

## RESEARCH

## Comparative Feeding Performance and Digestive Physiology of *Helicoverpa armigera* (Lepidoptera: Noctuidae) Larvae-Fed 11 Corn Hybrids

A. S. Hosseinijad, B. Naseri,<sup>1</sup> and J. Razmjou

Department of Plant Protection, Faculty of Agricultural Sciences, University of Mohaghegh Ardabili, Ardabil, Iran

<sup>1</sup>Corresponding author, e-mail: bnaseri@uma.ac.ir

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**ABSTRACT.** This study aimed to evaluate the feeding responses and digestive proteolytic and amylolytic activity of *Helicoverpa armigera* (Hübner) on 11 corn (*Zea mays* L.) hybrids at  $25 \pm 1^\circ\text{C}$ ,  $65 \pm 5\%$  relative humidity (RH), and a photoperiod of 16:8 (L:D) h. The fourth- and fifth-instar larvae fed on hybrid K47\*K19 had the highest weight of food consumption and those reared on hybrid KSC705 had the lowest value of food consumption. The highest weight gain of the larvae was observed when *H. armigera* were fed hybrid KLM78\*MO17 and lowest when they were fed hybrids K36 \* MO17, KSC705, and K35 \* K36. Pupal weight of *H. armigera* was heaviest when larvae were fed hybrid K47\*K19 and lightest when they were fed hybrid KSC705. The highest proteolytic activity of the fourth-instar larvae was observed when they were fed hybrid KSC705, and the lowest activity was observed when they were fed hybrid K47\*A67. Fifth-instar larvae that fed on hybrid K47\*K19 showed the highest proteolytic activity. Fourth-instar larvae that fed on hybrid K36\*MO17 showed the highest amylase activity. The fifth-instar larvae fed on hybrid K47\*A67 showed the maximum amylase activity and those reared on the K48\*K18 showed the minimum activity. Our results indicated that K36 \* MO17, KSC705, and K48 \* K18 were the most unsuitable hybrids for feeding *H. armigera*.

**Key Words:** cotton bollworm, digestive enzyme, feeding response, corn

Corn grain (*Zea mays* L.) is not only important in terms of energy supply but also its green forage is high in quality and possible for silage making. Because of the importance of corn in humans and animals' nutrition as well as in industrial applications, identifying the barriers to its production seems to be necessary. One of the factors limiting corn production is the cotton bollworm, *Helicoverpa armigera* (Hübner), a common agricultural pest in Iran (Farid 1986; Naseri et al. 2011) and in many countries of the world (Liu et al. 2004; Harsulkar et al. 1999). Some important characteristics such as a broad host range (Zalucki et al. 1994), high mobility through flight (Drake 1991), high reproductive potential, and a facultative diapauses has allowed *H. armigera* to survive under unstable habitats (Fitt 1989). Application of chemical pesticides has led to a variety of environmental hazards and resistance in *H. armigera* to the pesticides, especially to synthetic pyrethroids (Gunning et al. 1984). Such hazards in the ecosystem and the increase of acute and chronic toxicity exposure in humans have led to notable changes in pest management guidelines (Naseri et al. 2009).

Host plant resistance serves an important tool, which is satisfactory in terms of economic and environmental health (Kennedy et al. 1987). Growth, development, and reproduction of insects strongly depend on the quality and quantity of the food eaten by them (Scriber and Slansky 1981). The primary and secondary metabolites of the host plants significantly affect the population parameters of herbivores (Bernays and Chapman 1994). The variation in growth is even more remarkable when different food constituents are involved (Ashfaq et al. 2003). Nutritional regulation by an insect represents the integrated outcome of a highly complex set of interacting processes. Acquisition and allocation of different nutrient molecules required for survival and reproduction of the insects is central to these processes (Simpson and Raubenheimer 1999). For availability of the major nutrients, regulation of digestive enzymes gains priority. Activities of the insects' digestive enzymes are affected by various factors, such as the stage of individuals (larval or adult stage), the midgut pH, and secondary metabolites in the ingested tissues of the plants (Amorim et al. 2008).

Digestion of dietary protein is an essential process for herbivorous insects. During their development, insect larvae liberate free amino acids and nitrogen by the action of endopeptidases and exopeptidases. In response to insect feeding, plants produce inhibitory proteins to interfere with the insects' digestive system (Green and Ryan 1972). The proteolysis in the lepidopteran larvae gastrointestinal tract is inhibited by these inhibitors. Proteolysis inhibition which interfere with amino acid metabolism is a key target to control insect pests (Hilder et al. 1992), leading to decreased utilization of food resources, growth delay, reduction in insect survival, size or weight, and reproduction of the new generation adults (Gatehouse et al. 1999). Amylases ( $\alpha$ -1, 4 glucan 4 glucanohydrolases; EC 3.2.1.1) are of the most important enzymes in insects' digestion biochemistry including the lepidopteran larvae. These enzymes catalyze hydrolysis of  $\alpha$ -D-(1, 4)-glucan linkage in starch components, glycogen, and other related carbohydrates to serve as an energy source (Franco et al. 2000).

