Journal of Applied Biological Sciences
E-ISSN: 2146-0108 12 (3): 26-32, 2018

Laboratory Rearing of Cotton Bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera:Noctuidae) Wild Colony on Different Artificial Diets

Seda YÜCEL[1] Hanife GENÇ[1]*
[1]Çanakkale Onsekiz Mart University, Faculty of Agriculture, Department of Agricultural Biotechnology, 17100, Çanakkale, TURKEY

*Corresponding Author
E-mail:hgenc@comu.edu.tr

Received:July 27, 2018
Accepted:October 11, 2018

Abstract

The cotton bollworm, *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) is a major pest in agricultural areas in the world. It is crucial to study the pest's biology, behaviour, insecticides resistance and control methods in the laboratory conditions in all year around without depending on the natural host plant. In order to do that, the pest should be reared on artificial diet continously. The aim of the study is to test known artificial diets for laboratory rearing and adaptation of the cotton bollworm and also modify them if necessary to find out the best larval diet. The bollworm larvae were brought to the laboratory with infested tomatoes from Çanakkale provinces. Six artificial diets were prepared with basic ingredients including pinto bean flour, soybean meal, corn meal, chickpea meal, wheat germ, brewer's yeast, torula yeast, sucrose, vitamins and mold inhibitors. Larvae also reared on sliced tomato fruits as control during the experiments. Some biological parameters such as number of larval stages, survival of larval and pupal stages and development times, pupal weights and adult emergence rate were determined on six different artificial diets in the laboratory. As a result, Diet I, originally developed for *Grapholita molesta* that supported larval development succesfully and completed 11.6 ± 1.5 days and had 331.8 mg pupal weights. Another diet called as Diet II, originally developed for Lepidopteran species, also succesfully used to rear the cotton bollworm larvae having 16.6 ± 1.4 days of larval durations. The cotton bollworm was reared consecutive four generations on both diets. The study results showed that out of six tested diets, Diet I and Diet II were sufficient and nutrionally suitable for laboratory uses and adaptation of wild colony.

Keywords: Artificial diet, *Helicoverpa armigera*, Lepidoptera, cotton bollworm, rearing

INTRODUCTION

Helicoverpa armigera Hübner (Lepidoptera: Noctuidae) is a major pest and well-known polyphagous insect in agricultural areas in worldwide [1,2]. The common names are known as old world bollworm, African bollworm, cotton bollworm, tomato fruit worm, the gram pod borer and American bollworm [1,3]. Cotton bollworm was first recorded in 1913 in Bergama, Turkey [4]. This pest has a wide geographical distribution, reported in Asia, Africa, Ocenia, Europe, Australia and recently in South America [1,5,6]. Larvae of *H. armigera* have been reported more than 181 cultivated and over 67 hosts, including Fabaceae, Malvaceae, Poaceae, Asteraceae and Solanaceae [1,7,8,9]. It has caused damages on economically important agricultural crops such as tomato, maize, cotton, sorghum, soybean, legumes and ornamental plants [1,10,11].

H. armigera feeds on various parts of the host plants, leaves and also reproductive structures [10,12]. It is easily adapted to environmental changes, having high mobility and surviving in different habitats [2]. Females deposit numerous eggs with high fecundity causing population increases [6].

It is a well known pest and several studies have been published on the cotton bollworm such as genetic diversity [13,14,15,16,17,18], biology [2,8,19], pesticide resistances [20,21,22], life table [1,6,23], geographic distributions [24,25] and rearing on different artificial diets [3,26,27,28]. These studies help to understand pest's behaviour, population dynamics analysis, life cycle and other control approaches.

Artificial diets are important to know the basic biology of the pest and to understand the factors involved in insects nutrition and insect rearing studies. Therefore, such informations could be critical for the pest nutritional requirements. Several diets slightly modified and reported by Vanderzant et al. [29], have been developed for the cotton bollworm. Understanding of pest nutritional requirements could allow accelerate laboratory rearing, adaptation and developing artificial diets to study without depending on the host plants. It is important to have large numbers of larvae in health and uniformly developed insects with low cost.

