

Inhibitory effects of an extract from non-host plants on physiological characteristics of two major cabbage pests

M. Dastranj¹, E. Borzoui², A. R. Bandani^{1*} and O. L. Franco^{3,4}

¹Department of Plant Protection, College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran: ²Department of Plant Protection, Faculty of Agriculture and Natural Resources, University of Mohaghegh Ardabili, Ardabil, Iran: ³S-Inova Biotech, Pos-Graduação em Biotecnologia, Universidade Católica Dom Bosco, Campo Grande, MS, Brazil: ⁴Centro de análises Proteômicas e Bioquímicas, Universidade Católica de Brasília, Brasília-DF, Brazil

Abstract

The diamondback moth (*Plutella xylostella*) and small white cabbage butterfly (*Pieris rapae*) are the two main serious pests of cruciferous crops (Brassicaceae) that have developed resistance to chemical control methods. In order to avoid such resistance and also the adverse effects of chemical pesticides on the environment, alternative methods have usually been suggested, including the use of plant enzyme inhibitors. Here, the inhibitory effects of proteinaceous inhibitors extracted from wheat, canola, sesame, bean and triticale were evaluated against the digestive α -amylases, larval growth, development and nutritional indices of the diamondback moth and small white cabbage butterfly. Our results indicated that triticale and wheat extracts inhibited α -amylolytic activity in an alkaline pH, which is in accordance with the moth and butterfly gut α -amylase optimum pH. Dose-dependent inhibition of two crucifer pests by triticale and wheat was observed using spectrophotometry and gel electrophoresis. Implementation of specificity studies showed that wheat and triticale-proteinaceous extract were inactive against Chinese and purple cabbage amylase. Triticale and wheat were resistant against insects' gut proteases. Results of the feeding bioassay indicated that triticale-proteinaceous extract could cause a significant reduction in survival and larval body mass. The results of the nutritional indices also showed larvae of both species that fed on a Triticale proteinaceous inhibitor-treated diet had the lowest values for the efficiency of conversion of ingested food and relative growth rate. Our observations suggested that triticale shows promise for use in the management of crucifer pests.

Keywords: enzyme–inhibitor interactions, α -amylase, *Plutella xylostella*, *Pieris rapae*, Nutritional indices

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Introduction

Cruciferous crops are subjected to attack by lepidopteran larvae, which cause significant yield losses. The diamondback moth, *Plutella xylostella* (Lep: Plutellidae), and small white cabbage butterfly, *Pieris rapae* (Lepidoptera, Pieridae), are the two most important pests of cruciferous crops (*Brassicaceae*), causing direct damage through feeding on leaves and indirect

*Author for correspondence
 Tel.: +98-263-2818705
 Fax: +98-263-2238529
 E-mail: abandani@ut.ac.ir

damage by depositing excremental materials (Zhao *et al.*, 2002). Although the use of synthetic pesticides has been shown to be effective in controlling both these pests, the increasing concerns over resistance development, pesticide residues in agricultural products and environment, and adverse effects of chemical pesticides on beneficial organisms have motivated entomologists to seek alternative pest control strategies (Sayyed, Omar & Wright, 2004; Damalas, 2011).

Insect pests utilize α -amylases for carbohydrate digestion and metabolism, and due to their importance in producing energy, different forms of amylases have been found in insects' digestive system. Thus, amylase can be considered as a potent candidate for bio-insecticide development using enzyme inhibitors (Franco *et al.*, 2002). Small protein molecules, known as amylase inhibitors, are able to interfere with the normal growth and development of insects by inhibiting the amylolytic activity in their digestive system (Walker *et al.*, 1998; Saadati & Bandani, 2011; Mehrabadi *et al.*, 2012). These molecules, found in the seeds of several plant species, particularly in cereals and legumes, play a central role in plant defense against pathogens and pests (Franco *et al.*, 2002). α -Amylase inhibitors ectopically expressed in plants have been seen to provide protection against insect pests due to their involvement in plant natural defense systems (Schroeder *et al.*, 1995; Svensson *et al.*, 2004).

However, insect-resistant transgenic crops decrease the demand for chemical pesticides and provide a compatible system with other alternative control means such as biological control (Dias *et al.*, 2010). For instance, Dias *et al.* (2010) indicated that the rye α -amylase inhibitor (α -AI), expressed in transgenic tobacco (*Nicotiana tabacum*) seeds, caused a significant mortality in *Anthonomus grandis* first-instar larvae. Based on the study reported by Barbosa *et al.* (2010), an artificial diet containing transgenic seed extract derived from transformant *Coffea arabica* with the common bean α -AI1 caused a considerable reduction in α -amylase activity in the coffee berry borer pest (*Hypothenemus hampei*). These studies clearly demonstrate that ectopic expression of α -AI1 can confer stable resistance against a wide range of insect pests.

Host plants have various degrees of nutritional quality and this has an effect on the population dynamics of different pests (Borzoui & Naseri, 2016). Several pieces of research have determined that the availability of suitable food plays the main role in population outbreaks for oligophagous and polyphagous pests (Mehrkhou *et al.*, 2013; Kianpour *et al.*, 2014; Borzoui *et al.*, 2015). The study of nutritional indices of *P. xylostella* and *P. rapae* has some practical applications, such as a comparison of the performance of two pests in the treatment and nontreatment diet, an evaluation of consumed food by larvae with the aim of forecasting damage, and an assessment of the negative effects of triticale-proteinaceous inhibitor on their nutritional performance.

