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FULL LENGTH ARTICLE

Feeding indices and enzymatic activities of carob moth *Ectomyelois ceratoniae* (Zeller) (Lepidoptera: pyrallidae) on two commercial pistachio cultivars and an artificial diet

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Abstract Feeding indices and enzymatic activities of *Ectomyelois ceratoniae* (Zeller) were studied in a growth chamber under controlled conditions (29 ± 2 °C, relative humidity of 70 ± 5% and a photoperiod of 16:8 (L:D) hours) on two commercial Pistachio cultivars (Akbari and Kalequchi) and an artificial diet. Feeding indices of *E. ceratoniae* larvae differed significantly on three hosts ($P < 0.05$). The relative consumption rate was calculated to be 5.36 ± 0.009, 11.10 ± 1.49 and 10.631 ± 0.599 (mg/mg/day) on artificial diet, Akbari and Kalequchi cultivars, respectively. Carob moth larvae reared on Akbari cultivar showed the highest efficiency of conversion of digested food (ECD) (5.64 ± 0.43). The highest amount of efficiency of conversion of ingested food (ECI) was obtained on artificial diet but approximate digestibility (AD) was the lowest on this diet. The highest enzymatic activities of alpha-amylase, general proteases and lipase were observed in the midgut of larvae reared on artificial diet. Total protein and lipid value were highest in larvae that were reared on artificial diet.

1. Introduction

Wild pistachio (*Pistacia vera* L.) belongs to the sumac (Anacardiaceae) plants. The genus *Pistacia* has 11 species, all of which secrete turpentine oil. Various pests attack Pistachio out of which carob moth is considered as the most serious one. Halperin (1986) and Rice (1978)reported damage of/caused by *Ectomyelois ceratoniae* (Zell) and *Apomyelois transitella* Wal. on Pistachio.

The carob moth *E. ceratoniae* (Zeller), also known as date moth, is an important pest attacking fruit trees and nut crops throughout the world. It is also a major field pest of pomegranate, *Punica granatum* L., date, *Phoenix dactylifera* L. and almond, *Prunus dulcis* (Mill.) (Norouzi et al., 2008). Adult carob moths begin to emerge early in May in Iran and preferably attack pomegranate. Apparently, pomegranate

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fruits provide suitable conditions for oviposition of these moths. After completing some generations on pomegranate, they attack Pistachio (Mehrnejad, 1992). This insect is able to continue its damage during storage. Its maximum activity is found to be during September and November in Rafsanjan, a main region of Pistachio cultivation in Iran. Adult moths cannot attack un-cracked hull nuts, however the larvae can penetrate into un-cracked shell from stem end. It appears that the carob moth spends several generations on alternate hosts, mainly pomegranate before attacking pistachio nuts (Mehrnejad, 1992).

Metabolic efficiency of insect feeding on plant varieties (Waldbauer, 1968), and the effect of plant on insect metabolism and interactions between insects and their food sources are shown by using feeding indices (Bhat and Bhattacharya, 1987). For example in *Spodoptera frugiperda* Smith (Lep.: Noctuidae) feeding indices were calculated on 9 bermuda grass types and based on these results, resistance and susceptible varieties were distinguished (Jamjanyn and Quisenberry, 1988). Feeding indices demonstrate the digestion efficiency or utilization of diet or diet ingredients and in fact illustrate the conversion of food to the biomass of insects. These indices can provide valuable information about the positive or negative impact of ingredients or total food (Cohen, 2005). The general feeding indices used are: approximate digestibility (AD), Efficiency of conversion of ingested food (ECI), Efficiency of digested food (ECD) and relative Consumption rate (RCR) (Waldbauer, 1968). One of the easiest methods of control of carob moth is the use of resistant varieties (Shakeri, 2004). Feeding indices may be used as the methods for establishing resistant varieties.

Growth of insect is influenced by biotic and abiotic factors such as temperature, humidity, food quality and quantity (Jansen and Groot, 2004). These factors will also affect insect physiological processes. So the activity of digestive enzymes depends on the nature of food and chemicals ingested (Mendiola-Olaya et al., 2000; Silva et al., 2009). Digestive enzymes are commonly found in the salivary secretions and various regions of the digestive tract of insects. Digestive enzymes play a major role in the body of insects by converting complex food materials into smaller molecules necessary to provide energy and metabolites (Wigglesworth, 1984).The major digestive enzymes in the midgut of insects consist of amylases, lipases and proteases that are similar in their hydrolytic nature. α-Amylases are the hydrolytic enzymes that catalyze the hydrolysis of α-D-(1,4)-glucan linkages in glycogen and other related carbohydrates (Franco et al., 2000). Lipases catalyze the hydrolysis of fatty acid ester bonds, and are widely distributed among animals, plants and microorganisms (Grillo et al., 2007). Peptidases act on peptide bonds and include endopeptidases and exopeptidases (Terra and Ferreira, 2005).

