eggs laid on seeds</th>
<th>No. of eggs on Jar wall</th>
<th>Total no. of eggs</th>
<th>No. of adults emerged</th>
<th>Development time (Day)</th>
<th>Weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Male</td>
</tr>
<tr>
<td>Control untreated</td>
<td>40.3±0.8</td>
<td>2.0±0.0</td>
<td>42.6±0.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Partially purified α-AI</td>
<td>28.3±1.2</td>
<td>3.0±0.5</td>
<td>31.3±0.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Purified α-AI</td>
<td>22. ±1.0</td>
<td>2.0±0.5</td>
<td>24.0±1.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

B)

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Group</th>
<th>Longevity (Day)</th>
<th>Fecundity (eggs/female)</th>
<th>Adults Emerged</th>
<th>Development Time (Day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>PP-α-AI</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>P-α-AI</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The data presented is average of triplicate independent experiments ± SD
Figure 3.18: Pictorial representation of effect of α-AI on grain damage and eggs laying by *C. chinensis* with reconstituted grains of soybean
Table 3.11: Profiles of biochemical composition of the five grain legumes.

<table>
<thead>
<tr>
<th>Grain legume type</th>
<th>Protein (g%)</th>
<th>Amino acids (g%)</th>
<th>Fats (g%)</th>
<th>Fibre (g%)</th>
<th>Carbohydrate (g%)</th>
<th>Reducing sugar (g%)</th>
<th>Germination (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chickpea</td>
<td>17.1 ± 0.1</td>
<td>2.8 ± 0.1</td>
<td>5.4 ± 0.1</td>
<td>3.7 ± 0.1</td>
<td>60.7 ± 0.6</td>
<td>6.5 ± 1</td>
<td>88.3 ± 4.1</td>
</tr>
<tr>
<td>Pigeon pea</td>
<td>22.3 ± 0.1</td>
<td>3.6 ± 0.1</td>
<td>1.8 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>57.6 ± 0.2</td>
<td>5.7 ± 1</td>
<td>90.0 ± 0.0</td>
</tr>
<tr>
<td>Blackgram</td>
<td>23.9 ± 0.6</td>
<td>3.8 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>59.6 ± 0.1</td>
<td>4.3 ± 0.5</td>
<td>90.0 ± 0.0</td>
</tr>
<tr>
<td>Greengram</td>
<td>24.1 ± 0.2</td>
<td>3.9 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>4.2 ± 0.0</td>
<td>56.7 ± 0.2</td>
<td>4.7 ± 0.5</td>
<td>81.7 ± 4.1</td>
</tr>
<tr>
<td>Soyabean</td>
<td>42.3 ± 0.2</td>
<td>6.9 ± 0.1</td>
<td>20 ± 0.4</td>
<td>3.8 ± 0.1</td>
<td>20.6 ± 0.6</td>
<td>5.5 ± 0.5</td>
<td>90.0 ± 0.0</td>
</tr>
</tbody>
</table>

The data presented is average of three independent experiments ± SD
Table 3.12: A profile of physical characteristics of the grain legume seeds.

<table>
<thead>
<tr>
<th>Grain legume type</th>
<th>Shape</th>
<th>Colour</th>
<th>Texture</th>
<th>Size (mm) (l x w)</th>
<th>Weight (mg)</th>
<th>Seed coat thickness (µm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chickpea</td>
<td>Subglobose beaked</td>
<td>Yellowish brown</td>
<td>Smooth</td>
<td>8.0 x 6.0</td>
<td>190</td>
<td>24</td>
</tr>
<tr>
<td>Pigeon pea</td>
<td>Subglobose biconvex</td>
<td>Brown</td>
<td>Smooth</td>
<td>6.0 x 5.0</td>
<td>43</td>
<td>21</td>
</tr>
<tr>
<td>Black gram</td>
<td>Subglobose</td>
<td>Black</td>
<td>Smooth</td>
<td>4.5 x 3.0</td>
<td>72</td>
<td>23</td>
</tr>
<tr>
<td>Greengram</td>
<td>Subglobose</td>
<td>Green</td>
<td>Smooth</td>
<td>4.5 x 3.5</td>
<td>53</td>
<td>20</td>
</tr>
<tr>
<td>Soybean</td>
<td>Spherically biconvex</td>
<td>White</td>
<td>Hard</td>
<td>8.0 x 5.0</td>
<td>197</td>
<td>28</td>
</tr>
</tbody>
</table>