The aim of this study was to assess the effect of canola, sesame, bean, triticale, and wheat seed extracts on some physiological, biochemical, and biological parameters of the diamondback moth and small cabbage butterfly larvae. The fate of the inhibitor during the feeding and digestion process was subsequently pursued using interaction experiments. To consider the specificity of selected inhibitors, the proteinaceous extracts were also tested against the plant's own amylases. Thus, after the selection of the most efficient inhibitor under *in vitro* assays, the *in vivo* experiments were conducted in order to evaluate the insecticidal effects of the proteinaceous extracts on the growth and development of the two main crucifer pests, *P. xylostella* and *P. rapae* larvae.

Material and methods

Insect rearing

P. xylostella and *P. rapae* larvae were collected from the cabbage fields of Karaj region (Alborz Province, Iran) and transferred to a growth chamber under the conditions of 14:10 h photoperiod, a temperature of $25 \pm 1^\circ\text{C}$, and relative humidity of $65 \pm 5\%$. The emerged adults were transferred to a cage with fresh Chinese cabbage, *Brassica pekinensis* (Lour.). The cabbage plants with eggs were transferred to new cages daily (Ebrahimi *et al.*, 2013).

Enzyme extraction

Last larval instar guts were carefully dissected in NaCl solution (10 mM) under the stereomicroscope (Stemi SV6 ZEISS, Germany). Midguts were separated from the rest of the body by scalpel and homogenized in the pre-cooled homogenizer (Teflon pestle). The resulting homogenates were transferred into 1.5 ml centrifuge tubes and centrifuged at $15000 \times g$ for 15 min at 4°C . The supernatants were pooled, stored at -20°C , and then used as an enzyme source for subsequent analyses (Shi *et al.*, 2013).

Plant enzyme extraction

Plant amylase was extracted according to Biswas *et al.* (1978) with some modifications. Seeds of *B. napus* and *Brassica oleracea* were placed in Petri dishes on moistened paper and were left under germination conditions for 12 h. Subsequently, the germinated seeds were macerated thoroughly in liquid nitrogen using a pestle in a chilled mortar with 10 ml of cold phosphate buffer (0.1 M, pH 7.2). The homogenate was centrifuged at $10,000 \times g$ for 25 min at 4°C . The clean supernatant was collected and stored in -20°C for enzyme inhibition assay (Biswas *et al.*, 1978).

Seed inhibitor extraction

Proteinaceous seed extracts from common bean (*Phaseolus vulgaris*), pea (*Pisum sativum*), wheat (*Triticum aestivum*), canola (*B. napus*), sesame (*Sesamum indicum*), and triticale (\times Triticosecale wittmack) were extracted according to the method of Baker (1987) and Melo *et al.* (1999). Seeds (30 g) were ground and mixed with a solution of NaCl (0.1 M) and stirred for 1.5 h followed by centrifugation at $10,000 \times g$ for 30 min at 4°C . The supernatant was exposed to ammonium sulfate fractionation. The precipitate obtained between 20 and 80% saturation was dissolved in 2 mL of PBS (20 mM), dialyzed against the same buffer and heated at 70°C for 30 min in order to inactivate endogenous enzymes. The 80% pellet containing the highest fraction of amylase inhibitors was selected as a source of plant inhibitor (Dastranj *et al.*, 2013).

Insect gut pH determination

Eight standard indicator dyes were used in order to determine gut pH. These indicators include cresol red (pH 7.2–8.8), thymol blue (pH 8–9.6), bromophenol blue (pH 3–4.6), methyl orange (pH 3.1–4.4), bromocresol purple (pH 5.2–6.8), bromothymol blue (pH 6–7.6), neutral red (pH 6.8–8), and alizarin yellow (pH 10.2–12.1). The gut pH was determined based on the Bignell & Anderson (1980) procedure with some modifications.

Last-instar larvae were dissected and their midguts were separated and kept in the watch glass, and then 10 μ l of each pH indicator was added to each sample and the color was recorded.

Effect of temperature, pH, and inhibitors on α -amylase activity

α -Amylase activity was assayed using the dinitrosalicylic acid (DNS) procedure (Bernfeld, 1955), with 1% soluble starch solution as substrate. The extracted proteins were subjected to various temperatures (20, 25, 30, 35, 40, 45, and 50°C) for 30 min for thermal stability. Effects of pH were also assessed using the universal buffer at different pH (5, 6, 7, 8, 9, and 10). Enzyme and inhibitor were preincubated together for 15 min, and then starch was added to the reaction and OD read at 540 nm. All assays were performed with three technical replicates and three biological repetitions.

SDS-PAGE analysis

SDS-PAGE was performed according to Laemmli (1970). 10% acrylamide:bisacrylamide (37.5:1) was used for separating gel and 4% one was used for the stacking gel. Polyacrylamide gel was stained with 0.2% Coomassie Brilliant Blue R-250.