Because of the economic importance of *E. ceratoniae* as a pest species, there has been a considerable amount of research on various aspects of its developmental biology. In the present study, we have investigated some basic measures of fitness on field hosts and artificial diet. For a better control and improved management strategy, a better understanding of its digestive physiology is helpful, which hopefully will lead to new strategies for management of this important pest.

2. Materials and methods

2.1. Insect rearing

Approximately 1000 infested pomegranate fruits by carob moth larvae were originally collected from pomegranate orchards of agricultural research center of Yazd city, Yazd province (Center of Iran) and were transported to the laboratory. Then the larvae of carob moth were separated from infested fruits. They were reared in transparent plastic jars ($30 \times 20 \times 13$ cm) on Akbari and Kalequchi cultivars and artificial diet (containing wheat flour 72 g, honey 12, glycerin 10 g, yeast 1 g, distilled water 5 ml) in controlled condition (29 ± 2 °C, 70 ± 5% RH, and 16:8 L:D photoperiod). The emerged adults from infested fruits were transferred into transparent jars (18×7 cm) and were provided with cotton wool soaked in 10% honey for feeding. The carob moths were reared on each diet in the laboratory for three generations before the experiments.

2.2. Feeding efficiency

Newly ecdysed fifth instar larvae were collected from the stock culture and transferred into plastic jars $30 \times 20 \times 13$ cm with a hole covered by a fine mesh net for ventilation, and containing artificial diet or Akbari or Kalequchi cultivars. Experiments were carried out for 3 days. The experiments were conducted with eight replicates for each diet. A gravimetric technique was used to determine weight gain, food consumption, and the amount of feces produced. The newly ecdysed fifth instar larvae were starved 4 h prior to the start of experiments to exude gut contents. Nutritional indices were measured on the dry weight basis. After measuring the weight of the fifth instar larvae, they were introduced to each host diet, and the weights of the larvae were recorded before and after feeding until they stopped feeding. Efficiency indices were calculated as described by Huang and Ho (1998):

Approximate digestibility (AD) = 100 (E – F)/F, Efficiency of conversion of ingested food (ECI) = 100 P/E, Efficiency of digested food (ECD) = 100 P/(E – F), Relative Consumption rate (RCR) = E/TA, where: A = dry weight of larvae in the start of experiment, E = dry weight of consumed food, F = dry weight of produced feces, P = dry weight of the biomass of larvae, T = duration of the experiment (3 days).

2.3. Preparation for enzymatic activities

The fifth instar larvae (2 days old) were dissected under a stereomicroscope in an ice-cold saline buffer (0.15 M NaCl). Their midguts were removed from the insect body, rinsed in ice cold distilled water. Then placed in a pre-cooled homogenizer with a known volume of distilled water and ground before centrifugation. The homogenates were then centrifuged at 13,000 rpm for 20 min at 4 °C. The resulting supernatants were transferred to new micro tubes and frozen at −20 °C until used.

2.4. Assay for α-amylase activity

α-Amylase activity was assayed according to Bernfeld (1955), by dinitrosalicylic acid (DNS) as the reagent and 1% soluble

starch as the substrate. Fifteen microliters of the enzyme was incubated for 30 min at 35 °C with 100 μL of phosphate buffer (0.02 M, pH 7.1) and 30 μL soluble starch. The reaction was stopped by the addition of 100 μL DNS and heating in boiling water for 10 min prior to reading the absorbance at 540 nm. One unit of α-amylase activity was defined as the amount of enzyme required to produce 1 mg maltose in 30 min at 35 °C. All assays were performed in duplicate and each assay was repeated at least three times.

2.5. Assay for lipase activity

The lipase assays were carried out as described by Tsujita et al. (1989). Twenty microliters of gut extract and 40 μL of *p*-nitrophenyl butyrate (50 mM) as substrate were incorporated with 100 μL of phosphate buffer (1 M, pH = 7) and incubated at 37 °C. After 1 min, 100 μL of distilled water was added to each tube (control and treatment) and absorbance was read at 405 nm. One unit of enzyme will release 1.0 nmol of *p*-nitrophenol per min at pH 7.2 and 37 °C when *p*-nitrophenyl butyrate is used as substrate. The standard curve was used to calculate the specific activity of enzyme.