* Measured by a Vernier caliper
The biochemical composition was found to be varying in different grain legumes. Protein and amino acid contents were found to be highest in soybean and lowest in chick pea. Fats were highest in soybean and lowest in green gram. Crude fibres were the highest in green gram and the lowest in black gram. Chick pea was richest in carbohydrates while lowest levels were recorded in soybean. Reducing sugars were highest in chickpea and pigeon pea whereas lowest in green gram and black gram. Besides the grain legumes under study were found to have good germanability which varied between 80 – 90% (Table 3.11).

Similarly, physical characteristics like shape, colour, texture, size, weight and seed coat thickness of different grain legumes differed considerably. Seed size of chickpea was biggest and green gram was smallest. Weight of seeds varied between 43 to 309 mg with chickpea having the highest weight/seed. Seed coat was thickest in green gram followed by black gram and soybean, respectively (Table 3.12). Earlier studies by some other researchers demonstrated that host preference and behaviour of C. chinensis females were determined by a complex interactive external (e.g. environmental factors, spatial configuration of potential host plants, chemical and physical characteristics of each host species) and internal (e.g. female genotype, recent oviposition experience and physiological conditions) factors. In addition to discriminating seeds having different sizes, shapes or surfaces, there seems to be a correlation with variations in concentration of some biochemicals (nutritional and antinutritional factors) in the seeds (Salunke, 2006). In order to be useful for large scale application, the treatment not only should be economical but it should be easily administrable also. The grains under study (500 gm each) were treated topically with Rajgira seed and leaf powder (@ 1.5 gm/500 gm) and purified inhibitor protein (@ 0.5 ml/500 gm containing 0.7 mg of purified protein), force infested with a pair of
freshly emerged *C. chinensis* and monitored for marker biochemical parameters, seed damage and microbial load for a period of six months. None of the treatments could protect any of the grain types from pest infestation (causing grain damage), decline in quality (as a function of biochemical parameters) and increase in microbial load. A representative data of the three treatments on green gram is shown in Table 3.13 and more or less similar trends were seen with other grain types (therefore data not shown to avoid repetition).

After forced infestation of a pair of *C. chinensis* adults, the females started depositing eggs on seeds. Initial damage of seeds in terms of number of insects observed and % grains damaged was found to be low after first month but it gradually increased as period increased. Damage rate of seeds attained level of 50% within first 3-4 months and increased up to 100% within 7-8 months after infestation of *C. chinensis*. Insect number was found to increase after every month but it relatively stayed constant after 8 months till 12 months. Bacterial infestation was observed since first month itself, which increased as the period increased but stayed relatively constant after 6 months. Actinomycetes infestation started 2 month onwards which also relatively stayed constant after 6 months like bacteria. However, during this period fungal infestation took a pick which started from 3 month onwards and overcame all other infestants including insects, bacteria and actinomycetes within 6 months (Figure 3.19).

### 3.8.3 Effect on *H. armigera:*

Effect of α-AI isolated from *A. paniculatus* (Rajgira) seeds on growth of larvae of *H. armigera* was also studied. In this, Purified α-AI, equal to 200 mg/ml was mixed in artificial diet and laboratory reared early 3rd instars of *H. armigera* were allowed to feed, one each in separate jar. The diet was changed every alternate day.
Table 3.13: Effect of rajgira seed, leaf powder and purified $\alpha$-AI on biochemical, vital (germinability) parameter, and microbial load and seed damage as function of time in forced infested green gram.