In-gel amylase inhibition assay

The inhibitory effect of prepared inhibitors on insects' α -amylase activity was detected by native gel electrophoresis (PAGE) at a concentration of 10% under non-denaturing conditions. Extracted enzyme was pre-incubated with different concentration of Triticale and wheat proteinaceous extracts (5.6, 2.8, 1.4, 0.7, 0.35 μ g protein) for 30 min at 35°C, then the remaining amylase activity was determined by Native SDS-PAGE. Native SDS-PAGE was performed using the procedure described by Laemmli (1970). The gel was rinsed with distilled water and treated by 1% (v/v) Triton X-100 for 15 min after electrophoresis. Then, the gel was incubated in Tris-HCl buffer pH 8.0 containing 1% starch solution, 2 mM CaCl_2 and 10 mM NaCl for 1.5 h. Finally, the gel was transferred into a solution of 1.3% I2 and 3% KI to stop the reaction and to stain the unreacted starch background. Light band zones indicated α -amylase activities which appeared against the dark background.

Interaction between the inhibitors and digestive enzymes

Plant proteinaceous extracts were pre-incubated with midgut enzymes for 3 h in order to evaluate whether gut proteases inactivate amylase inhibitors. The period of 3 h is more or less the time an ingested meal spends in a larval gut. Then, these samples were subjected to gel electrophoresis (SDS-PAGE) and compared with the control to see if any band(s) were removed.

In vivo inhibitory bioassays

Effects of triticale-proteinaceous extract on *P. xylostella* and *P. rapae* larval weight and survival were evaluated using dipping assay (Bandani & Butt, 1999). Triticale proteinaceous extract at 0.85 mg ml⁻¹ concentration was used as a working solution and water as the control. Leaf discs (3 cm in diameter) from *B. pekinensis* leaves were dipped for 30 s in the working solution and control, then were air-dried for 1 h at room temperature. The leaf discs were then shifted to 9-cm diameter

petri dishes and early second-instar larvae (0–24 h) were put on the fresh leaves. The leaves were prepared in the same way every 24 h for 4 days. Then larval growth and development, as well as each larval stage weight up to adult emergence, were monitored.

Feeding efficiency assays

Feeding efficiency, as described by Waldbauer (1968) with some modifications from Hemati *et al.* (2012), was determined for four days larval feeding. Seventy replications of newly hatched fourth-instar larvae (<24 h) were prepared. The initial weight of larvae was measured and they were transferred separately onto *B. pekinensis* leaves supplemented (0.85 mg ml⁻¹) or not (control) with triticale inhibitor in 30-ml transparent plastic cups (diameter 10 cm, depth 3 cm) with the top end open and blocked with cloth mesh (diameter 10 cm) to prevent the larvae from escaping. Each day, larvae and their frass were weighed separately to the nearest 0.00001 g. Each larva was provided each day with weighed fresh food. To establish the percentage of dry weight of the larvae, their frass, and food, 20 specimens of larvae, 20 specimens of frass and 20 specimens of food were weighed, oven-dried (48 h at 60°C), and subsequently re-weighed. Nutritional indices of *P. xylostella* and *P. rapae* larvae were calculated using the formulae described by Waldbauer (1968):

(AD) = E - (F/E); Efficiency of conversion of ingested food (ECI) = P/E; Efficiency of conversion of digested food (ECD) = P/(E - F); Relative consumption rate (RCR) = E/(A × T); and Relative growth rate (RGR) = P/(A × T). Where A = mean dry weight of insect over unit time, E = dry weight of food consumed, F = dry weight of feces produced, P = dry weight gain of insect, and T = duration of feeding period.

Protein determination

Protein concentrations were determined by the method of Bradford (1976), with bovin serum albumin as the standard.

Statistical analysis

All experiments were conducted in a factorial arrangement with three technical replications. Data analyses were performed using SAS 9.1 (SAS, 2004), and means were compared with Duncan's multiple range test (DMRT) at $P < 0.05$ following the analysis of variance (ANOVA).

Results

Gut pH, optimum conditions for α -amylase

Several factors affect midgut protein content and enzyme activity, including diet, temperature, and pH. *P. xylostella* and *P. rapae* midgut content were alkaline (pH 8.3–8.6) and the activity of α -amylases could be maximized at pH 8.0. The effect of temperature on α -amylases was also examined, showing that in both cases tested α -amylases were thermolabile, with optimum temperatures at 30°C. Above and below these temperatures, the α -amylases' activities sharply decreased (data not shown).

Table 1. The effect plant proteinaceous extract on the α -amylase two major cabbage pests.

Plant extract	<i>P. rapae</i>		<i>P. xylostella</i>	
	Amylase inhibition (%)	Optimum pH of inhibition	Amylase inhibition (%)	Optimum pH of inhibition
Pea	27c	6	12e	6
Bean	21d	5	9.7e	6
Canola	16f	7	19d	8
Sesame	18.6e	8	23c	8
Triticale	95a	10	95a	10
Wheat	92.6b	9	83b	8

Means followed by different letters in the same column are significantly different (Duncan, $P < 0.01$).

