Bioefficacy of α-amylase inhibitors from the seeds of *macrotyloma uniflorum* and *vigna unguiculata* against *sitophilus oryzae*

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A R T I C L E   I N F O

A B S T R A C T

In the present study the bioefficacy of the α-amylase inhibitor from the seeds of *Macrotyloma uniflorum* and *Vigna unguiculata* against *Sitophilus oryzae* larvae has been studied. *M. uniflorum* α-amylase inhibitor (MUAI) was purified using ion exchange chromatography on a CMC column while *V. unguiculata* α-amylase inhibitor (VUAI) was purified on a Poros HS- 50 column. Both amylase inhibitors were tested against the fourth instar larvae for their insecticidal activity at different concentrations. Both MUAI and VUAI inhibited the *S. oryzae* α-amylase in a non-competitive manner, with a *Ki* value of 2.2 and 1.17 μM respectively. Significant mortality was observed only at higher concentration of the inhibitors.

INTRODUCTION

The biopesticides have been extensively studied because the chemical pesticides used for the control of the insect pests of the stored food grain can be harmful due to the persistence of the toxic residues on the food grains and development of resistance in the insects. (Nakakita and Ikenaga, 1997). Such insects cause food spoilage and consequent economic loss (Downes *et al.,* 2008). *Sitophilus oryzae* (L.) commonly known as rice weevil is considered as a cosmopolitan pest. It is one of the most resistant stored grain pest (Athanassioua *et al.,* 2003). *S. oryzae* adults feed on the rice grains while the larvae develop inside the rice kernel (Lucas and Riudavets, 2002). Such insects live on a polysaccharide rich diet for which they are dependent on their α-amylases (Mehrabadi *et al.,* 2011). Legume seeds are known to contain compounds that are either toxic or act as feeding deterrents to the insect pests (Hou and Taylor, 2006).

α-Amylase inhibitors are particularly abundant in the seeds of cereals and legumes and are known to be plant defense proteins. The α-amylase inhibitors impede digestion through their action on insect gut digestive α-amylases (Khan N, 2011). Further these α-amylase inhibitors are target specific, biodegradable and non-toxic. Therefore, such bioactive substances can be effectively used in the integrated pest management (Kim *et al.,* 2003).

Presence of α-amylase inhibitors in the seeds of *Macrotyloma uniflorum* (Horse gram) and *Vigna unguiculata* (Cowpea) has been reported (Gupta *et al.,* 2011 and John *et al.,* 2005). The present study was therefore carried out to study the bioefficacy of these α-amylase inhibitors against *S. oryzae* larvae.

Preparation of the seed extract and isolation of the α-amylase inhibitor

The isolation and purification of *M. uniflorum* α-amylase inhibitor (MUAI) was carried out by ion exchange chromatography on CM cellulose essentially as described by Sabharwal and Devanhalli (2012). The *V. unguiculata* α-amylase inhibitor (VUAI) was isolated from a saline extract (100 g seeds in 500 ml 0.85% NaCl) and purified by ion exchange chromatography on Poros HS-50 column followed by gel filtration on Sephadex G-75 column.

Insect culture

S. oryzae adults (mixed sex and age) were reared on white rice in glass jars in the laboratory at $25 \pm 2°C$ and $60 \pm 5\%$ relative humidity with a photoperiod of 14 h/10 h light and dark cycle. The larvae were collected after 20 days with the help of a 2.0 mm sieve.

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Bioassay

MUAI and VUAI were lyophilized to powder. Rice was ground to a fine powder. The amylase inhibitor (MUAI/VUAI) was mixed with powdered rice to make a final concentration of 0.01, 0.05, 0.1 and 0.5% (w/w) respectively. This mixture was then fed to the fourth instar larvae (10 larvae per experiment). The feed was changed every 2 days for a period of 10 days. Larval mortality was checked every 2 days. Corresponding controls were prepared without the inhibitor in the feed. Each experiment was conducted in 5 replicates.