2.6. Assay for general protease activity

General proteases were assayed using azocasein (1%) as substrate according to García-Carreno and Haard (1993) with slight modifications. 30 μL of 1% azocasein solution was added to 80 μL of 40 Mm phosphate buffer of specified pH. The reaction was started by adding 15 μL of enzyme extract and incubating at 30 °C for 120 min. For reaction termination, 150 μL of 30% TCA (trichloroacetic acid) was added to the reaction mixture. Precipitation was achieved by cooling at 4 °C for 45 min and the reaction mixture was centrifuged at 13,000 rpm for10 min. Then 100 μL of supernatant was mixed with 100 μL of NaOH and absorbance was read at 440 nm.

2.7. Assay for triacylglyceride

A diagnostic kit from PARS-AZMOON® CO, Iran was used to measure the amount of triacylglyceride in the fourth instar larvae. Reagent solution contained phosphate buffer (50 mM, pH 7.2), 4-chlorophenol (4 mM), Adenosine Triphosphate (2 mM), Mg^{2+} (15 mM), glyserokinase (0.4 KU/L), peroxidase (2 KU/L). lipoprotein lipase (2 KU/L), 4-aminoantipyrine (0.5 mM) and glycerol-3-phosphate-oxidase (0.5 KU/L). Samples (10 μL) were incubated with 10 μL distilled water and 70 μL of reagent for 20 min at 25 °C (Fossati and Prencipe, 1982). ODs of samples and reagent as standard were read at 546 nm. The following equation was used to calculate the amount of triacylglyceride:

$$mg/dl = \frac{\text{OD of sample}}{\text{OD of standard}} \times 0.01126$$

2.8. Electrophoresis

Activity of the enzymes that existed in crude homogenates of the midgut was studied by non-denaturing polyacrylamide gel electrophoresis (PAGE) described by Laemmli (1970). Electrophoresis was conducted at room temperature and 120 V. For gel preparation for the α-amylase assay, the gel was rinsed with water and washed by shaking gently with 1% (v/v) Triton X-100 in phosphate buffer containing 2 mmol/l $CaCl_2$ and 10 mmol/l NaCl for1 h. The gel was then rinsed with water and treated with a solution of 1.3% I2, 3% KI to stop the reaction and to stain the unreacted starch background. Zones of α-amylase activity appeared as light bands against a dark background (Campos et al., 1989).

Zymogram analysis of lipase was carried out using 10% resolving and 4% stacking gels. After loading the samples, gel was run at 4 °C and constant voltage of 100 mV. The gel was gently separated from glasses and immediately immersed in 5 mMof MU-butyrate solution as fluorescent substrate. After 30 min, the gel was placed on a UV trans-illuminator to observe white bands in a dark background.

Electrophoretic detection of proteolytic enzymes was performed according to the method described by García-Carreno and Haard (1993). Non-reducing SDS–PAGE was carried out at 4 °C using gels containing 0.5% gelatin. When the dye reached the end of the glass, the gel was gently removed, washed with distilled water and immediately fixed and stained with 0.1% Coomassie brilliant blue R-250 in methanol–acetic acid–water (50:10:40) overnight. Destaining was done in methanol–acetic acid–water (50:10:40).

2.9. Protein determination

Protein concentration was measured according to the method of Bradford (1976), using bovine serum albumin (BSA; Bio-Rad, biorad.com) as standard.

2.10. Statistical analysis

The data were analyzed separately for each experiment and were subjected to analysis of variance (ANOVA) using SAS software. Difference between the treatments was determined by Turkey's multiple range tests (SAS, 1997). Differences among means were considered significant at $P \leqslant 0.05$.

3. Results

The results of the nutritional indices of fifth instar larvae of *E. ceratoniae* reared on two commercial Pistachio cultivars and an artificial diet are provided in Table 1. The larvae reared on artificial diet showed the highest value of efficiency of conversion of digested food. The lowest value of ECD was observed on Kalequchi cultivar. The highest and lowest efficiency conversion of ingested food values of *E. ceratoniae* were on artificial diet and Akbari cultivar respectively. The approximate digestibility was highest on Kalequchi, followed by that on Akbari cultivar and the lowest on artificial diet. The relative consumption rate of larvae was higher when the larvae reared on artificial diet than when reared on either Akbari or Kalequchi cultivars.