<table>
<thead>
<tr>
<th>Months</th>
<th>Carbohydrate (gm/100gm)</th>
<th>Protein (gm/100gm)</th>
<th>Germination (%)</th>
<th>Seed damage (%)</th>
<th>Reducing sugar (%)</th>
<th>Microbial infestation X $10^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Bacteria</td>
</tr>
<tr>
<td>0</td>
<td>55.36</td>
<td>24.58</td>
<td>100</td>
<td>-</td>
<td>17.77</td>
<td>11</td>
</tr>
<tr>
<td>1</td>
<td>53.16</td>
<td>23.10</td>
<td>90</td>
<td>10</td>
<td>16.36</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>50.16</td>
<td>22.10</td>
<td>80</td>
<td>36</td>
<td>16.01</td>
<td>21</td>
</tr>
<tr>
<td>3</td>
<td>49.14</td>
<td>21.95</td>
<td>50</td>
<td>52</td>
<td>14.08</td>
<td>28</td>
</tr>
<tr>
<td>4</td>
<td>45.36</td>
<td>18.35</td>
<td>40</td>
<td>73</td>
<td>8.23</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>43.54</td>
<td>15.25</td>
<td>20</td>
<td>89</td>
<td>8.32</td>
<td>30</td>
</tr>
<tr>
<td>6</td>
<td>42.12</td>
<td>13.45</td>
<td>00</td>
<td>91</td>
<td>6.52</td>
<td>34</td>
</tr>
</tbody>
</table>

b) Seed powder of Rajgira

<table>
<thead>
<tr>
<th>Months</th>
<th>Carbohydrate (gm/100gm)</th>
<th>Protein (gm/100gm)</th>
<th>Germination (%)</th>
<th>Seed damage (%)</th>
<th>Reducing sugar (%)</th>
<th>Microbial infestation X $10^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Bacteria</td>
</tr>
<tr>
<td>0</td>
<td>55.36</td>
<td>24.58</td>
<td>100</td>
<td>-</td>
<td>17.77</td>
<td>13</td>
</tr>
<tr>
<td>1</td>
<td>54.21</td>
<td>24.10</td>
<td>90</td>
<td>09</td>
<td>15.89</td>
<td>17</td>
</tr>
<tr>
<td>2</td>
<td>54.21</td>
<td>22.16</td>
<td>80</td>
<td>29</td>
<td>15.23</td>
<td>21</td>
</tr>
<tr>
<td>3</td>
<td>50.11</td>
<td>22.16</td>
<td>50</td>
<td>57</td>
<td>14.12</td>
<td>25</td>
</tr>
<tr>
<td>4</td>
<td>46.71</td>
<td>19.30</td>
<td>30</td>
<td>63</td>
<td>8.12</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>44.28</td>
<td>18.43</td>
<td>20</td>
<td>78</td>
<td>8.23</td>
<td>31</td>
</tr>
<tr>
<td>6</td>
<td>42.06</td>
<td>15.61</td>
<td>00</td>
<td>90</td>
<td>6.23</td>
<td>31</td>
</tr>
</tbody>
</table>