Previously, Cohen and Patana (1984) evaluated the efficiency of food utilization by *Heliothis zea* (Boddie) -fed artificial diets or green beans. Food consumption and digestive enzymatic activity of *H. armigera* on different host plants have recently been studied by several authors (Kotkar et al. 2009; Naseri et al. 2010; Fathipour and Naseri 2011; Hemati et al. 2012a,b; Rahimi Namin et al. 2014). However, little information is available about nutritional responses and digestive physiology of *H. armigera* on seeds of various corn hybrids when incorporated into artificial diets (Arghand et al. 2011; Naseri and Razmjou 2013). Therefore, we studied feeding performance, digestive proteolytic, and amylolytic activities of *H. armigera* fourth and fifth instar on 11 corn hybrids to better understand the digestion physiology of the pest. Such information will be important for identifying efficient proteinase inhibitors (PIs) from different corn hybrids and their subsequent use for transgenic expression in host plants to supply stable resistance against *H. armigera*.

### Materials and Methods

**Plant Sources.** In this study, 11 hybrids of corn including K47/2-2-1-22-1-1-1\*K19, K3547/3\*K3615/2, K3653/2\*MO17, K3615/2\*K19/1,

KSC705, KLM77002/10-1-1-1-1-6-1\*K19/1, K47/22-1-22-1-1-1\*A679, K48/3-1-1-3-2-1-1-1\*K18, KLM78018/6-1-1-1-3-2 \* MO17, KSC720, and KSC704 were obtained from Gorgan Center of Agricultural Researches and Natural Resources in the suburbs of Gorgan, Iran, in summer 2012. Hybrid corn is a strain produced by fertilizing one variety of corn plant with the pollen from another. The source of our plant was a national project started 5 years ago in Iran which consisted of two steps. The first step was to inbreed a desirable strain of corn for five generations. Inbreeding was done by pollinating the young ear with pollen from its own plant. To prevent pollen from other plants from contacting the silk, the tassels and young ears were enclosed in bags. The next step was to select two strains of inbred plants, each with certain desirable qualities, and to crossbreed them. This was done by placing pollen from one strain on the silk of a plant of the other strain. The tassels were removed from the plants that were to bear the hybrid ears, so that the female-parent plants did not receive their own pollen but got pollen only from the male-parent plants in the adjoining rows. This crossbreeding produced hybrid ears.

**Insect Collection and Rearing.** Fifth-instar larvae of *H. armigera* were collected from cotton fields in Gorgan (North of Iran) in July and August 2012. For this study, the grains of different corn hybrids were detached from different hybrids and transferred to a controlled growth chamber ( $25 \pm 1^\circ\text{C}$ ,  $65 \pm 5\%$  relative humidity [RH], and a photoperiod of 16:8 [L:D] h). The corn tassel was used for feeding of first and second larval instars, and the immature corn kernels were used for feeding of the third to fifth larval instars. Fresh food material was provided every 12 h. The insects tested on different hybrids of corn had already been reared for two generations on the same hybrids they were fed during experiments.

Neonate larvae were gathered from the stock culture and separated into five replicates (10 larvae in each) and transferred into plastic containers (diameter: 19.5 cm, depth: 7.5 cm) with a hole covered by a mesh net for aeration, containing the fresh corn tassel of each examined hybrid. The end of detached tassel was inserted in water-soaked cotton to maintain freshness. A fine camel's hair brush was used to transfer the younger larvae. The first- and second-instar larvae were reared in groups until the third instar, after which they were divided onto individual plastic plates (diameter: 8 cm, depth: 1 cm) to prevent cannibalism (Twine 1971). Head capsules or exuviae from molting larvae were used to distinguish the larval instars. For prepupation and pupation, fifth-instar larvae were kept in small plastic tubes (diameter: 2 cm, depth: 5 cm).

**Feeding Responses of *H. armigera*.** For this examination, a gravimetric method described by Waldbauer (1968) was used to evaluate feeding responses of *H. armigera* on different corn hybrids. Larval weight, food consumed, and feces produced by fourth- and fifth-instar larvae fed on different corn hybrids were measured. The prepupa and pupa from the larvae reared on each hybrid were weighed as well. To obtain the percentage of dry weights of the foods, feces, larvae, prepupa, and pupa, 20 extra specimens for each were weighed, oven-dried (48 h at  $60^\circ\text{C}$ ), and subsequently reweighed.