Larval period

The time taken by the fourth instar larvae to emerge as adults was observed in the inhibitors treated and the control groups.

Repellent assay

Repellent assay was carried out according to the method described by Viglianco et al. (2008) with slight modification. Filter-paper discs of 9 cm in diameter, cut in halves were used for the assay. One half of the disc was uniformly coated with different concentrations (0.01, 0.05, 0.1 and 0.5%) of MUAI or VUAI respectively and air dried. The other half of the disc was left untreated. One treated and one untreated half was placed in Petri dishes. 10 fourth instar larvae were released in the Petri dishes. The number of larvae present in each half of the disc was counted on an hourly basis for a period of 8 hours. The control consisted of the Petri dish containing both halves of the untreated disc. The data were expressed in terms of percent repulsion (PR) and calculated by using the following formula,

$$PR\ (\%) = (Nc - 50) \times 2$$

Where, N_c = percentage of larvae present on the control half.

Positive values indicated repellent while negative values indicated non-repellent nature.

on Sephadex G-50 column. 0.5 ml fractions were collected. Each fraction was monitored for its protein content by monitoring the absorbance at 280nm on a spectrophotometer (Jasco V-630 spectrophotometer, Japan) and the α-amylase activity was determined according to the dinitro salicylic acid (DNSA) method (Sadasivam and Manickam, 2005). The fractions showing high α-amylase activity were pooled, lyophilized and used for the kinetic studies. One unit of α-amylase activity was defined as the amount of α-amylase that liberates one μmole of maltose equivalent under assay conditions.

Enzyme kinetic studies

α-amylase (0.2 Units) was incubated with MUAI (0.1 ml, 100 μg) or VUAI (0.1 ml, 80 μg) in acetate buffer (pH 6.0, 10 mM) at 37°C for 1 h in a total reaction volume of 0.25 ml, before initiating the reaction with different concentrations of the substrate (0.5-2.0% starch solution). The inhibitory activity of MUAI and VUAI against the *S. oryzae* α-amylase was studied essentially according to the procedure of Bernfeld (1955) with slight modification. Lineweaver Burk plots were drawn for the uninhibited and the partially inhibited α-amylase and the values of K_i were obtained from the plots.

Statistical analysis

The data was expressed as mean ± S.D. Statistical analysis was carried out by repeated measure ANOVA followed by *post hoc* Dunnett's multiple comparison test (GraphPad InStat version 3.00 for Windows Vista[TM] BASIC). *P<0.05* was considered statistically significant.

RESULTS

Both MUAI and VUAI treated fourth instar larvae showed significant mortality (about 43 and 52% respectively) at higher concentrations (0.1 and 0.5%) of the inhibitors, while low mortality was observed in the groups treated with lower concentrations of the inhibitors (Tables 1, 2) even after 10 days of treatment. A slight increase in the larval period (11 ± 1 and 13 ± 1 days) in the MUAI and VUAI treated groups respectively as compared to the

Table 1 Effect of MUAI on the larval mortality and larval period of *S. oryzae* fourth instar larvae

No. of Days	Control	0.01% MUAI	0.05% MUAI	0.1% MUAI	0.5% MUAI
2	0.0 ± 0.0	0.0 ± 0.0[ns]	0.0 ± 0.0[ns]	0.0 ± 0.0[ns]	0.0 ± 0.0[ns]
4	0.0 ± 0.0	0.0 ± 0.0[ns]	0.0 ± 0.0[as]	3 ± 1.45*	9 ± 1.41*
6	0.0 ± 0.0	0.0 ± 0.0[ns]	4 ± 1.41*	9 ± 1.43*	16.5 ± 0.7*
8	0.0 ± 0.0	0.0 ± 0.0[ns]	9 ± 1.44*	14 ± 1.41*	29 ± 1.41**
10	0.0 ± 0.0	0.0 ± 0.0[ns]	11.66 ± 10.4*	27.33 ± 5.03**	43.33 ± 4.06**
Larval period (days)	7.33 ± 0.57	7.33 ± 1.15[ns]	7.66 ± 0.57[ns]	9.33 ± 0.58**	11 ± 1**

Values are mean ± S.D.; n=10; Statistical analysis by ANOVA followed by Post hoc Dunnett's multiple comparison test; ns- not significant, *** p<0.001; ** p<0.01; * p<0.05 compared to the control.