c) Leaf powder of Rajgira

<table>
<thead>
<tr>
<th>Months</th>
<th>Carbohydrate (gm/100gm)</th>
<th>Protein (gm/100gm)</th>
<th>Germination (%)</th>
<th>Seed damage (%)</th>
<th>Reducing sugar (%)</th>
<th>Microbial infestation X $10^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Bacteria</td>
</tr>
<tr>
<td>0</td>
<td>55.36</td>
<td>24.58</td>
<td>100</td>
<td>-</td>
<td>17.77</td>
<td>08</td>
</tr>
<tr>
<td>1</td>
<td>54.31</td>
<td>24.21</td>
<td>90</td>
<td>12</td>
<td>15.87</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>54.31</td>
<td>22.21</td>
<td>80</td>
<td>38</td>
<td>15.35</td>
<td>22</td>
</tr>
<tr>
<td>3</td>
<td>50.61</td>
<td>21.21</td>
<td>50</td>
<td>56</td>
<td>13.96</td>
<td>28</td>
</tr>
<tr>
<td>4</td>
<td>47.12</td>
<td>20.55</td>
<td>30</td>
<td>78</td>
<td>8.24</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>45.26</td>
<td>18.65</td>
<td>20</td>
<td>84</td>
<td>8.25</td>
<td>31</td>
</tr>
<tr>
<td>6</td>
<td>42.26</td>
<td>15.65</td>
<td>00</td>
<td>92</td>
<td>6.30</td>
<td>32</td>
</tr>
</tbody>
</table>

d) Purified $\alpha$-AI from seeds of Rajgira

<table>
<thead>
<tr>
<th>Months</th>
<th>Carbohydrate (gm/100gm)</th>
<th>Protein (gm/100gm)</th>
<th>Germination (%)</th>
<th>Seed damage (%)</th>
<th>Reducing sugar (%)</th>
<th>Microbial infestation X $10^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Bacteria</td>
</tr>
<tr>
<td>0</td>
<td>55.36</td>
<td>24.58</td>
<td>100</td>
<td>-</td>
<td>17.77</td>
<td>07</td>
</tr>
<tr>
<td>1</td>
<td>53.56</td>
<td>23.12</td>
<td>80</td>
<td>08</td>
<td>15.77</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>53.56</td>
<td>23.12</td>
<td>80</td>
<td>21</td>
<td>15.01</td>
<td>19</td>
</tr>
<tr>
<td>3</td>
<td>52.16</td>
<td>22.92</td>
<td>60</td>
<td>42</td>
<td>14.00</td>
<td>24</td>
</tr>
<tr>
<td>4</td>
<td>50.86</td>
<td>21.92</td>
<td>40</td>
<td>61</td>
<td>13.00</td>
<td>28</td>
</tr>
<tr>
<td>5</td>
<td>47.67</td>
<td>16.45</td>
<td>30</td>
<td>78</td>
<td>8.23</td>
<td>27</td>
</tr>
<tr>
<td>6</td>
<td>42.67</td>
<td>12.95</td>
<td>10</td>
<td>89</td>
<td>7.91</td>
<td>30</td>
</tr>
</tbody>
</table>
Figure 3.19: Chronology of events leading to grain deterioration in green gram due to *C. chinensis* and microbial infestation during 12 month storage.
The amount of diet consumed, fecal matter produced, larval weight and mortality were recorded for both control and treated sets ($n = 20$).

Incorporation of $\alpha$-AI from seeds of Rajgira in the artificial diet had a significant effect on both growth and development of $H.\ armigera$ larvae. One day before pupation, the average larval weight in control set was almost double the average larval weight in treated set (Table 3.14 A & B and Figure 3.20). Effect of $\alpha$-AI on larval period, mortality and extended larval growth was also recorded. It was observed that treatment of $\alpha$-AI significantly affected the survival of larvae. Larval period in $\alpha$-AI treated set was extended by two days as compared to control. Moreover, there were no mortalities in control against over 60% in control set. Time taken by larvae to enter pupation phase was also altered after treatment with $\alpha$-AI, larvae in control set behaved normally and took usual period for entering pupation phase, while in the larvae utilizing $\alpha$-AI through their diet remained in larval stage and could not enter pupa stage.

