The Effect of Plant Quality on Survival of *Lymantria dispar* L. (Lepidoptera: Lymantriidae) Larvae Infected by Nucleopolyhedrovirus

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ABSTRACT

In this study the influence of plant protein and secondary compounds on the survival rate of *Lymantria dispar* L. which was infected with *Lymantria dispar* nucleopolyhedrovirus (LdNPV), was investigated by using four different plant species, *Elaeagnus rhamnoides* (L.) A. Nelson, *Quercus cerris* L. 1753, *Corylus maxima* Mill. and *Crataegus monogyna* Jacq. The highest survival rate has been observed on the larvae feeding on the *E. rhamnoides* (L.) A. Nelson that had the highest protein rate. On the other hand, the highest mortality rate was recorded on the larvae which fed on the *C. monogyna* Jacq. containing the lowest protein rate. We have also discovered that the survival rate was related to gallotannin amounts. The survival rates of the infected larvae which fed on *E. rhamnoides* (L.) A. Nelson with the highest gallotannin amount were higher than the other diets. Our results showed that the survival rates in the larvae infected with *Lymantria dispar* nucleopolyhedrovirus were positively related to the proanthocyanidin (condensed tannin) and total phenolic content of foods.

Key words: Lymantria dispar, nucleopolyhedrovirus, secondary compounds, insect survival, mortality.

INTRODUCTION

Various microbial factors can cause natural infections in insects (Tanada and Kaya, 1993; Boucias and Pendland, 1998; Yılmaz *et al.*, 2009; Charles *et al.*, 2000; Gökçe *et al.*, 2010; Sevim *et al.*, 2010; Tanyeli *et al.*, 2010; Danışmazoğlu *et al.*, 2012). Insect viruses can be considered as the most effective natural factor that causes insects to get sick and die (Hunter-Fujita *et al.*, 1998; Înce *et al.*, 2008; Demir *et al.*, 2009, 2013). The infection which is caused by pathogens negatively affects the survival rate and reproductive output of the hosts (Moore, 2002).

Plant diet can affect the interactions between insects and their pathogens (Duffey *et al.*, 1995). The food consumption in which the quality has changed as a consequence of either previous or current plant defoliation by herbivores is highly related with a

change in insect fitness (Larsson, 2002; Howe and Schaller, 2008). There are some studies that put forward the positive (Felton and Duffey, 1990; Hoover *et al.*, 1998a; Martemyanov *et al.*, 2006) and negative (Cook *et al.*, 2003) effects between the content of secondary compounds in an insect's diet and its resistance to pathogens. One of these secondary compounds is tannin. Tannins are known aspolyphenolic compounds which are able to bind to proteins and decrease the activity of many enzymes (Swain, 1979). Tannins can be categorized into two main groups: condensed tannins (proanthocyanidins) and hydrolysable tannins (including ellagitannins and gallotannins). Tannins are capable of inhibiting a great number of microorganisms such as viruses, bacteria, and fungi. By this way, the organisms are protected against the effects of pathogens (Swain, 1979). Tannins, probably, provide this protection by binding to the microbial proteins (Cadman, 1960).

The host is able to struggle against and withstand infection and this is highly related to the host's nutritional state (Chandra, 1996; Keating *et al.*, 1988; Coop and Kyriazakis, 2001). Protein is considered as the most important substrate for producing immunological components which are used to resist viral infections (Swain and Hillis, 1959; Trudeau *et al.*, 2001; Keating *et al.*, 1990).

The previous studies have proved that phenolic compounds of diets frequently restrain the viral infection (Felton *et al.*, 1987; Keating *et al.*, 1990; Hoover *et al.*, 1998b), yet it is revealed that this doesn't always show proper efficiency. Therefore, this study makes a search of how protein and tannins in plants have an effect on *L. dispar* larvae, which are exposed to viral infection, and it checks whether total phenolic amount in plants has any effects on the larvae or not.

MATERIALS AND METHODS

Virus and plants

Lymantria dispar nucleopolyhedrovirus (LdNPV) which isused in this study was isolated from field collected *L. dispar* larvae, in Bafra, Turkey, in May, 2012. After detecting the baculovirus infection under light microscope from dead insects, occlusion bodies (OBs) were purified according to the procedure described by O'Reilly *et al.* (1992). Viral propagation was performed in healthy *L. dispar* larvae in the laboratory. The larvae which were placed in plastic dishes, fed with a few leaves contaminated with OBs isolated from the infected larvae, and maintained at 25°C to develop infection. OBs from newly infected larvae were purified and stored at -20°C.