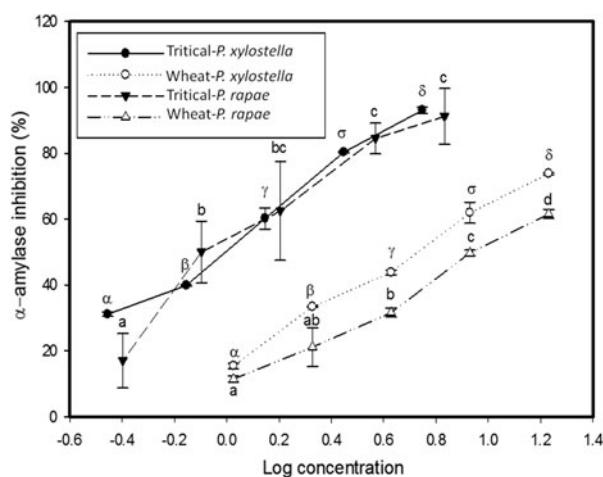


Fig. 1. Dose-response curve of inhibition of *P. xylostella* and *P. rapae* α -amylase by triticale and wheat seed proteinaceous extracts. Each point represents the average of at least three independent measurements. The means with same lowercase letters are not significantly different according to Duncan's multiple range test (DMRT) at $\alpha = 0.05$.

α -Amylase inhibition studies

The inhibitory effects of proteinaceous plant extract on the α -amylase of *P. xylostella* and *P. rapae* at different pH were determined *in vitro*, and the highest inhibition occurred at alkaline pH by triticale and wheat (table 1). A dose-dependent trend was observed for *P. xylostella* amylase with I_{50} values of 0.88 and 5.5 μ g for triticale and wheat, respectively (fig. 1). *P. rapae* amylase was also inhibited by triticale and wheat proteinaceous extract in a dose-dependent manner, with an I_{50} value of 0.78 and 1.16 μ g protein, respectively (fig. 1).

Specificity of inhibition

In order to investigate the effects of plants' inhibitors on their own α -amylase activity, we designed an experiment in which the inhibitors were assayed against plants' amylase. The triticale and wheat inhibitors, which strongly inhibited α -amylase of *P. rapae* and *P. xylostella* induced no inhibition on *B. rapa* and *B. oleracea* amylase (data not shown).

Zymogram analysis

The larvae of *P. xylostella* and *P. rapae* possess two α -amylase isoforms as determined by gel assays. The *P. xylostella* and *P. rapae* larvae fed on the *B. rapa* expressed two amylase isoforms, one minor and one major band (fig. 2, lane 6). Using a spectrophotometric assay, a strong inhibition of wheat and triticale extracts was revealed quantitatively; in the gel inhibition assay of the extract they were also investigated qualitatively. *P. xylostella* and *P. rapae* α -amylase were subjected to a serial concentration of triticale and wheat extracts. The low concentrations of triticale inhibitors caused an impressive reduction in the intensity of the two amylolytic bands and complete disappearance of both bands (fig. 2a, c). The reduced intensity of amylolytic bands was also induced by the wheat, but the bands did not disappear even when a high inhibitor concentration was applied (fig. 2b, d).

Insect bioassay

The effects of triticale inhibitor on larval weight and survival of *P. xylostella* and *P. rapae* were monitored using dipping assay. Changes in the body weight of different instar larvae feeding on treated and control leaves are shown in fig. 3. A significant decrease in body weight of *P. xylostella* third- and fourth-instar larvae fed on the treated leaves was noted when compared with those reared on control leaves (fig. 3a). The triticale inhibitor caused an overall 30% reduction in the larval weight at the early pupal stage ($P < 0.0001$). The survival of *P. xylostella* larvae was negatively affected by triticale-proteinaceous extract, so that extracted proteins caused an overall 55% mortality of *P. xylostella* larvae (fig. 3b). Survival of the triticale-fed larvae of *P. rapae* was not affected significantly (fig. 2d), but the range of 8–16% weight reduction was observed at different larval instars (fig. 3c).

Feeding efficiency assays

Fourth-instar *P. xylostella* and *P. rapae* that continuously fed on triticale-proteinaceous inhibitor containing diet for 5 days showed some statistically significant reductions in the nutritional indices (AD, ECI, ECD, RCR, and RGR) in comparison with control larvae (table 2). In most cases and both *P. xylostella* and *P. rapae*, the highest and lowest values of the nutritional indices were in control and treated larvae, respectively.

Interaction of triticale-proteinaceous extract with digestive enzyme

The stability of the inhibitor during digestion against proteolytic activities is an important trait that defines the inhibitor's efficacy. The results showed that although incubation of triticale with *P. xylostella* and *P. rapae* midgut enzyme reduced part of an inhibitory activity, the bands present in the triticale extract were not affected by digestive enzymes (fig. 4a, b).

Discussion

α -Amylase is the main carbohydrate digestive enzyme prevailing in the insect gut, which hydrolyzes starch and glycogen and helps to regulate larval energy metabolism (Terra *et al.*, 1996). Several environmental properties affect the efficiency of digestive enzyme activity, including temperature and pH. In this study, results showed gut lumen