Simultaneously, gut $\alpha$-amylase activity of larvae of $H.\ armigera$ was also determined qualitatively and quantitatively as a function of treatment for 13 days (Table 3.15, Figure 3.21). It was observed that that the amylase activity of larvae in control set was increasing marginally day by day and it reached to 280 $\mu$mol/min on 9$^{th}$ day from 260 $\mu$mol/min on 3$^{rd}$ day and larvae entered pupation phase on 11$^{th}$ day, which is normal. On the contrary, amylase activity was observed to be significantly affected in treated larvae. On 3$^{rd}$ day of treatment the amylase activity was observed to be 169.4 $\mu$mol/min, which was almost 40% lower as compared to control. Gut $\alpha$-amylase activity continued to be reduced drastically and it reached to as low as 2.7 $\mu$mol/min on 13$^{th}$ day (Table 3.15, Figure 3.21).
Table 3.14: Effect of α-AI isolated from *A. paniculatus* (Rajgira) seeds on larvae of *H. armigera* (A) on growth and (B) on development parameters.

A)

<table>
<thead>
<tr>
<th>Growth Parameter</th>
<th>Weight of larvae (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Age of larvae (Day)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>32.0 ± 1.2 (20)</td>
</tr>
<tr>
<td>5</td>
<td>173 ± 2.5 (20)</td>
</tr>
<tr>
<td>7</td>
<td>379.3 ± 4.0 (20)</td>
</tr>
<tr>
<td>9</td>
<td>396 ± 0.8 (20)</td>
</tr>
<tr>
<td>11</td>
<td>Pupation</td>
</tr>
<tr>
<td>13</td>
<td>--</td>
</tr>
</tbody>
</table>

*Value in parenthesis indicate no. of surviving larvae on that day. n=20

B)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Larval period (Day)</td>
<td>9-11</td>
<td>11-13</td>
</tr>
<tr>
<td>Mortality</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>Extended larval growth (day)</td>
<td>Normal</td>
<td>No Pupation</td>
</tr>
</tbody>
</table>
Figure 3.20: Effect of exposure of purified α-AI through diet on development of *H. armigera*. The picture is taken after 7 days in 3rd instar.
Table 3.15: Effect of α-AI isolated from *A. paniculatus* (Rajgira) seeds on larval gut amylase activity of *H. armigera*.

<table>
<thead>
<tr>
<th>Days</th>
<th>Amylase activity (µmol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>3</td>
<td>260</td>
</tr>
<tr>
<td>5</td>
<td>272</td>
</tr>
<tr>
<td>7</td>
<td>275</td>
</tr>
<tr>
<td>9</td>
<td>280</td>
</tr>
<tr>
<td>11</td>
<td>Pupation</td>
</tr>
<tr>
<td>13</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 3.21: Effect of purified α-AI on expression of amylase in 3rd instar larvae *H. armigera* as a function of exposure time.
3.8.4 Effect on nutritional indices of *H. armigera*:

The effect of purified $\alpha$-AI incorporated in the artificial diet on the nutritional indices of larvae feeding on it is shown in Table 3.16. It is observed that incorporation of $\alpha$-AI in the artificial diet affected almost all the indices but the effect on approximate digestibility (AD) and efficiency of conversion of ingested food (ECI) were found to be significantly affected in larvae feeding on test diet compared to those feeding on control diet and it seems to be on expected lines. Whereas ECI is an overall measure of the ability of the insect to utilize the ingested food for growth, ECD indicates overall increase or decrease in the proportion of digested food metabolized for energy (Koul et al., 1997; 2004). The incorporated $\alpha$-AI appears to be inhibiting the gut amylase (s) which is reflected in significantly reduced AD and ECI values. Since amylase (s) are inhibited, less energy per unit of diet consumed could be realized and channelized for biomass accumulation as seen in figure 3.20.

To validate insect $\alpha$-amylase for target crop protection, it is important to research their variety and understand the mechanism and regulation of their expression. Studies in this area are in infancy and some early breakthroughs have been made with the demonstration of different forms of $\alpha$-amylases from the midgut of *C. maculates* and *Z. subfasciatus* (Franco et al., 2002). Recently, Kotkar et al (2009) reported the variations in the amylase isoforms in *H. armigera* in response to the host plant. This study proved to be important, as it demonstrates the logical reason for *H. armigera* to be a polyphagous pest. As many as eleven different $\alpha$-amylase isoforms are reported which indicate the flexibility of the insect towards sustaining itself in wide host range.