In this study, four plant species belonging different families, *Elaeagnus rhamnoides* L. (Fam.: Elaeagnaceae), *Quercus cerris* L. (Fam.: Fagaceae), *Corylus maxima* Mill. (Fam.: Betulaceae) and *Crataegus monogyna* Jacq. (Fam.: Rosaceae) were used. All plant samples were collected daily and the larvae fed on them.

Obtaining larvae

The eggs of *L. dispar* were collected from the Cernek Lake area, which is within the borders of Kızılırmak Delta in Bafra, Samsun. To provide the disinfection, the eggs

were treated with 10% of sodium hypochlorite. Then, they were washed with pure water and put into the refrigerator at 5°C. After six months, the eggs were taken out of the refrigerator and put into the climate cabin. Thiswas adjusted to the temperature of 22°C and 70% of humidity during a period of 16 hours of light and 8 hours of dark. The larvae that came out of the eggs were put into the plastic containers (sized 5×10×2 cm) for each food group and they were fed with fourplants that were indicated in the study until the 4th instar.

Feeding experiment

The larvae of the 4th instar were put into the plastic containers (sized 5×10×2 cm) which contained 30 larvae in each experimental group. Fifteen of these were control groups and others were infected by virus, all of which were put into each food group in the experiment. This process was carried out with 30 larvae and repeated 3 times in each experiment group. During the feeding experiment, as there were fourplants, 360 larvae in total were put into the containers. The plastic containers had six holes so that the larvae could get air.

The control group and the larvae, which were infected by virus, were fed for 10 days in different incubators having the same temperature and humidity. During the feeding experiments fresh leaves of each plantwere given each day and the remaining was dried in incubator. The survivor larvae which were both in control and infected groups were fed until they became pupae.

Drying and grinding leaves

In order to determine the amount of totalphenolics, protein, gallotannin and proanthocyanidin, the leaf samples were taken from the plants which the larvae were fed. Then, they were wrapped inside the aluminum folio and were dried for two months under laboratory conditions, and then for 5 days in incubator at 50°C. After the dried leaves were taken out and ground, they were kept in nylon bags.

Plant analysis

The protein content of the leaf samples were measured by semi-micro Kjeldahl method with Kjeltec Auto 1030 analyzer (Tecator, Sweden). The method which wasused to determine gallotannin contents of the leaf samples was described by Bate-Smith (1977). Proanthocyanidin contents of the leaf samples were determined spectrophotometrically by a method which was described by Bate-Smith (1975). The total phenolic contents of the leaf samples were determined by a method originally used by Swain and Hillis (1959). The nitrogen content of each sample which was obtained by Kjeldahl method was multiplied with 6.25 to calculate the total protein content of the plant sample (Monk, 1987).

Infection of larvae with LdNPV

In order to infect the larvae with virus, concentration of LdNPV was adjusted to 10⁵ OB/ml by using a Neubauer haemocytometer. Each of the plant samples taken from

the four plants used in feeding process was treated with 1 ml of virus suspension. After the surface sterilization of each leaf in control group was carried out with 50% of ethyl alcohol, they were treated with 1 ml of pure/distilled water and put into the containers for the experiment.

Determination of pupal protein contents

Pupae were left to dry to constant mass in aincubator at 50°C, weighed to the nearest 0.1 mg and lipid-extracted in three, 24-hour changes of chloroform before being re-dried and re-weighed. Nitrogen content of the lipid-free pupae was measured by semi-micro Kjeldahl method with Kjeltec Auto 1030 analyzer (Tecator, Sweden). The nitrogen content of each sample obtained by Kjeldahl method was multiplied with 6.25 to calculate the total protein content of the pupae (Monk, 1987).

Statistical analyses

The comparison of the amounts of protein, gallotannin, proanthocyanidin and total phenolic in plants was made by using ANOVA Duncan Test. According to fourdifferent plants; Kaplan-Meier Survival Analysis Test was applied to determine the relationship between the survival rates of the larvae which were infected by the virus and the larvae of the control group. The total food consumption amounts of the larvae in the control group and the larvae which were infected by virus were compared by parid-T test. In this comparison, the data of the survivor larvae were used. In order to compare the effect of the amounts of protein, gallotannin, proanthocyanidin and total phenolic in plants on survival larvae Cox-Regression analysis test was used.