In the present study, larvae that reared on artificial diet showed higher activity of α-amylase, lipase and protease significantly in comparison to Kaleguchi and Akbari cultivars (Table 2). Also, the amount of triacylglycerides and total protein in larvae reared on Kaleguchi and Akbari cultivars decreased compared to artificial diet (Table 3).

The analysis of gut homogenates by electrophoresis showed that all three enzymatic activities in artificial diet were more than the two hosts (Fig. 1).

Table 1 Nutritional indices of fifth instar larvae of *Ectomyelois ceratoniae* on artificial diet, Akbari cultivar and Kalequchi cultivar.

Nutritional indices/host	AD [%]	ECD [%]	ECI [%]	RCR(mg/mg/days)
Artificial diet	46.643 ± 0.037b	10.29 ± 0.011a	4.52 ± 0.002a	15.05 ± 0.009a
Akbari cultivar	50.756 ± 0.061b	5.64 ± 0.433b	2.409 ± 0.091b	11.62 ± 1.485b
Kalequchi cultivar	68.803 ± 1.566a	4.20 ± 0.367b	2.866 ± 0.197b	10.63 ± 0.599b

Within columns, means followed by the same letter do not differ significantly ($p \leqslant 0.05$); AD – approximate digestibility; ECI – efficiency of conversion of ingested food; ECD – efficiency of conversion of digested food; RCR – Relative Consumption Rate.

Table 2 The amount of digestive enzymes of *Ectomyelois ceratoniae* on artificial diet, Akbari cultivar and Kalequchi cultivar.

Enzymes/host	α-Amylase (means ± S.E.)	Protease (means ± S.E.)	Lipase (means ± S.E.)
Artificial diet	6.351 ± 0.329a	6.036 ± 0.519a	5.586 ± 0.352a
Akbari cultivar	3.035 ± 0.502b	2.568 ± 0.306b	3.878 ± 0.307b
Kalequchi cultivar	4.379 ± 0.551ab	2.184 ± 0.208b	1.862 ± 0.207c

Within columns, means followed by the same letter do not differ significantly ($p \leqslant 0.05$).

Table 3 The total amount of protein and lipid of *Ectomyelois ceratoniae* on artificial diet, Akbari and Kalequchi cultivar.

Diet	Total protein (mg)	Lipid
Artificial diet	0.014 ± 0.00099a	3.958 ± 0.342a
Akbari cultivar	0.0096 ± 0.00099b	2.244 ± 0.223b
Kalequchi cultivar	0.007 ± 0.00055b	1.809 ± 0.241b

Within columns, means followed by the same letter do not differ significantly ($p \leqslant 0.05$).

4. Discussion

Resistant varieties are considered as means of controlling carob moth (Shakeri, 2004). Feeding indices could be used to identify resistant varieties and then implement them for pest control strategies. The quality of larval food may affect the pupal and adult phenotypic characteristics. Obvious effects of larval diets are pupal distortions and wing malformations in the imago (Rosenthal and Dahlman, 1975). The fecundity, longevity, and forewing area of lepidopteran adults are the most commonly used parameters for determining the effect of larval diet on adult stage. Additionally, the insect's ability to store energy (e.g., pupal weight and lipids and glycogen levels) varied depending on the larval host plants (Liu et al., 2007).

Our data show that carob moth larvae had the highest relative consumption rate on artificial diet when compared with other hosts. The RCR value did not show any differences between the two determined hosts. The relative consumption rate is used for measurement exploitation of food by insect. This index shows the rate of feed connected weight in insects at certain time. Rate of feeding in insect depends on water and physicochemical properties of food (Srinivasan and Uthamasamy, 2005).

Reduction of RCR, ECI, ECD lead to delay in larval growth and formation of smaller pupae which have a direct relationship to fecundity and longevity of the adult insect and make them susceptible to diseases and natural enemies. The ability of an organism to convert nutrients, especially protein, will positively influence its growth and development (Sogbesan and Ugwumba, 2008).

The highest ECI and ECD values of the carob moth larvae were on artificial diet. Significant variations on the value of ECI and ECD did occur in the present study which reveals the differences in nutritional value of the hosts. The chemical composition of host plants significantly affects survival, growth, and reproduction of phytophagous insects (Bernys and Chapman, 1994). Food quality links plant attributes with insect performance (Slansky, 1990). For polyphagous insects, the availability of different host plants plays an important role in triggering population outbreaks (Singh and Parihar, 1988). Growth, development, and reproduction of insects strongly depend on the quality and quantity of food consumed (Scriber and Slansky, 1981).