In order to be of practical use for the development of transgenic plant, the $\alpha$-AI should have appropriate profile. Ideally the $\alpha$-AI should be effective against the
Table 3.16: Nutritional indices of larvae *H. armigera* feeding on α-AI containing artificial diet.

<table>
<thead>
<tr>
<th>Days</th>
<th>Parameter</th>
<th>FC (mg)</th>
<th>FP (mg)</th>
<th>DW (mg)</th>
<th>CI</th>
<th>AD</th>
<th>ECI</th>
<th>ECD</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Control</td>
<td>417.3±1.1</td>
<td>36.6±1.2</td>
<td>33.7±0.5</td>
<td>12.11±0.7</td>
<td>0.93±0.01</td>
<td>0.08±0.0</td>
<td>0.085±0.0</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>369.6±1.1</td>
<td>23.04±1.1</td>
<td>31.04±0.8</td>
<td>12.28±0.1</td>
<td>0.92±0.02</td>
<td>0.08±0.0</td>
<td>0.079±0.0</td>
</tr>
<tr>
<td>5</td>
<td>Control</td>
<td>614.3±1.2</td>
<td>55.44±4.2</td>
<td>175.4±0.2</td>
<td>5.88±0.0</td>
<td>0.94±0.01</td>
<td>0.17±0.01</td>
<td>0.22±0.1</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>317.6±2.5</td>
<td>19.35±0.5</td>
<td>133.8±0.3</td>
<td>9.5±0.5</td>
<td>0.74±0.01</td>
<td>0.10±0.1</td>
<td>0.12±0.3</td>
</tr>
<tr>
<td>7</td>
<td>Control</td>
<td>869±3.8</td>
<td>78.21±2.1</td>
<td>276.7±3.1</td>
<td>2.60±0.06</td>
<td>0.96±0.01</td>
<td>0.57±0.03</td>
<td>0.52±0.6</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>293.6±3.2</td>
<td>16.6±0.4</td>
<td>188.7±1.6</td>
<td>1.71±0.01</td>
<td>0.75±0.02</td>
<td>0.37±0.02</td>
<td>0.61±0.4</td>
</tr>
<tr>
<td>9</td>
<td>Control</td>
<td>769.3±3.3</td>
<td>69.03±1.7</td>
<td>298.3±2.8</td>
<td>1.82±0.02</td>
<td>0.97±1.5</td>
<td>0.52±0.3</td>
<td>0.69±0.05</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>153.1±1.6</td>
<td>9.8±0.5</td>
<td>216.3±3.8</td>
<td>0.63±0.06</td>
<td>0.77±0.1</td>
<td>0.42±0.6</td>
<td>0.75±0.0</td>
</tr>
<tr>
<td>11</td>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>99.1±1.3</td>
<td>6.3±0.3</td>
<td>152±1.2</td>
<td>0.46±0.02</td>
<td>0.78±0.6</td>
<td>0.60±0.3</td>
<td>0.6±0.0</td>
</tr>
<tr>
<td>13</td>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>56.3±0.5</td>
<td>4.2±0.2</td>
<td>138.3±0.6</td>
<td>0.29±0.1</td>
<td>0.89±0.8</td>
<td>0.73±0.7</td>
<td>0.67±0.5</td>
</tr>
</tbody>
</table>

The data presented in an average of triplicate independent experiments ± SD

**FC** = Dry weight of food consumed.

**FP** = Dry weight of faeces produced.

**DW** = Mean dry weight of larvae.

**CI** = Consumption index.

**AD** = Approximate digestibility.

**ECI** = Efficiency of conversion of ingested food.

**ECD** = Efficiency of conversion of digested food.
wide range of predatory insects; it should not inhibit endogenous α-amylase of physiological significance (Kadziola et al., 1998). Lack of activity against mammalian α-amylase though less significant can be considered as an additional quality. Although biochemical screening will continue to play important role, search of inhibitor with desirable characteristics through understanding of structural basis of α-amylase and α-amylase inhibitor interaction will enable specific mutation in existing inhibitor or design of synthetic peptide which will be specific to the α-amylase of small number of pests.