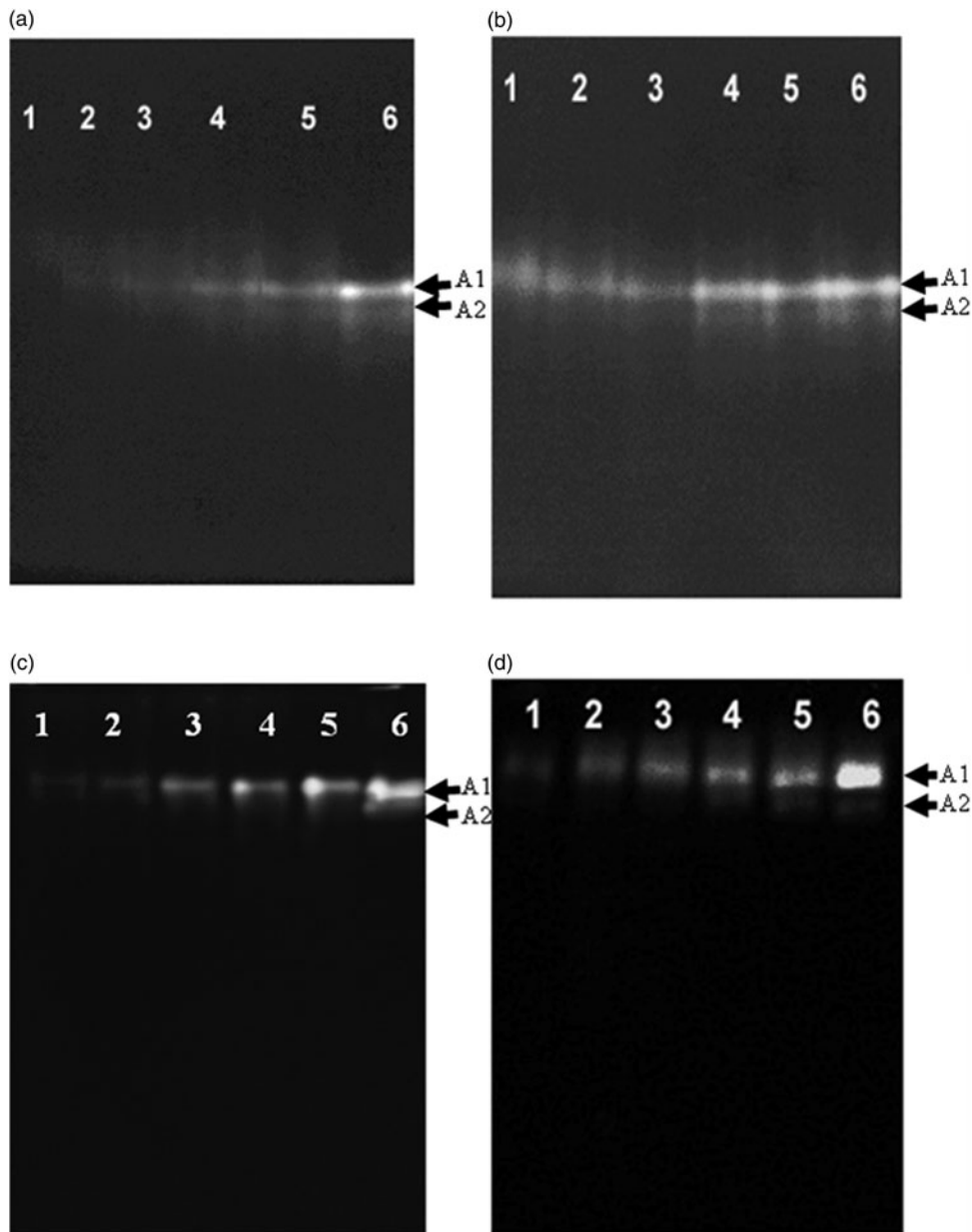


Fig. 2. Cabbage pests gut extract zymogram using 1% starch as substrate. Extracted enzymes were pre-incubated with Triticale and wheat proteinaceous extracts for 30 min at 35°C, then the remaining amylase activity was determined by SDS-polyacrylamide gel electrophoresis (Native Page). In gel amylase inhibition assay was carried out using (a) triticale on *P. xylostella*, (b) wheat on *P. xylostella*, (c) triticale on *P. rapae*, and (d) wheat on *P. rapae*. Numbers 1–6 correspond to 5.6, 2.8, 1.4, 0.7, 0.35 μ g protein and control, respectively. A1 and A2 are defined as an amylase isozyme.

pH and maximum amylase activity of two main lepidopteran cabbage pests, *P. xylostella* and *P. rapae*, which occurred in alkaline pH. The optimal pH for enzyme activity has been reported to be correlated with the prevailing pH of the midgut from which the enzyme has been isolated (Terra *et al.*, 1996; Terra & Ferreira, 2012). The alkaline midgut pH in lepidopteran insects enables them to feed on plant materials rich in tannins, which bind to proteins at acidic pH and consequently cause a reduction in digestion efficiency (Dow, 1992).

Initial observation revealed that *P. xylostella* and *P. rapae* amylase have two isoenzymes. Expression of multi-isoenzymes in insect pests is a strategy to escape from inhibitor toxicity present in the host (Sivakumar *et al.*, 2006). A mixture of different α -amylase isoenzymes has been reported for other insects such as *Sitophilus oryzae*, *Tribolium castaneum*, *A. grandis*, *Callosobruchus maculatus*, *Rhyzopertha dominica*, *Sitophilus granarium*, and *Eurygaster intergriceps* (Terra *et al.*, 1977; Chen *et al.*, 1992; Oliveira-Neto *et al.*, 2003; Kazzazi *et al.*, 2005; Mehrabadi *et al.*, 2011).