**Chemicals.** The general proteolytic substrate azocasein, Bradford reagent, the dinitrosalicylic acid (DNS), and the amylolytic substrate starch were obtained from Sigma Chemical Co. (St. Louis, MO).

**Preparation of Larval Midgut Homogenates.** Fourth- and fifth-instar larvae reared on different corn hybrids (for 24 h) were cold incapable and quickly dissected under a stereomicroscope in cold distilled water. The midguts of the larvae were homogenized on ice by a hand-held glass homogenizer. The homogenates were then centrifuged at  $16,000 \times g$  for 10 min at  $4^\circ\text{C}$ . The resulting supernatant was gathered, frozen in aliquots, and stored at  $-20^\circ\text{C}$  until needed for protease and amylase assays.

**Protein Quantification of Larvae.** General protein concentrations in the midgut of fourth- and fifth-instar larvae of *H. armigera* were determined using bovine serum albumin as a standard according to the method of Bradford (1976).

**Proteolytic Activity.** General proteolytic activity present in the midgut of *H. armigera* larvae fed on different corn hybrids was assayed using azocasein as a substrate at the optimal pH = 12. To evaluate the azocaseinolytic activity, the reaction mixture containing  $80 \mu\text{l}$  of 1.5% azocasein solution in 50 mM universal buffer system (50 mM sodium phosphate-borate) (pH 12) and  $50 \mu\text{l}$  of crude enzyme was incubated at  $37^\circ\text{C}$  for 50 min. Proteolysis was finished by the addition of  $100 \mu\text{l}$  of 30% trichloroacetic acid (TCA), continued with cooling at  $4^\circ\text{C}$  for 30 min and centrifugation at  $16,000 \times g$  for 10 min. An equal quantity of 2M NaOH was added to the supernatant, and the absorbance was read at 440 nm. Appropriate blanks that TCA had been added before the substrate were prepared for each examination. Unit activity was represented as an increase in optical density per milligram protein of the tissue per minute due to azocasein proteolysis (Elpidina et al. 2001). All experiments were carried out in three replicates.

**Amylolytic Activity.** Digestive amylolytic activity of *H. armigera* larvae fed on different corn hybrids was determined using the DNS method, with 1% soluble starch as substrate at the optimal pH = 9 (Bernfeld 1955). A quantity of  $50 \mu\text{l}$  of the enzyme was incubated with  $250 \mu\text{l}$  of universal buffer system (10 mM succinate-glycine-2, morpholinoethan sulfonic acid) (pH 9) and  $20 \mu\text{l}$  of soluble starch for 30 min at  $37^\circ\text{C}$ . The reaction was stopped by the addition of  $50 \mu\text{l}$  DNS and heating in boiling water for 10 min. The absorbance was determined at 540 nm after cooling on ice. One unit of amylase activity was defined as the amount of enzyme required to produce 1 mg of maltose in 30 min at  $37^\circ\text{C}$  under the given assay conditions. All experiments were carried out in three replicates.