Isolation of α-amylase from S. oryzae larvae

The α-amylase was isolated from the untreated fourth instar larvae essentially according to the procedure described by Gupta et al. (2011) with slight modifications. 10 larvae were homogenized in 1.5 ml of phosphate buffer (pH 6.0, 10 mM) containing 10 mM sodium chloride and 10 mM calcium chloride, followed by centrifugation at 10,000 g for 15 min at 4°C. The supernatant obtained was dialyzed against distilled water followed by gel filtration

control (7.33 ± 0.57 days) for the fourth instar larvae (Tables 1, 2) was observed. MUAI was found to be moderately repellent at the concentration of 0.5%, while at lower concentrations it was observed to be a non-repellent (Figure 1). VUAI was found to be a non-repellent at all concentrations (Figure 1). Both MUAI and VUAI were found to inhibit the α-amylase in a non-competitive manner with K_i values of 2.2 and 1.17 μM respectively (Figures 2a, 2b).

Table 2 Effect of VUAI on the larval mortality and larval period of *S. Oryzae* fourth instar larvae

| No. of Days	Control	0.01% VUAI	0.05% VUAI	0.1% VUAI	0.5% VUAI
2	0.0 ± 0.0	0.0 ± 0.0^{ns}	0.0 ± 0.0^{ns}	0.0 ± 0.0^{ns}	0.0 ± 0.0^{ns}
4	0.0 ± 0.0	0.0 ± 0.0^{ns}	0.0 ± 0.0^{ns}	$7 \pm 2.82^{*}$	$12 \pm 1.41^{*}$
6	0.0 ± 0.0	0.0 ± 0.0^{ns}	$2.5 \pm 0.7^{*}$	$15.5 \pm 3.53^{*}$	$26 \pm 2.82^{**}$
8	0.0 ± 0.0	0.0 ± 0.0^{ns}	$3.5 \pm 0.7^{*}$	$26.5 \pm 0.7^{**}$	$40 \pm 1.41^{**}$
10	0.0 ± 0.0	0.0 ± 0.0^{ns}	$5.33 \pm 2.51^{*}$	$40.33 \pm 2.51^{**}$	$52 \pm 2.0^{**}$
Larval period (days)	7.33 ± 0.57	7.66 ± 0.57^{ns}	$9.66 \pm 0.58^{**}$	$10.33 \pm 0.58^{**}$	$13 \pm 1^{**}$

Values are mean $\pm$ S.D.; n=10; Statistical analysis by ANOVA followed by Post hoc Dunnett's multiple comparison test; ns- not significant, *** $p<0.001$; ** $p<0.01$; * $p<0.05$ compared to the control.
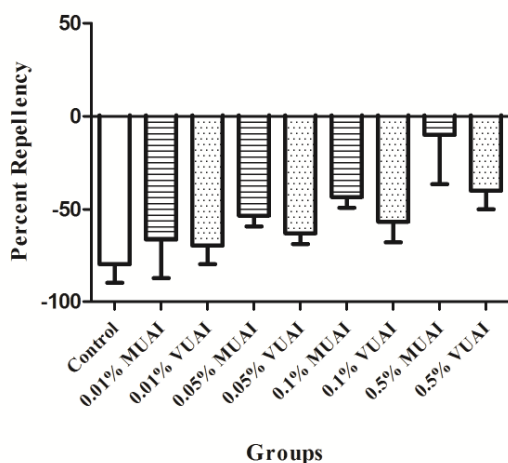
The graph represents average of the percent repellency and the bars indicate standard deviation