Rearing of cotton bollworm in the laboratory depends mostly on natural host plants. Susceptibility of larval rearing, maintaining of host plant year around are important issues to be considered [30] as well as the labor and cost.

The objective of the present study is to test known artificial diets to rear the cotton bollworm in the laboratory and to study some biological parameters by comparing natural host in order to find out the best larval diet.

MATERIALS AND METHODS

The present study was carried out at Çanakkale Onsekiz Mart University, Faculty of Agriculture, Insect Molecular Biology Laboratory, to study the effect of host plants and artificial diets on the development of the different biological stages of cotton bollworm. All experiments were conducted in controlled laboratory conditions, at 24±1˚C, 65 % RH and 16:8h (light: dark) photoperiod.

Cotton Bollworm Colony

Bollworm larvae were collected from infested tomato orchards in Çanakkale province and brought to the laboratory. They were reared about 7 generations on tomato fruits at 24±1˚C, 65 % RH and 16:8h (light: dark) photoperiod in the growth chamber (Figure 1A). The larvae were reared on sliced tomatoes in Tupperware® plastic container (30cm x 18 cm x 7 cm) having sterilized moist soil and a paper towel at the bottom (Figure 1B). Pupae were collected with soft forceps and transferred to petri dish (9 cm diameter) and incubated until adult emergence. Emerged adults were sexed and kept in rearing cages (30 x 17 cm) with 10% honey solution. Eggs were laid individually on tomato leaves in adult rearing cages. The eggs were collected daily and incubated in moisturized black filter paper in a petri dish. Newly hatched (neonate) larvae were used to set up the experiments.

Tested Artificial Diets and Ingredients

Six artificial diet formulations were experimentally tested. The composition of the diets and their quantity in a diet mixture were shown in Table 1. The tested diets were offered to newly hatched cotton bollworm larvae. Diet formulations reported by Diet I, originally developed for *Grapholita molesta* [31], Diet II formulated for Lepidopteran species [32], Diet III was a commercial diet developed for Gypsy Moth Diet; (https://www.mpbio.com), Diet IV developed for noctuid species [27], Diet V [33] and Diet VI were originally also developed for noctuid species [28]. All ingredients were weighed and mixed until having homogeneous mixture. Mold inhibitors and vitamins were added lastly. Diets were freshly prepared before each experiment and poured into plastic cups then kept at 4˚C until used. The tomato fruit was used as control. Sliced tomatoes were used to rear larvae until third instar then whole fruits were given to test and complete their developments.

Table 1. Composition of artificial diets used to rear *Helicoverpa armigera* larvae in this study.

Diet Ingredients	Diet I (Ivaldi-Sender, 1974)	Diet II (Poitout and Bues, 1970)	Diet III (MP #960293)	Diet IV (Hamed and Nadeem, 2008)	Diet V (Daguang et al., 2002)	Diet VI (Jha et al., 2012)
Pinto Bean	-	-	-	-	25 g	37.5 g
Soybean Meal	-	-	-	-	25 g	-
Corn Meal	-	56 g	-	-	100 g	-
Corn Semolina	25 g					
Chickpea Meal	-	-	-	-	-	37.5 g
Brewer's Yeast	-	15 g	-	-	45 g	30 g
Agar	10 g	10 g	6 g	4.5 g	6 g	18.75 g
Wheat Germ	25 g	14 g	-	6 g	-	27.5 g
Torula Yeast	25 g	-	-	6 g	-	-
Sucrose	-	-	-	6 g	20 g	-
Methyl paraben	0.9 g	-	-	0.75 g	-	0.875 g
Sorbic Acid	-	-	-	0.45 g	1 g	0.75 g
Cholesterol	-	-	-	0.06 g	-	-
Ascorbic Acid	2.25 g	2 g	-	1.2 g	-	3 g
Benzoic Acid	0.9 g	0.4 g	-	-	-	-
Streptomycin	-	-	-	0.01 g	-	-
Formaldehyde (10%)	-	-	-	1.5 ml	1 ml	-
Vitamin Mix USDA	-	-	-	0.12 g	-	-
Choline Chloride (20%)	-	-	-	3 ml	-	-
Vanderzant Vitamin Mixture	-	-	-	-	2 g	-
Olive Oil	-	-	-	0.06 ml	2 ml	-
Nipagine	-	0.5 g	-	-	3 g	-
L-cysteine	-	-	-	-	-	0.3 g
Diet Mix Gypsy Moth (MP #960293)	-	-	66.4 g	-	-	-
Water	390 ml	340 ml	400 ml	220 ml	650 ml	650 ml