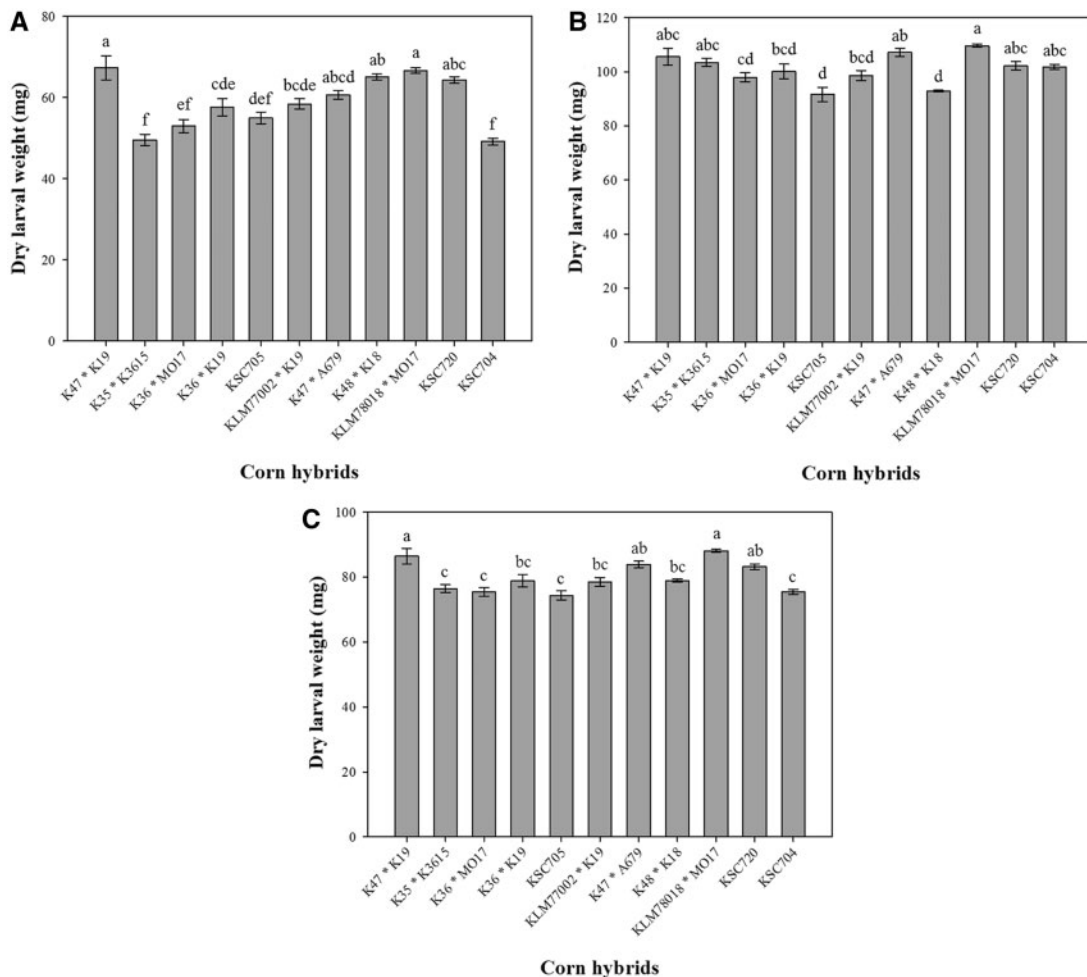
**Statistical Analysis.** Data were analyzed with one-way analysis of variance followed by comparison of the means with Tukey HSD test at  $\alpha = 0.05$  using statistical software Minitab 16.0 (Minitab Inc. 2000). All data were tested for normality before analysis.

## Results

**Feeding Responses of *H. armigera*.** The highest larval weight of the fourth instar ( $F = 19.16$ ;  $df = 319, 329$ ;  $P < 0.05$ ) was on hybrids K47 \* K19 ( $67.35 \pm 2.95$  mg per larva) and KLM78 \* MO17 ( $66.69 \pm 0.70$  mg per larva) and the lowest was on hybrids K35 \* K36 ( $49.57 \pm 1.41$  mg per larva) and KSC704 ( $49.19 \pm 0.84$  mg per larva). In the fifth-instar larvae, the highest larval weight ( $F = 8.80$ ;  $df = 319, 329$ ;  $P < 0.05$ ) was observed when *H. armigera* were fed hybrid KLM78 \* MO17 ( $109.66 \pm 0.64$  mg per larva), and the lowest was observed when they were fed hybrids K48 \* K18 ( $92.85 \pm 0.34$  mg per larva), and KSC705 ( $91.63 \pm 2.56$  mg per larva). The weight of whole fourth and fifth instars ( $F = 12.89$ ;  $df = 318, 328$ ;  $P < 0.05$ ) was heaviest when larvae were fed hybrids KLM78 \* MO17 ( $88.17 \pm 0.48$  mg per larva) and K47 \* K19 ( $86.47 \pm 2.43$  mg per larva) and lightest when they were fed hybrids K35 \* K36 ( $76.50 \pm 1.18$  mg per larva), KSC704 ( $75.52 \pm 0.73$  mg per larva), K36 \* MO17 ( $75.48 \pm 1.32$  mg per larva), and KSC705 ( $74.39 \pm 1.40$  mg per larva) (Fig. 1).

Figure 2 shows feeding responses of *H. armigera* whole fourth- and fifth-instar larvae-fed 11 corn hybrids. The fourth- and fifth-instar larvae consumed more food ( $F = 56.85$ ;  $df = 318, 328$ ;  $P < 0.05$ ) when they were fed hybrid K47 \* K19 ( $1,170.90 \pm 31.90$  mg per larva). The feces produced ( $F = 85.51$ ;  $df = 319, 329$ ;  $P < 0.05$ ) was the highest when larvae fed hybrid K36 \* MO17 ( $193.75 \pm 4.79$  mg per larva) and lowest when they were reared hybrids KSC720 ( $91.02 \pm 3.92$  mg per larva) and KSC705 ( $87.70 \pm 2.79$  mg per larva). The highest weight gain of the larvae ( $F = 67.39$ ;  $df = 317, 327$ ;  $P < 0.05$ ) was on hybrid KLM78 \* MO17 ( $37.69 \pm 0.50$  mg per larva) and the lowest was on hybrids K36 \* MO17 ( $16.22 \pm 0.81$  mg per larva), KSC705 ( $17.51 \pm 0.63$  mg per larva), and K35 \* K36 ( $18.01 \pm 0.74$  mg per larva) (Fig. 2).