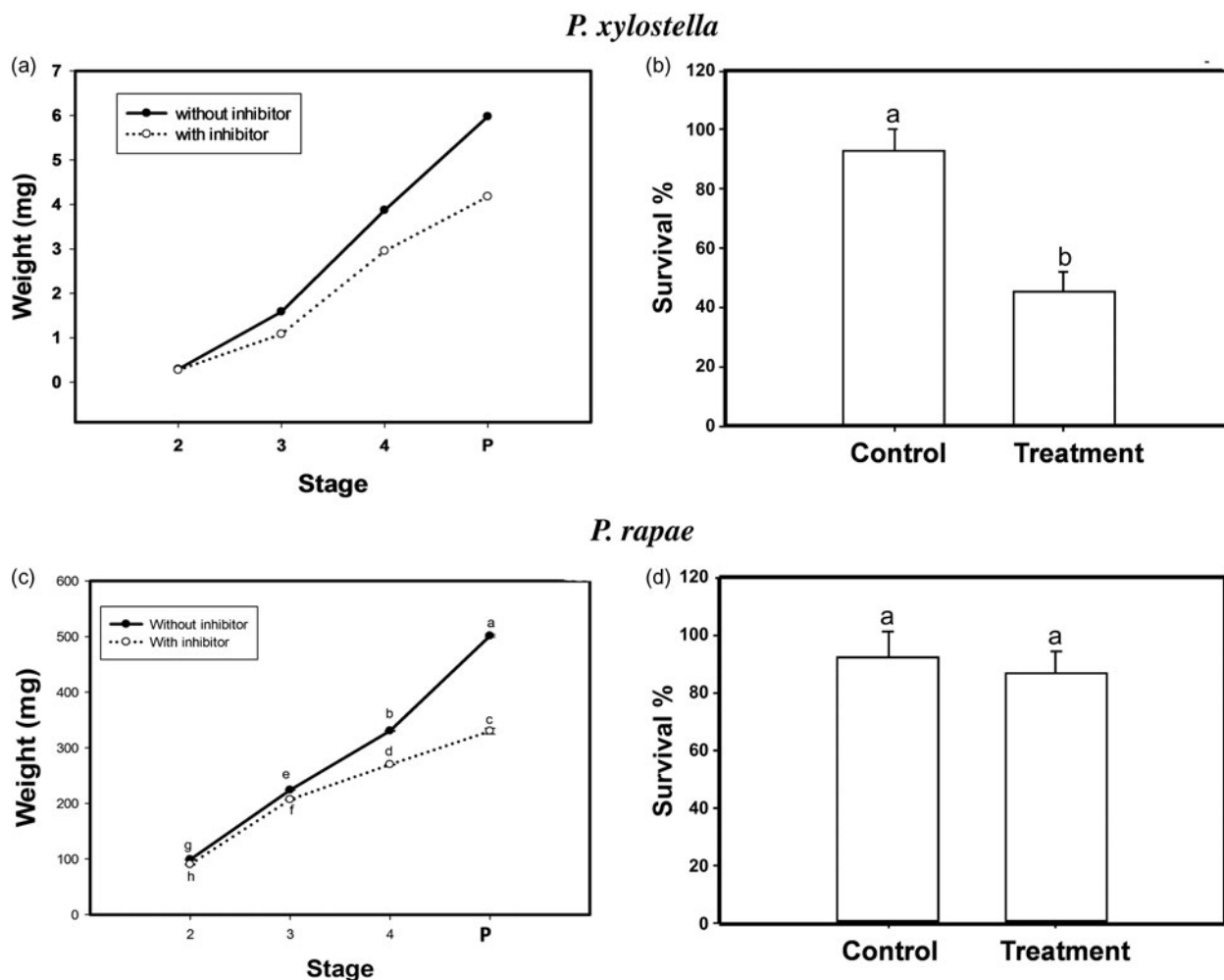


Fig. 3. The effects of triticale-proteinaceous extract on weight and survival of *P. xylostella* (a,b) and *P. rapae* (c,d). The cabbage leaves were painted with the highest concentration of the inhibitor (5.6 μg) used in the previous *in vitro* inhibition assay. Numbers 2, 3, 4 are respectively second, third, and fourth larval instars and the letter P indicates pupa. The means with same lowercase letters are not significantly different according to Duncan's multiple range test (DMRT) at $\alpha = 0.05$.

The amylase inhibitors, which naturally belong to the plant's natural defense systems, impede digestion by acting on insect gut amylase (Jongsma & Bolter, 1997). Different classes of plant amylase inhibitors have been identified, based on their structural varieties, which cause different modes of inhibition and specificity (Franco *et al.*, 2002). Recently, the use of enzyme inhibitors has received a great deal of attention in pest management using ectopic expression in transgenic plants (Carrillo *et al.*, 2011).

Initial efforts in this study were made to screen the inhibitory effects of canola, sesame, bean, triticale and wheat proteinaceous extract against the α -amylase of *P. xylostella* and *P. rapae*. The highest inhibition among the inhibitors was related to triticale and wheat. Comparison of estimated I_{50} values for triticale and wheat indicated that *P. xylostella* and *P. rapae* α -amylase is more sensitive to triticale than wheat. Like other cereal grain, wheat and triticale are relatively rich in amylase inhibitors. The proteins extracted from some cereal seeds, such as finger millet, have been shown to induce inhibitory effects against a wide range of insect species, including

S. oryzae, *T. castaneum*, *C. chinensis*, *Carcyra cephalonica*, *Spodoptera litura*, *Helicoverpa armigera*, *Acaea janata*, and *P. xylostella* (Sivakumar *et al.*, 2006). The amylase-inhibiting activity of triticale and wheat, which has also been investigated in other studies (Mehrabadi *et al.*, 2010; Ashouri *et al.*, 2015). Mehrabadi *et al.* (2010, 2012), showed that the inhibitors extracted from triticale reduced the activity of midgut and salivary gland α -amylase of the sunn pest (*E. integriceps*).