Since insect α-amylases play an important role in carbohydrate metabolism, an effective pest control strategy would require understanding of α-AI activity and its specificity (Laskowski and Kato 1980; Franco et al., 2000). In spite of the presence of inhibitors, insect feed on seeds and overcome plant defense. Two factors seem to be responsible to this phenomenon. First, many plants suffer reduction in defense compounds during domestication (Hilder et al., 1987). Secondly, just as plants evolve defenses, their predators evolve means to evade those defense mechanisms (Franco et al. 2002). Several insect pests synthesize at least two α-amylase isozymes in the digestive tract (Silva et al., 1999; Franco et al., 2002). Shivakumar et al. (2006) reported presence of more than one isozymes of α-amylase in midgut extracts of eight insect pests. The presence of large number of α-amylase isozymes could be an efficient strategy for insects to escape the toxicity due to inhibitors (Silva et al., 1999). Moreover, since, both the plants and insect have co-evolved, the latter have adapted themselves to many phytochemicals by modifying their gut physiology and expression of digestive enzyme including amylases (Hivrale et al., 2011). Because of changing midgut physiology, host plant α-AI can become ineffective in inhibiting the enzyme α-amylase in target insect pest (Giri and Kachole, 1998) and excellent alternative
approach to counter this problem is to search for non-host plant as a source of proteinaceous α-AI. Another desirable feature in the α-AI is its ability to have same inhibitory potential against all the isoforms of enzyme of target pest.

*Helicoverpa armigera* is an agronomically and economically important pest infesting more than 3000 plant species worldwide (Arora et al., 2007; Rajapakse and Walter, 2007). Polyphagy, high mobility, fecundity and a facultative diapause are important physiology and ecological characteristics of the pest that help it survive even in the challenging habitats (Kotkar et al., 2009). Conventional chemical based approaches to control this pest have failed due to variety of reasons including development of resistance against many insecticides (Kranthi et al., 2002). While developing sustainable approaches for control of this economically important pest, it becomes necessary to characterize digestive enzymes and their inhibitors from different sources. Studies indicate that specificity of insect amylases and inhibitors obtained from different plant sources varies. Similar to proteinases and their inhibitor (Franco et al., 2000; Kotkar et al., 2009). Whereas, latter have been investigated thoroughly, the farmer have not been exploited to that extent. Though, several inhibitor of amylases have been characterized and proved their effectiveness against a variety of storage pests (Fronco et al., 2002; Kotkar et al., 2009; Gupta et al., 2012), it is necessary to continue to study and explore novel host and non-host plant α-AIs as potential sources to overcome the adaptive strategies of insect pests and in spite of remarkable structural and functional variety naturally found among α-AIs, screening for inhibitor(s) with desirable characteristics is always an attractive option. The study reports a new, fairly stable α-AI from a non-host species with good inhibitory potential against α-amylases from larval gut/ larvae of two economically important pests which can be gainfully utilized in further pest control strategies.
4.0 Conclusions:

- Of the various members of family Amaranthaceae and their parts studied, α-AI activity was found to be maximum in the seeds of *Amaranthus paniculatus*, both qualitatively and quantitatively.

- The α-AI protein from the seeds of *A. paniculatus* was purified by a four stage process and about 16 fold purification was achieved.

- The Molecular weight of purified protein was determined to be 14.3 kea by both SDS-PAGE and gel permeation chromatography.

- The purified α-AI was found to be a glycoprotein with the carbohydrate content of about 3.5%.