Rearing Procedure

Diets were cut into small pieces as 3 cm in diameter, weighing and placed into petri dish having white filter papers at the bottom. To test each diet, 10 neonate larvae were used with three replications. Neonate larvae were transferred with a camel hair brush to each diet (Figure 1C). Diets and tomato fruits were renewed every other day. Larval development and survivorship were monitored daily under Olympus SZX9 stereozoom microscope. Biological stages and development periods were observed, measured and photographed. Mature larvae left tomato fruits or diets and pupated in sterilized moist soil. The pupae were sexed by examining externally the last abdominal segments [34,35,36]. Newly emerged adults were kept in adult cages (30 x 17 cm) containing fresh tomato leaves for oviposition (Figure 1D).

For each diet, the following biological parameters were reported: (1) larval development time (from hatching to pupation), (2) pupal development time (from the onset of pupariation of the mature larvae until the emergence of the first adult), (3) pre-pupa duration (day), (4) pupal recovery (total number of pupae produced from the number of original larvae), (5) percentage of adult emergence, (6) pupal width, length and weight (at day 5 after pupariation) and (7) the sizes of the head capsules. Additionally, after testing each artificial diet based on biological parameters, if the diets support larval development and growth, larvae keep rearing on that diet for four continous generations in order to adapt and see any nutritional deficieny.

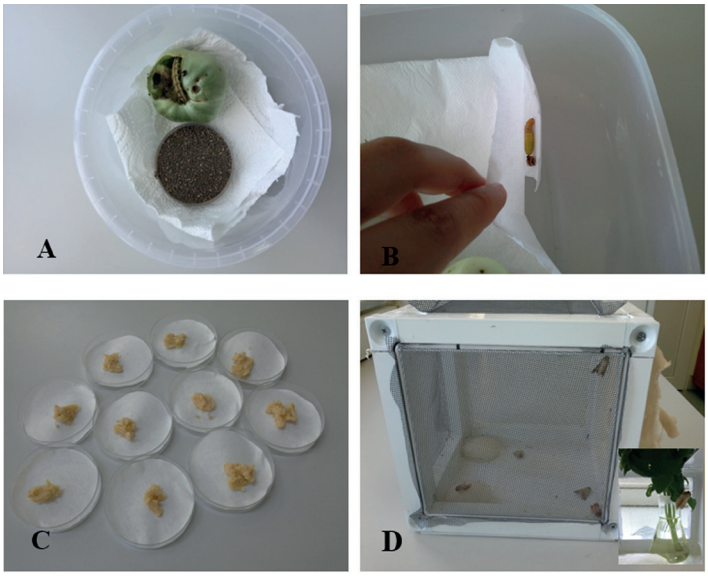

Figure 1. A view of *H. armigera* larvae reared on tomato as positive control (A), pupae on paper towel at the bottom (B), tested larval diets on petri dishes (C) and adult rearing cages (D).

Statistical Analysis

Descriptive statistics as mean values ± standard error (SE) were calculated. The differences among the diets quality control parameters were determined by analysis of variance (ANOVA) and Tukeys's test was used. Significance was accepted with $p \leq 0.05$. The biological parameters were analyzed with Minitab 17 statistical software.