Figure 3 shows prepupal and pupal weight of *H. armigera* fed different corn hybrids. Our results indicated that the prepupa ( $F = 7.55$ ;  $df = 319, 329$ ;  $P < 0.05$ ) and pupa were heaviest ( $F = 28.69$ ;  $df = 319, 329$ ;  $P < 0.05$ ) when larvae were fed hybrid K47 \* K19 ( $360.30 \pm 9.39$



**Fig. 1.** Mean larval weight of fourth (A), fifth (B), and whole fourth- and fifth- (C) instar larvae of *H. armigera*-fed different corn hybrids. Bars represent standard error of the means. The means followed by different letters are significantly different (Tukey HSD,  $P < 0.05$ ).

and  $305.10 \pm 8.11$  mg, respectively). However, the lowest prepupal weight was observed when larvae fed hybrid KLM77 \* K19 ( $302.10 \pm 2.87$  mg), and the lowest pupal weight was observed when they were fed hybrid KSC705 ( $220.73 \pm 5.19$  mg).

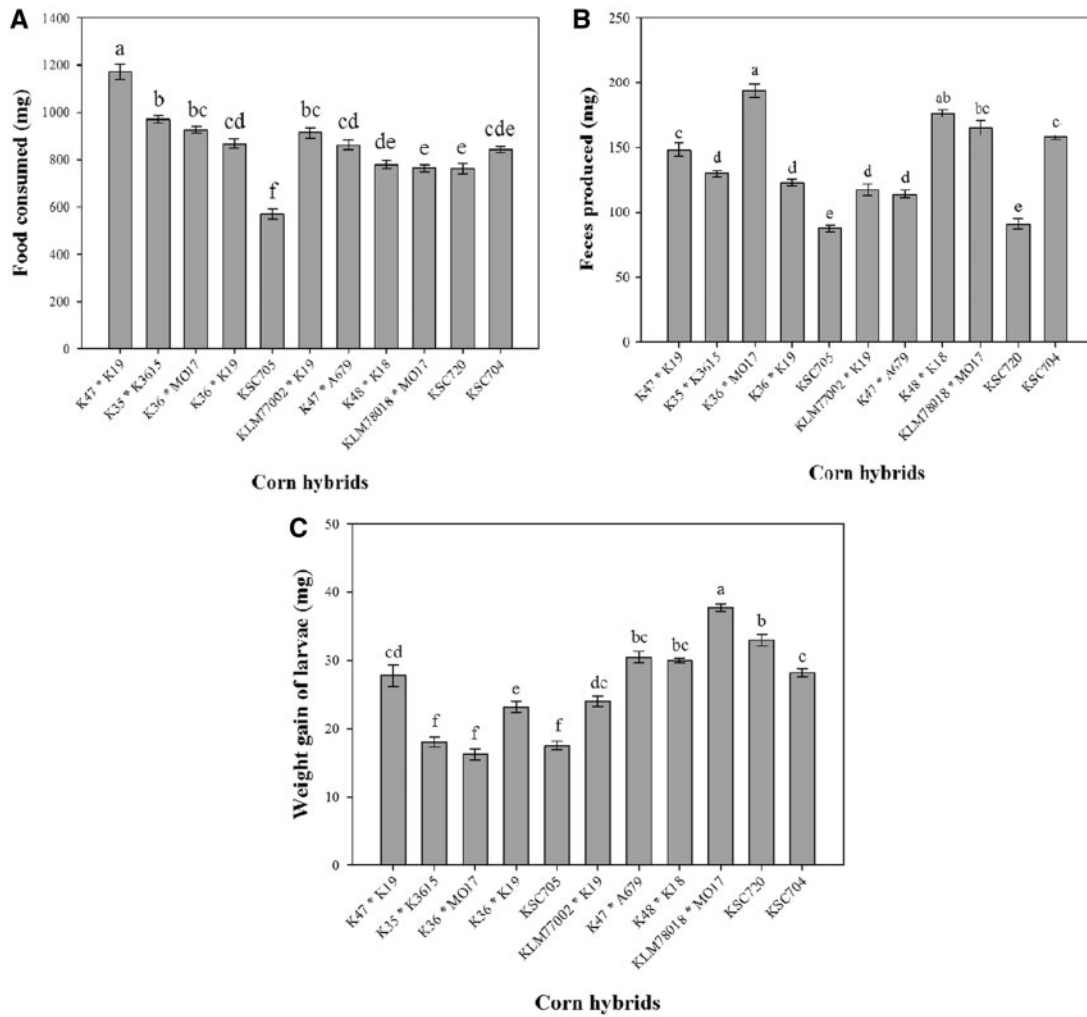
**General Proteolytic Activity.** The general proteolytic activity data from midgut extracts of *H. armigera* larvae-fed 11 corn hybrids are indicated in Fig. 4. The highest proteolytic activity of *H. armigera* fourth-instar larvae ( $F = 33.60$ ;  $df = 11, 21$ ;  $P < 0.05$ ) was observed when *H. armigera* were fed hybrid KSC705 ( $4.85 \pm 0.24$   $\text{Umg}^{-1}$ ), and the lowest activity was observed when they were fed hybrid K47 \* A67 ( $1.34 \pm 0.19$   $\text{Umg}^{-1}$ ). Fifth instars that fed on hybrid K47 \* K19 ( $8.38 \pm 0.06$   $\text{Umg}^{-1}$ ) showed the highest general proteolytic activity ( $F = 76.07$ ;  $df = 11, 21$ ;  $P < 0.05$ ), and those reared on hybrid K48 \* K18 ( $0.24 \pm 0.03$  ( $\text{Umg}^{-1}$ )) showed the lowest activity.