- The isolated α-AI from seeds of *A. paniculatus* was found to be stable over a pH range of 4-9 and temperature range of 25-50°C.

- It was found to be mono-functional, that is, not having protease inhibitory activity. The isolated α-AI was found to be non-competitive inhibitor of insect (*C. chinensis*) amylase. The inhibitor constant was calculated to be 2.06 µg.

- Amino acid arginine, disulphide bond(s) and sulphydryl groups were not found to be essential for the activity of isolated protein in our studies.

- The purified protein was found to inhibit α-AI from different sources. The maximum inhibition by the isolated protein was recorded against bacterial amylase (*Bacillus* species) and minimum against Human salivary amylase. The insect amylase from (*C. chinensis* and *H. armigera*) were inhibited to the extent of around 72%.
The inhibitory activity of the isolated protein against two insect amylases were also confirmed by SEM of starch granules incubated with enzymes both in presence and absence of inhibitor.

Similarly, the inhibitory activity of the isolated $\alpha$-AI against $\alpha$-amylases obtained from four different sources was also confirmed by starch gel profile.

PAGE profile revealed that same types of $\alpha$-amylases are expressed by larvae of *C. chinensis* feeding on different grain and mixture of them albeit, in different quantities. An additional fast moving (low molecular weight) isoform of amylase was observed in larvae feeding on green gram and mixture of all grains. The isolated $\alpha$-AI was found to inhibit all the amylases including the isoform.

The effect of $\alpha$-AI on the amylases of the target pests *in vivo*, was determined by allowing the larvae of *C. chinensis* and *H. armigera* to feed on reconstituted grains and artificial diet, respectively containing isolated $\alpha$-AI.

The $\alpha$-AI in different reconstituted grains was found to affect the eggs laying capacity, adult emergence, development time and weight of male and female adults to various extents. The maximum effect was observed in chickpea, black gram and soybean and minimum in green gram.

The F1 generation of the survived insects from the reconstituted grains was also found to be affected in terms of longevity, fecundity and development time compared to control.
Studies on plant alpha amylase inhibitor and its role in pest management

- The variation in activity $\alpha$-AI in reconstituted grains could be due to variation in physical characteristic, biochemical composition and presence of endogenous inhibitor in different grains.

- The Rajgira leaf, seed powder as well as the purified $\alpha$-AI, when applied topically on intact grains failed to preserve the grains which were found to be infested by insect, bacterial, fungal pathogens and were totally unfit for human as well as animal consumption due to severe deterioration of the biochemical and vital parameters.

- It established the fact that, insect amylases could overcome inhibitory effect of host $\alpha$-AI but could be inhibited by the $\alpha$-AI from a non-host species.

- Similarly, the isolated $\alpha$-AI incorporated into the artificial diet was found to significantly affect the growth and pupation of $H. \text{armigera}$. In fact, the gut amylase activity of the larvae from the treated set was found to be severely affected as a function of time.

- Of the various nutritional induces of $H. \text{armigera}$ larvae, approximate digestibility (AD) and efficiency of conversion of ingested food (ECI) were found to be affected as function of treatment. Because of this, probably, the growth and weight gain in larvae were found to be affected though the ECD was found to be equal or better in the larvae of the treated set compared to control.

- For developing sustainable approaches for control of economically important pests, it becomes necessary to characterize digestive enzymes and their inhibitors from different sources. Whereas, proteinases and their inhibitors have been investigated thoroughly, the amylases and
their inhibitors have not been exploited to that extent. The study reports a new, fairly stable α-AI from a non-host species with good inhibitory potential against α-amylases from larval gut/larvae of two economically important pests which can be gainfully utilized in further pest control strategies. Though, several inhibitors of amylases have been characterized and proved their effectiveness against a variety of storage pests it is necessary to continue to study and explore novel host and non-host plant α-AIs as potential sources to overcome the adaptive strategies of insect pests.

********