RESULTS AND DISCUSSION

Six different artificial diets along with tomato fruit as positive control were tested in this study. Diet evaluations based on the duration of larval and pupal stages, pre-pupa duration, pupal recovery (%), adult emergence (%), weight, width and length of pupae were given in Table 2. The results of all tested diets were compared to natural host to order to find out the best artificial diets.

Table 2. Biological parameters of cotton bollworm reared on artificial diets and natural host (tomato fruit) (mean±SD) (n=30).

Parameters	Diet Type					
	Diet I	**Diet II**	**Diet III**	**Diet V**	**Diet VI**	**Control (tomato)**
Larval duration (day)	11.6 ± 1.5 e	16.6 ± 1.4 d	25.1 ± 0.9 a	25.5 ± 3.2 a	22.1 ± 1.7 b	18.2 ± 0.8 c
Pupal duration (day)	9.1 ± 0.5 c	10.1 ± 1.6 c	31.2 ± 4.2 a	29.5 ± 4.8 a	19.9 ± 4.1 b	9.3 ± 0.4 c
Pre-pupal duration (day)	2.1 ± 0.3 b	2.2 ± 0.7 b	3.3 ± 0.5 a	3.4 ± 0.7 a	3.5 ± 0.9 a	2.3 ± 1.1 b
Pupal recovery (%)	76.6 ± 2.1 c	80.1 ± 1.1 b	76.6 ± 1.5 c	83.3 ± 1.5 b	83.3 ± 2.8 b	90.1 ± 1.1 a
Adult emergence (%)	85.9 ± 2.7 a	86.2 ± 5.4 a	73.3 ± 2.8 b	66.6 ± 1.2 c	83.3 ± 2.8 a	88.4 ± 11.1 a
Pupal weight (g)	0.33 ± 0.04 b	0.31 ± 0.03 ab	0.26 ± 0.04 c	0.35 ± 0.08 ab	0.34 ± 0.04 ab	0.36 ± 0.05 a
Pupal width (mm)	5.72 ± 0.21 ab	5.62 ± 0.27 b	5.5 ± 0.34 b	5.85 ± 0.44 ab	5.77 ± 0.31 ab	6.05 ± 0.3 a
Pupal length (mm)	19.45 ± 1.02 ab	19.2 ± 1.12 ab	18.8 ± 1.29 b	18.62 ± 1.34 b	18.97 ± 1.35 b	19.97 ± 0.58 a

Means within a row followed by the same letter do not differ significantly by Tukey test ($P<0.05$).

Larvae survived on Diet I, completed their development successfully and the larval feeding behaviours were shown in Figure 2. We clearly observed second instar (Figure 2A), third instar (Figure 2B), fourth instar (Figure 2C) and mature larvae (Figure 2D) on Diet I. They prepared silken filament and attached themselves to diet to feed on. The sizes of the head capsules on Diet I were 0.26±0.03 mm for the first instar, 0.45±0.04 mm for the second instar, 0.85±0.05 mm for the third instar, 1.52±0.04 mm for the fourth instar and 2.96±0.32 mm for the fifth instar (Table 3). Variance analysis of head capsule width among different larval stages in column indicated that the differences were significant ($p<0.05$). So, the cotton bollworm has five instars on Diet I. The larval duration period was 11.6±1.5 days and pupal duration was about 9.1±0.5 days. No significant differences in pupal duration between artificial diet and tomato, but larval development time on artificial diet was shorter than control. Pre-pupal duration was about 2.1±0.3 days. Pupae measured about 5.72±0.21 mm in width, 19.45±1.02 mm in length and weighed about 0.33±0.04 g on Diet I. Adult emergence rate was 85.9±2.7%. The number of emergenced adults was 21 (11♀:10♂). Adults were mated in rearing cages and females laid eggs on tomato leaflets. The hatched egg viability was about 87% (Table 2).