Insects' digestive tract determines the consequence of enzyme-inhibitor interaction by providing the conditions, such as pH (Valencia *et al.*, 2000; Mehrabadi *et al.*, 2012). The inhibitory activity of triticale and wheat proteinaceous inhibitors against amylases of *P. xylostella* and *P. rapae* has been demonstrated to be pH-dependent. As the gut lumen pH of both insects was determined to be alkaline, the interaction of the plant proteinaceous inhibitors and midgut enzymes occurs at alkaline pH, which is optimum for inhibition. Similar reports have been made in other studies in which the amylase-inhibitor complex was affected by the pH value of the interaction environment (Franco *et al.*, 2002; Morton *et al.*, 2000).

Table 2. Mean (\pm SE) nutritional indices of two major cabbage pests on diet treated with Triticale proteinaceous extract.

Insect (Treatment)	AD ¹ (%)	ECl ² (%)	ECD ³ (%)	RCR ⁴ (mg/mg/day)	RGR ⁵ (mg/mg/day)
<i>P. xylostella</i>					
Control	95.48 \pm 8.75a	1.49 \pm 0.11a	1.56 \pm 0.17a	34.66 \pm 2.17a	0.51 \pm 0.06a
Inhibitor	95.33 \pm 11.54a	1.00 \pm 0.12b	1.05 \pm 0.18b	36.17 \pm 2.34a	0.36 \pm 0.03b
<i>P. rapae</i>					
Control	74.40 \pm 9.64A	20.34 \pm 2.14A	27.34 \pm 3.06A	0.96 \pm 0.09A	0.19 \pm 0.009A
Inhibitor	80.33 \pm 6.82A	11.90 \pm 1.57B	14.82 \pm 2.78B	0.90 \pm 0.08A	0.10 \pm 0.007B

Means followed by different letters in the same column are significantly different (Duncan, $P < 0.01$).

¹Approximate digestibility.

²Efficiency of conversion of ingested food.

³Efficiency of conversion of ingested food.

⁴Relative consumption rate.

⁵Relative growth rate.