Figure 1 Comparative repellency of MUAI and VUAI with respect to the control against *S. oryzae* fourth instar larvae
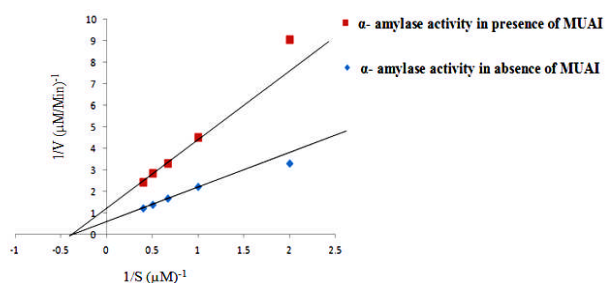
a). In presence and absence of MUAI

Figure 2 Lineweaver Burke plot for *S. oryzae* α-amylase
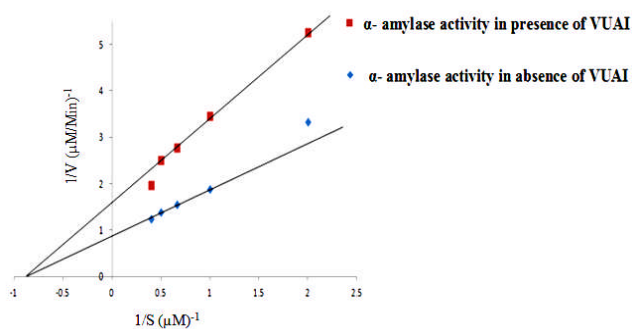
b). In presence and absence of VUAI

DISCUSSION

Enzyme inhibitors from plant source can act as growth inhibitors of insects and hence, the genes of these inhibitors can be used for increasing the resistance of cereals to store grain pests (Pueyo *et al.,* 1995). Some phytochemicals function as natural antifeedants. In integrated pest management, other than mortality of the target pest, the antifeedant and growth inhibiting activity of the insecticide is also important (Erturk *et al.,* 2004). Use of enzyme inhibitors for the control of stored grain pests is a safe method as these inhibitors have been present in the human foods without causing any detrimental effect on the human beings (Hubert *et al.,* 2007). The α-amylase inhibitor is not a contact poison and is involved in the impaired carbohydrate metabolism of the insect pests; it is thus of utmost importance that the inhibitor should not show repellent activity as it has to be ingested to show its effect. The overall non-repellent nature of MUAI and VUAI makes it suitable to be used as a biocontrol agent.

Due to the presence of high levels of α-amylase in *S. Oryzae,* it has an adaptive advantage of resisting the effect of naturally occurring α-amylase inhibitors present in the cereals (Baker and Woo, 1985). There are reports on the presence of two isoforms of α-amylase in *S.oryzae* (Mendiola-Olaya *et al.,* 2000). A single α-amylase inhibitor may not be able to inhibit all the isoforms of an α-amylase from an insect pest (Sivakumar *et al.,* 2006). The *in vitro* kinetic studies revealed that both MUAI and VUAI could significantly inhibit the *S. Oryzae* α-amylase, with VUAI having more affinity as compared to MUAI. However the decreased mortality observed in the *in vivo* studies at lower concentrations of these inhibitors suggests a possible resistance mechanism in the larvae to overcome the effect of the inhibitor.

CONCLUSION

In the present work the α-amylase inhibitory activity of a legume α-amylase inhibitor (MUAI and VUAI) has been tested against a cereal pest *in vitro* and *in vivo*. These studies demonstrate the detrimental effects of MUAI and VUAI on the development of *S. oryzae* larvae during feeding trials. The present findings are significant since an α-amylase inhibitor from a non-host would be more effective in conferring resistance to cereals against pests that commonly infest them. Further these studies offer new insights for the rational design of specific bioinsecticidal proteins in integrated pest management.

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