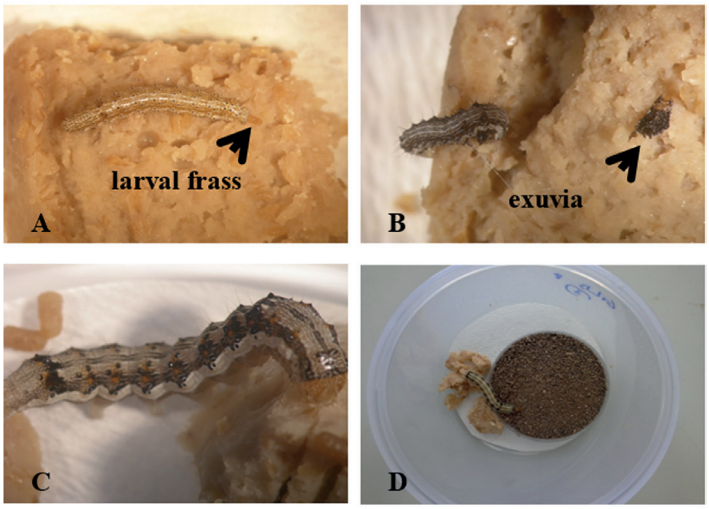

Figure 2. Larval feeding behaviour of *H. armigera* on Diet I, (A) 2nd instar, (B) 3rd instar, (C) 4th instar and (D) mature larva about to pupate.

Table 3. Head capsule width of bollworm larvae reared on artificial diets and tomato (n=15).

	Diet Type					
	Diet I	**Diet II**	**Diet III**	**Diet V**	**Diet VI**	**Control (tomato)**
1st instar	0.26±0.03 Aab	0.28±0.04 Aab	0.30±0.01 Aa	0.29±0.01 Aa	0.29±0.01 Aa	0.25±0.05 Ab
2nd instar	0.45±0.04 Bbc	0.41±0.03 Bd	0.47±0.03 Bab	0.47±0.03 Bab	0.50±0.01 Ba	0.42±0.02 Acd
3rd instar	0.85±0.05 Cbc	0.77±0.06 Cc	0.82±0.04 Cbc	0.80±0.12 Cbc	0.88±0.03 Cb	1.00±0.12 Ba
4th instar	1.52±0.04 Dab	1.63±0.08 Da	1.47±0.08 Db	1.37±0.16 Dc	1.56±0.06 Dab	1.48±0.13 Db
5th instar	2.96±0.32 Eb	1.85±0.08 Ec	3.13±0.23 Eab	1.81±0.07 Ec	3.27±0.19 Ea	1.85±0.10 Dc
6th instar	-	2.82±0.20 Fb	-	3.48±0.12 Fa	-	3.06±0.40 Eb

*Means within a row followed by the same small letter do not differ significantly by Tukey test ($P<0.05$).
**Means within a column followed by the same big letter do not differ significantly by Tukey test ($P<0.05$).

Bollworm larvae survived and completed their development with having six instars on Diet II. The larval frasses were observed during feeding inside the silky area of the diet. Larval duration was about 16.6±1.4 days which was longer than Diet I. The pupal recovery was 80.1±1.1%. Pre-pupal duration was 2.2± 0.7 days and there were no significant differences for pre-pupal duration between Diet II and control. Both diets were supported larval development. Pupal duration was about 10.1±1.6 days. The number of adult emergence was 21 (11♀:10♂) on Diet II. No significant differences in adult emergence rate were found between Diet II and tomato. Pupae were measured 5.62±0.27 mm in width, 19.2 ±1.12 mm in length and weighed about 0.31±0.03 g (Table 2). The larval frasses and head capsules were visible indicating that larvae were feeding (Figure 3). The sizes of the head capsules were 0.28±0.04 mm for the

first instar, 0.41±0.03 mm for the second instar, 0.77±0.06 mm for the third instar, 1.63±0.08 mm for the fourth instar, 1.85±0.08 mm for the fifth instar and 2.82±0.20 mm for the sixth instar (Table 3). Mean comparison revealed significant differences among the bollworm instars feeding on Diet II.