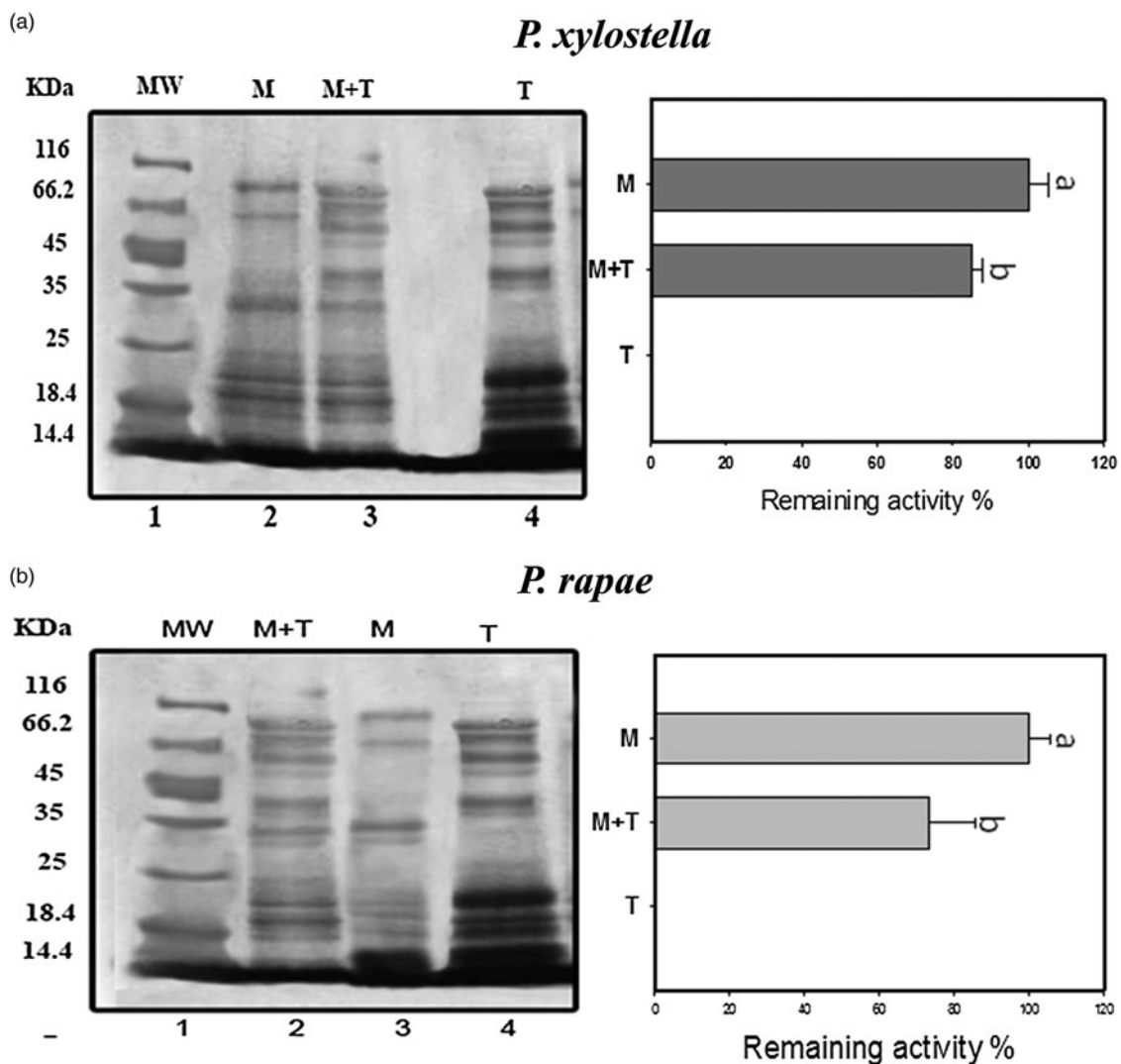


Fig. 4. Interaction of *P. xylostella* (a) and *P. rapae* (b) midgut extract and triticale-proteinaceous extract. The triticale-proteinaceous extracts were pre-incubated with *P. xylostella* and *P. rapae* midgut extracts for 3 h at 35°C. SDS-PAGE was performed in 10% (w/v) gel for separating gel and 4% for stacking gel, and the proteins in the polyacrylamide gel were stained with 0.2% Coomassie Brilliant Blue R-250. M, T and M+T respectively indicate Midguts, Triticale seed proteinaceous extract, and midgut mix with seed proteinaceous extract.

Another feature which affects the efficiency of an inhibitor is its stability against proteases after ingestion. Degradation of an inhibitor by proteases during feeding results in an reduction in the persistence of the inhibitory action in the gut. Yang *et al.* (2009) showed that insensitivity of *P. xylostella* to mustard trypsin inhibitor 2 (MTI2) was due to enzymatic specific degradation of MTI2 (Yang *et al.*, 2009). The stability of triticale after incubation for 3 h suggests that the triticale amylase inhibitor might be active during digestion in Lepidoptera. Additionally, the stability of triticale against insects from the Hemipteran order containing acidic pH gut lumen suggests that triticale can efficiently persist in a wide range of pH conditions (Mehrabadi *et al.*, 2012).

Taking the bioassay results into consideration, triticale-proteinaceous extract affected some fitness-related traits of larvae. The significant decreases in the body mass (30% for *P. xylostella* and 17% for *P. rapae*) were observed when the larvae fed on leaves contaminated by triticale inhibitor at 0.85 mg ml⁻¹. While triticale negatively affected the survival rate of *P. xylostella*, there was no alteration in *P. rapae* survival after ingestion of triticale-proteinaceous extract. Priya *et al.* (2010) showed that the incorporation of wheat α -amylase inhibitor at 1.5 and 2.0% concentrations in the diet of *R. dominica* caused a significant reduction in its development and survival, but did not affect the body weight of the treated insects. The expression of α -amylase inhibitors in transgenic plants led to decrease survival and block development in some insect pests (de Azevedo Pereira *et al.*, 2006; Barbosa *et al.*, 2010; Dias *et al.*, 2010; Priya *et al.*, 2010).

The variation in the nutritional indices, particularly ECI and ECD values, in both *P. xylostella* and *P. rapae* reared on treated- and control diets was strongly related to the weight of larvae, the weight of food consumed and weight of feces produced, suggesting that the triticale-proteinaceous inhibitor possesses antifeedant activity against both *P. xylostella* and *P. rapae* larvae. Amylolytic activity in fourth-instar *P. xylostella* and *P. rapae* fed on the diets treated with triticale-proteinaceous inhibitor was investigated to find the mechanism of action. The triticale-proteinaceous inhibitor decreased larval α -amylase activity in both insects' larvae. This suggests that the inhibitory effect of the proteinaceous inhibitor on amylolytic activity can be the reason for the reduction in ECI and ECD in both *P. xylostella* and *P. rapae* larvae, which is similar to the results of Borzoui *et al.* (2015) who reported that the inhibition of α -amylase affected the insect's nutritional indices.

Among different treatments, the highest RCR value was for control larvae, demonstrating that the weight of food consumed, relative to the mean weight of larvae over the feeding period, was the highest for these larvae, which suggests that larvae became impaired for feeding as a result of their intake of the proteinaceous inhibitor. Also, the RGR value of both *P. xylostella* and *P. rapae* larvae was highest for those larvae reared on the control diet and was lowest for those larvae reared on a treated diet. This demonstrates that the larvae fed on the control diet had higher efficacy than the larvae fed on a treated diet in the conversion of ingested food into growth. It seems that the proteinaceous inhibitor reduced the larvae's ability to convert ingested food into nutrients to growth (Borzoui *et al.*, 2015).

The use of non-host plant inhibitors is believed to enhance the efficiency and success of pest management programs, because these inhibitors are much more efficient in blocking enzymes from insects that have not already experienced them previously (Bolter & Jongsma, 1995; Broadway, 1997; Priya *et al.*, 2010).

We have shown that a Triticale seed proteinaceous extract effectively interfered with normal growth and survival of *P. xylostella* and *P. rapae*, and was inactive against plant amylases. Furthermore, Triticale was resistant to insect gut proteases indicating high stability during digestion. Altogether, given the Triticale inhibitor's ability to inactivate amylolytic activity in the insects, we conclude that Triticale has potential for application via plant molecular biology for the management of key crucifer pests.

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