**Amylolytic Activity.** Figure 5 shows mean amylolytic activity of midgut extracts from *H. armigera* larvae-fed different corn hybrids. The highest amylase activity in *H. armigera* fourth instar ( $F = 40.70$ ;  $df = 11, 21$ ;  $P < 0.05$ ) was detected when larvae were fed hybrid K36 \* MO17 ( $1.17 \pm 0.04$   $\text{Umg}^{-1}$ ), and the lowest activity was observed when they were fed hybrids K48 \* K18 ( $0.45 \pm 0.006$   $\text{Umg}^{-1}$ ) and KLM78 \* MO17 ( $0.42 \pm 0.03$   $\text{Umg}^{-1}$ ). Fifth-instar larvae fed on hybrid K47 \* A67 ( $F = 26.95$ ;  $df = 11, 21$ ;  $P < 0.05$ ) ( $4.49 \pm 0.47$   $\text{Umg}^{-1}$ ) showed the maximum amylase activity and those reared on K48 \* K18 ( $0.54 \pm 0.003$   $\text{Umg}^{-1}$ ) showed the minimum activity of the enzyme.

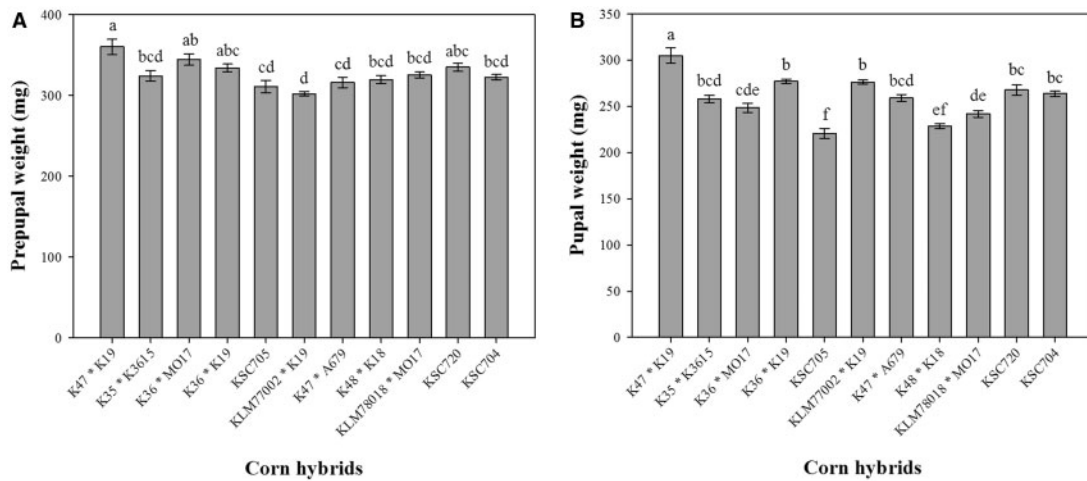
## Discussion

Larvae of *H. armigera* are voracious feeders and are highly adaptive to the kind of food ingested or available. Digestive gut proteinases of *H. armigera* revealed their complex diverse and flexible nature for protein digestion during larval development and upon feeding on various host plants (Patankar et al. 2001, Chougule et al. 2005). Efforts are being made to use PIs, as a class of plant defense proteins against herbivores, to develop insect resistance in susceptible crop plants. However, understanding of insect digestive enzymes is a prerequisite to plan strategies for successful and sustainable application of PIs (Franco et al. 2000).