ECI is an overall measure of an insect's ability to utilize the food ingested for growth and development (Koul et al., 2004). Results showed the highest value of ECI on artificial diet and the Kalequchi cultivar while the Akbari stand next. That is a sign of better quality and attractive material in artificial diet. It also shows that, although the insects had low food intake and low feces, they conspicuously enhanced their body weight. Another important feeding index is efficiency of digested food. ECD is a measure of the efficiency of conversion of digested food into growth (Senthil-Nathan et al., 2005). Change in ECD also indicates the overall increase or decrease of the proportion of digested food metabolized for energy (Koul et al., 2004). As can be seen in Table 2, the larvae fed on the Kalequchi cultivar had the lowest value of ECD, which suggests that these larvae were apparently not as efficient in turning digested food into biomass. It is well known that the degree of food utilization depends on the digestibility of food and the efficiency with which digested food is converted into biomass (Batista Pereira et al., 2002). The reduction in dietary utilization suggests that reduction in nutritional values may be resulted from both behavioral and physiological effects (Senthil-Nathan et al., 2005).

Approximate digestibility indicates ability of an insect to absorb food through the stomach wall. Increase in AD is indicative of more attempts made by insect to compensate for a lower nutritive value and reach the desired growth rate. Difference in value of AD shows differences in factors such as food shortages, lack of balance and higher levels of crude fiber and water shortages (Chapman, 1998). Digestibility of food with

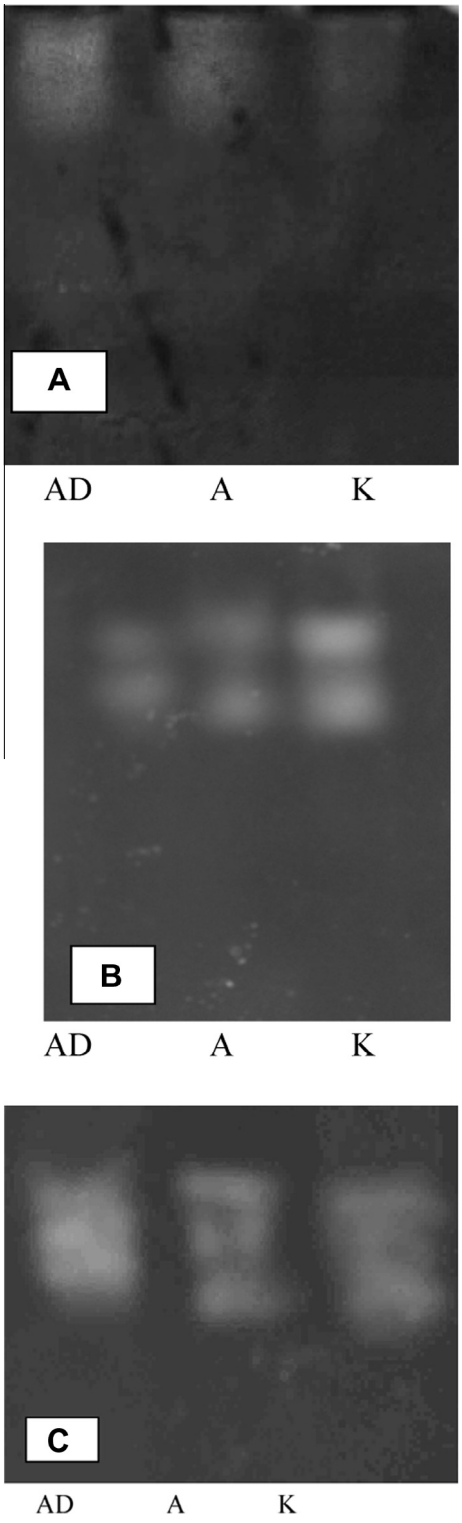

Figure 1 Non-denaturing gel electrophoresis of amylase (A), lipase (B), and protease (C) from midgut of fifth instar larvae from *E. ceratoniae* on two commercial Pistachio cultivars and artificial diet. (AD = Artificial diet, A = Akbari cultivar, K = Kalequchi cultivar).

those low subjects often contains compounds that reduce digestibility (Srinivasan and Uthamasamy, 2005). Our Study showed the highest value of AD on Kalequchi cultivar.

Undesirable food components that lack materials needed for the development of insect body bring about a higher index of AD and RCR but a lowest value index of ECD and ECI are expected (Cohen, 2001). In this study quality of Kalequchi cultivar was lower for insects, and index of AD was higher but the reverse was true for ECI.