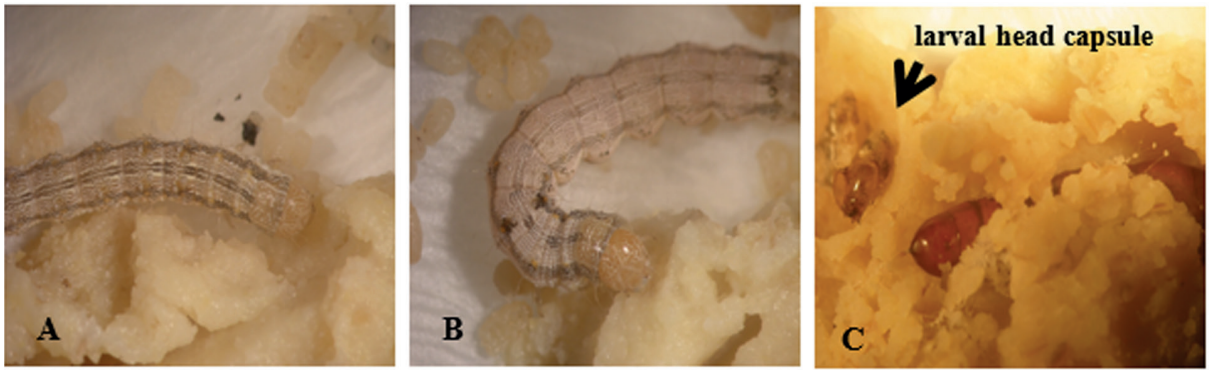

Figure 3. Larval feeding of *H. armigera* on Diet II, (A) 4th instar, (B) 5th instar and (C) pupa with the shredded larval head capsule.

Bollworm larvae had five instars based on head capsule measurements on Diet III (Table 3). The larval and pupal durations were longer than Diet I and Diet II. Larval duration was about 25.1±0.9 days. The pupal recovery was 76. ±1.5 %. Adult emergence rate was 73.3±2.8 %. Pupae were measured 5.5 ±0.34 mm in width, 18.8±1.29 mm in length and weighed about 0.26±0.04 g (Table 2). There were significant differences in pupal weights ($p<0.05$).

Larvae refused to feed on Diet IV and survived only first 10 days. Early instars had the highest mortality as 90%. This diet was not supported larval growth and development. Therefore, Diet IV was not suitable for rearing cotton bollworm larvae.

Diet V was also tested to rear the cotton bollworm. Larval development duration was 25.5±3.2 days. Larval development time on this diet was significantly longer than other diets. Pupal duration was about 29.5±4.8 days. Pre-pupal duration was 3.4±0.7 days and there were significant differences for pre-pupal duration between Diet I, Diet II and control. Pupae were measured 5.85±0.44 mm in width, 18.62±1.34 mm in length and weighed about 0.35±0.08 g. We observed that cotton bollworm has six instars on diet V (Table 2).

Bollworm larvae survived on Diet VI. Larval duration was about 22.1±1.7 days which was longer than Diet I and Diet II. Pupal development time was also longer compared to other diets. Adult emergence rate was 83.3±2.8 %. Pupae measured 5.77 ± 0.31 mm in width, 18.97±1.35 mm in length and weighed about 0.34±0.04 g. The cotton bollworm larvae had five instars on Diet V. Pre-pupal duration was about 2.3±1.1 days (Table 2).

The cotton bollworm larvae were also reared on tomato fruits as control and they had six instars. The adult emergence was 88.4 ± 1.1 %. Larval period lasted about 18.2±0.8 days, pupal period was about 9.3±0.4 days and pre-pupal duration was 2.3 ±1.1 days. Pupal recovery was 90.1± 1.1%. Pupae measured 6.05±0.3 mm in width, 19.97±0.58 mm in length and weighed about 0.36±0.05 g (Table 2).