The amount of ingested food gradually increases during growth of insects from earlier to last instars. Because of a high nutritional value of hybrids KLM78 \* MO17 and K47 \* K19, heaviest larvae were found on these hybrids. Also, the lowest weight of the larvae fed on hybrid KSC705 could be due to its low nutritional value. The weight of fifth instar *H. armigera*-fed KSC705 is 2-fold higher than that reported by Naseri and Razmjou (2013) for the larval weight of *H. armigera* fifth instar on artificial diet prepared by corn hybrid SC704 ( $45.77 \pm 6.16$  mg) as a suitable hybrid. Also, the food consumption by fifth instar *H. armigera*-fed KSC705 is approximately 3-fold higher than that reported for the food consumption by *H. armigera* fifth instar on artificial diet prepared by the seed of SC500 ( $219.56 \pm 11.40$  mg) as a suitable corn hybrid (Naseri and Razmjou 2013). It could be suggested that the corn hybrids used, in our research, for feeding of the larvae are more nutritive than those tested by aforementioned researchers.



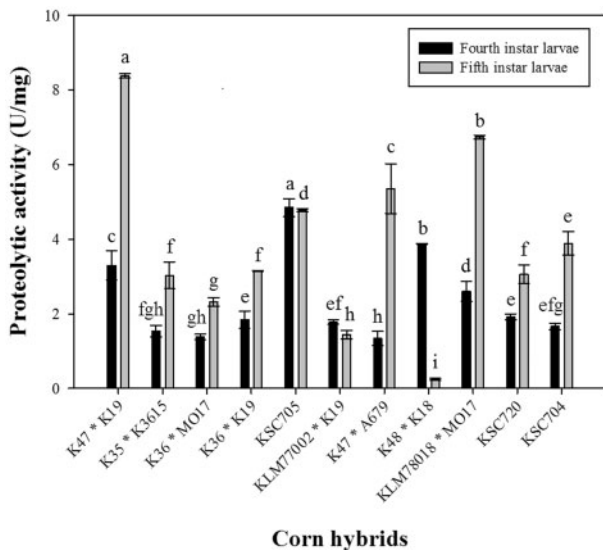
**Fig. 2.** Mean food consumed (A), feces produced (B), and larval weight gain (C) of whole fourth and fifth instars *H. armigera*-fed different corn hybrids. Bars represent standard error of the means. The means followed by different letters are significantly different (Tukey HSD,  $P < 0.05$ ).



**Fig. 3.** Mean prepupal (A) and pupal (B) weight of *H. armigera*-fed different corn hybrids. Bars represent standard error of the means. The means followed by different letters are significantly different (Tukey HSD,  $P < 0.05$ ).

According to Kotkar et al. (2009), artificial diets are complete foods for high insect performance and usually considered to be healthier than natural diets. However, our findings about larval weight and food consumption by *H. armigera* rearing on natural corn hybrids are not in

agreement with the results of Kotkar et al. (2009), indicating that natural corn diet for *H. armigera* larvae is more nutritive than artificial diet prepared by corn seeds. Differences in the tested corn hybrids, variation in the type of the diet used for larval feeding (natural or artificial diets),

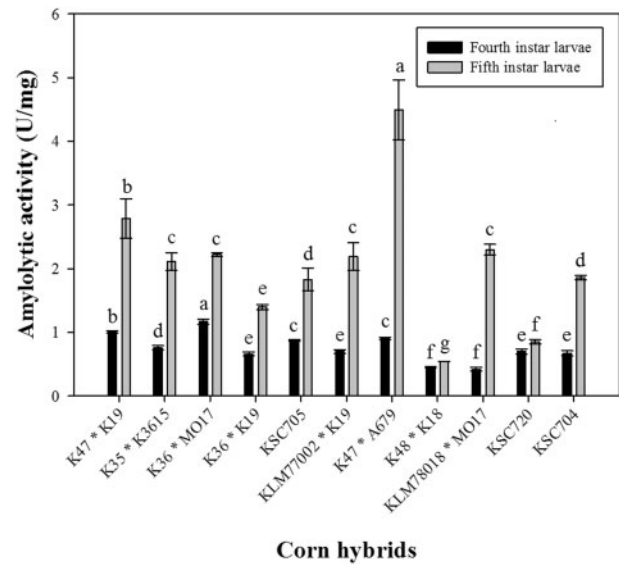


**Fig. 4.** Mean proteolytic activity ( $\text{Umg}^{-1}$ ) of midgut extracts from *H. armigera* fourth and fifth instars fed different corn hybrids. Bars represent standard error of the means. The means followed by different letters are significantly different (Tukey HSD,  $P < 0.05$ ).

and the genetic diversity of the examined *H. armigera* populations are probable explanations for such discrepancy.