Alpha-Amylase is a midgut and salivary enzyme that is involved in starch and other carbohydrate metabolism and its activity level depends on feeding diet (Shekari et al., 2008). The α-amylase converts starch to maltose, which is then hydrolyzed to glucose by glucosidase. The activity of α-amylase is dependent on food sources. The midgut amylase activity of *E. ceratoniae* fed on artificial diet was higher than those reared on two other hosts. Merkx-Jacques and Bede (2005) also demonstrated a higher activity of amylase in *Spodoptera exigua* Hübner (Lep.: Noctuidae) larvae fed on artificial diets in comparison with the larvae fed on legume, *Medicago truncatula* L.

The initial digestion of protein is carried out by proteinases (endopeptidases) that break internal bonds in proteins. Different proteinases are necessary to carry out this because the amino acid residues vary along the peptide chain. Proteolysis enzymes that are important in digestion of food and food proteins are broken down into amino-acids for the body needs. The enzyme activity in artificial diet of the present study represents the highest value. Increased activity of proteases in the present study is probably due to the need of insects for protein. Hemati et al. (2012) found significant differences in proteolytic and amyloletic activities in *Helicoverpa armigera* Hübner (Lep.: Noctuidae) larvae reared on different host plants. They claimed that various host plants have different amount of total protein and carbohydrate contents, therefore the observed differential activities of proteases or amylases in the larvae feeding on these host are reasonable.

Lipases are enzymes that preferentially hydrolyze the outer links of fat molecules and have been studied in few insects. The lipase mainly hydrolyzes the triglycerides to diglycerides and fatty acids. Previous observations suggested that the enzyme preferentially releases fatty acids from the alpha-positions (Terra and Ferreira, 2005). Lipases play an important role in the physiology of insects, particularly at the non larval life stages. Increased activity of lipase in the present study is probably due to the need of insects for lipids in the body. Its value in artificial diet is more than the other two hosts.

The ability to hydrolyze a substrate such as a protein, carbohydrate, or lipid means that the insect is prepared to use the food material is likely to be a useful part of the insect's nutritional capacity. The fact that the higher activity of α-amylase, lipase and proteinase is an indication that these insects encounter starch, lipid and protein in their diet. They are capable of breaking down these substrates into smaller units that can readily be ingested. They can use starch, lipids and proteins as a nutrient if it is included in their diet (Agusti and Cohen, 2000; Zeng and Cohen, 2000).

The application of such basic techniques as analysis of digestive enzymes has proved to be useful in the development or improvement of artificial diets for several species of insects (Cohen, 2001). However, this and other basic science techniques that help us understand the feeding adaptations and characteristics of the specific insects that we are trying to rear have not been as fully exploited as the potential of these techniques promise.

In Physiological studies measuring total protein in insects is important (Etebari and Matindoost, 2004). It was observed that the total amount of protein in larvae grown on artificial diet is more than the other two hosts and differed significantly with Akbari and kalequchi cultivars. No significant differences were observed between Akbari and kalequchi cultivars. Lipids have specific functions. Lipids are an important source of energy for insects. Lipids are important source of fat stored in bodies of insects and this is variable in developmental stages and nutritional status of insect. Usually fat stores increase during periods of dietary and decrease when feeding stops. A large proportion of carbohydrates digested during larval development are converted to lipids (Candy, 1985). The amount of lipids in insects depends on many factors such as development, nutrition, environmental temperature, sex, starvation, diapause and exposure to cold weather. Storage lipids in the larval stage are analyzed because the lipids will provide high energy levels for metamorphosis. Therefore, any changes in biochemical composition of insect create direct or indirect effects in adult. In the present study lipid levels in larvae reared on artificial diet showed highest amount compared with the larvae reared on two other hosts.

Lowest activity of lipase and amylase enzymes was observed in Kaleghuchi cultivar and sharpness of bands drastically declined in other hosts and one or two isoenzymes have been removed. Three bands of protease were observed in larvae that reared on artificial diet but on two varieties one of protease bands almost disappeared and the remaining bands showed less clarity and in the case of Akbari cultivar deletion of one of bands is evident.

5. Conclusion

The present information could be used to manage the pest population below the economic injury level. These results could provide information for establishing conditions for best rearing of *E. ceratoniae* as also reported by Mediouni and Dhouibi (2007) in Tunisia. For instance, mass culture methods could be improved by selecting artificial diet for rapid development, or high fecundity in order to use these individuals for laboratory research and mass rearing of natural enemies.

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