The development larval period of the bollworm on tomato fruits was reported as 19.23 days by Kumar et al. [37], 16.17 days by Gupta et al. [38], 17 days by Guerra and Ouye [39] and 15.04 days by Casimero et al. [40]. According to Tamer [41], larval period was 12.7 to 13.5 days. Barbosa et al. [3] and Mironidis and Savopoulou-Soultani [2] tested the rearing of cotton bollworm on artificial diets and larval duration were ranged from 12.7 ± 0.3 days to 15.5 ± 0.2 days.

Kumar et al. [37] observed pupal period of bollworm on tomato fruits as 11.57 days. Additionally, it was reported as 11.34 days by Devi and Singh [42]. Liu et al. [8] and Tamer [41] reported pupal duration were 9.35 ± 0.14 days and 11.2 days respectively. Guerra and Ouye [39] were reported as 14 days and Brewer [43] results indicated as 9.56 days when bollworm were reared on artificial diets. Our results were similar to previous studies.

According to Kumar et al. [37], pre-pupal duration of bollworm was 2.27 days on tomato fruit and 1.13 days on artificial diet. Similar results have also been reported by Liu et al. [8,23], as 2.35 days.

Pupal weights are an important parameter in terms of artificial rearing studies. The weight of bollworm pupae exhibited significant differences among tested different diets. The highest weight of pupae was 0.361 g when the larvae fed on tomato. On the other hand, the lowest pupal weight was 0.267 g observed on Diet III. According to Barbosa et al. [3], pupal weight of cotton bollworm was 0.358 g on the artificial diet. Similar findings have also been reported by Amer and Sayed [26], as 0.325 g and Kumar et al. [37] recorded that pupal weight as 0.237 g on tomato fruit and 0.321 g on artificial diet.

Data were presented in Table 2 showed that pupal recovery was 90.1 % on tomato. The average pupal recovery was 76.6 % and 80.1 % on Diet I and Diet II, respectively. These results were similar to Kumar et al. [37] recorded that pupal recovery was 91.86%. In addition to that, Devi and Singh [42] found pupal recovery as 75.05%. Ahmed [44] reported that the highest pupal recovery was 83.7 % in the first generation and 71.2% in the field generation.

Ahmed [44] and Ahmed et al. [45] also reported bollworm adult emergence as 92.8% in the fourth generation and 78.4% in the first generation, respectively. Amer and Sayed [26] reported adult emergence rate was 92.73% on artificial diet. Kumar et al. [37] also reported highest adult emergence were 70.61% on tomato and 83.46% on artificial diet.

The cotton bollworm had different number of instars depending on larval food. We determined 6 instars on Diet II, Diet V and control, whereas, it had 5 instars on Diet I, Diet III and Diet VI. The other investigators also reported different number of instars such as Kumar et al. [22] observed 5 instars Jha et al. [46] reported 6 instars whereas Barbosa et

al. [3] observed 3 to 7 instars and Liu et al. [8] observed 7 instars on different diets.

There are many factors which effect on the biological parameters as larval feeding on artificial diet [47,48] which are mostly depended on the host plants, temperatures, humidities, rearing conditions, mating behaviours as well as the structure, composition and nutritional values of artificial diet. The nutritional content of a diet can considerably affect development time, fecundity, survival and growth of lepidopteran species [49]. In this study, Diet I was tested for the first time to rear the cotton bollworm larvae. Diet I and Diet II were suitable and sufficient for adaptation of wild colony in the laboratory conditions.

CONCLUSION

In this study, we evaluated six different artificial diets in the controlled laboratory conditions. Diet I and Diet II were sufficient and nutrionally suitable for laboratory uses and adaptation of wild colony. Diet I was the best diet out of six tested artificial diets which was also used the first time to rear cotton bollworm larvae. Diet I is simple to prepare, low costed and has a good texture supporting larval development of cotton bollworm.

Acknowledgments

This research was based partly on the M.Sc. thesis of Seda Yücel, supervised by Hanife Genç and financially supported by Canakkale Onsekiz Mart University, Scientific Research Projects Commission (FYL-2018/2466). The abstract of this research was published on IV. International Congress on Applied Biological Sciences (May, 3-5 2018, Eskişehir).

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