It is reported that the body weight is one of the main biological indices of insect population dynamics (Liu et al. 2004). The value for the pupal weight of *H. armigera* on hybrid K47 \* K19 was heavier than that reported for *H. armigera* reared on corn (285.2 mg) (Liu et al. 2004) and on artificial diet prepared by seeds of different corn hybrids (260.75 mg) (Arghand et al. 2011). This finding emphasizes that the corn hybrids evaluated in our study are more nutritious for pupal stage than those used in the previous studies.

The amount of food, temperature, and acidity are important factors directly affecting the activity of digestive enzymes and the energy production needed for growth and development (Sivakumar et al. 2006). The larval stage of *H. armigera* is actively feeding stage responsible for accumulation of nutrients to complete its life cycle. Previous studies showed that disturbance in protein metabolism due to the presence of PIs in *H. armigera* larval diet results in reduced growth and fecundity (Telang et al. 2003). Insects can quickly alter their midgut composition in response to the protease inhibitors through regulating the proteases secretion (Jongsma and Bolter 1997, Harsulkar et al. 1999). The highest proteolytic activity of *H. armigera* fourth-instar larvae on hybrid KSC705 could be due to the presence of some PIs in this hybrid, which inhibit certain proteinases and make the insects to produce more digestive enzymes by the midgut cells. Furthermore, the food consumption, feces produced, and weight gain of the fourth-instar larvae were the lowest on KSC705 as compared to any other hybrid. Hyperproduction of proteinases in response to the PIs can cause more energy demand and essential amino acid production and finally results in developmental delay and decrease in the insect feeding (Broadway and Duffy 1986). The fifth-instar larvae *H. armigera* reared on hybrid K47 \* K19 had the highest proteolytic activity, which might be due to the higher food consumed by the larvae (Fig. 4). Variations in digestive enzymatic activities of *H. armigera* on different corn hybrids could be because of the presence of isoenzymes. The insects gut and complexity of different protease specificities has a set of diverse protease isoforms. For example, the gut of *H. armigera* approximately includes 20 different types of active serine protease isoforms (Purcell et al. 1992). Proteolytic activity of *H. armigera* fifth-instar larvae fed on hybrid K47 \* K19 shows approximately 2-fold higher activity than those fed on cowpea-based artificial diet (Naseri et al. 2010) and is nearly similar to those larvae



**Fig. 5.** Mean amylolytic activity ( $\text{Umg}^{-1}$ ) of midgut extracts from *H. armigera* fourth and fifth instars fed different corn hybrids. Bars represent standard error of the means. The means followed by different letters are significantly different (Tukey HSD,  $P < 0.05$ ).

reared on artificial diet prepared by corn hybrids SC500 and DC370 (Naseri and Razmjou 2013).

In this study, we measured  $\alpha$ -amylase of *H. armigera* larvae in midgut, and our results showed that this enzyme exists in high levels. Amylolytic activity of the fifth-instar larvae fed on hybrid K47 \* A67 is lower than that reported by Naseri and Razmjou (2013) for amylolytic activity of *H. armigera* on corn-based artificial diet.

In polyphagous insects, different diet items can lead to various negative effects including reduced food consumption, decreased weight gain, lighter larva, or pupa, which we found the majority of these effects in larvae fed on hybrids K36 \* MO17, KSC705, and K48 \* K18, suggesting unsuitability of these hybrids for *H. armigera* growth and development. For a better understanding of the *H. armigera*-corn interaction to its control, more attention should be allocated to investigate life table parameters of this pest on various corn hybrids under laboratory and field conditions. Results obtained from such experiments regarding choosing and developing resistant corn hybrids could be helpful to comprehensively manage *H. armigera* population under field conditions. Using resistant varieties means reducing pesticide use, reducing producer, and consumers costs as well as reducing environmental risks posed by using these chemicals (Da Silva et al. 2004). However, the cost of developing resistant varieties should also be considered. In the future studies, *H. armigera* response to the PIs should be necessarily considered for selection of proper PIs that could be used in transgenic expression for the insect resistance.

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