RESULTS

Chemical composition of the leaf samples

The total protein contents of the leaf samples were 15.1% in *E. rhamnoides*, 10.0% in *Q. cerris*, 10.9% in *C. maxima* and 8.3% in *C. monogyna*. While the highest total protein content was obtained from the leaves of *E. rhamnoides*, *C. monogyna* leaves contained the lowest protein content. A significant difference within the protein content of host plant species was found (Table 1).

The gallotannin contents of the plant samples observed in the present study were 5.2% for *E. rhamnoides*, 2.6% for *Q. cerris*, 4.3% for *C. maxima* and 1.8% for *C. monogyna* (Table 1). These results put forward that *E. rhamnoides* had much higher gallotannin content in its leaves than that of others. There were important differences in the proanthocyanidin contents of the leaf samples (Table 1). The proanthocyanidin contents of the leaf samples (Table 1). The proanthocyanidin contents of the leaf samples (Table 1). The proanthocyanidin contents of the leaf samples (Table 1). The proanthocyanidin contents of the leaf samples (Table 1). The proanthocyanidin contents of the leaves of *E. rhamnoides*, *Q. cerris*, *C. maxima* and *C. monogyna* were 3.9%, 7.6%, 11.5% and 7.2% respectively. The total phenolic contents of the leaves of *E. rhamnoides*, *Q. cerris*, *C. maxima* and *C. monogyna* were 10.6%, 7.9%, 9.9% and 6.2%, respectively. Results from statistical data analysis revealed that the host plant species had a significant difference in their total phenolic content (Table 1).

Teste	Dianta			05		ANOVA	
Tests	Plants	N	Mean	SE	*Significant groups	F	Р
Total Protein (%)	Elaeagnus rhamnoides	10	15,1	0,06	а		
	Quercus cerris	10	10,0	0,09	b]	
	Corylus maxima	10	10,9	0,08	с	1577,5	< 0,001
	Crataegus monogyna	10	8,3	0,07	d		
Gallotannin (%)	Elaeagnus rhamnoides	10	5,2	0,04	а		
	Quercus cerris	10	2,6	0,04	b		
	Corylus maxima	10	4,3	0,04	с	1508	< 0,001
	Crataegus monogyna	10	1,8	0,03	d		
Proanthocyanidin (%)	Elaeagnus rhamnoides	10	3,9	0,03	а		
	Quercus cerris	10	7,6	0,04	b		
	Corylus maxima	10	11,5	0,02	с	11542,5	< 0,001
	Crataegus monogyna	10	7,2	0,03	d		
Total phenolic (%)	Elaeagnus rhamnoides	10	10,1	0,03	а		
	Quercus cerris	10	7,9	0,03	b		
	Corylus maxima	10	9,9	0,04	с	3208,7	< 0,001
	Crataegus monogyna	10	6,2	0,03	d		

Table 1. The total	protein, gallotannin	, total phenolic and	proanthocvanidin	contents of the leaf samples.

*Different letters stand for significantly different group means (p < 0.05). (The groups named as a, b, c and d have statistically significant means according to Duncan's Multiple Range Test).

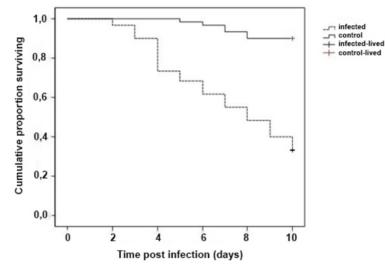
Consumption amounts of larvae in regard to plants

A decrease in total food consumption amounts in the larvae infected by LdNPV was found when it was compared to control groups. In all four plants, there was a significant difference between the larvae in control groups and the larvae infected by virus in terms of total food consumption. Moreover, the highest consumption amount in control groups and virus-infected groups was obtained from the larvae feeding on *E. rhamnoides*. The comparison of the total food consumption amounts of virus-infected larvae with the ones in control group in regard to plants is shown in Table 2.

The survival rates of infected larvae in regard to plant

The larvae in control groups and the larvae infected by the virus had been compared by through Log-Rank test and it was found that there was a significant difference (df=1, P<0,001). The survival rates were checked with Kaplan-Maier Test and it was found that the survival rate of virus-infected larvae was 65% while the survival rate of the control groups was 90%. The possibility of cumulative survival is shown in Fig. 1. The survival rates of virus-infected larvae and those in control groups in regard to plants are shown in Fig. 2. The survival rates of the larvae feeding on *E. rhamnoides,* which were both virus-infected and were in control groups,were higher than in other food groups. Table 2.The comparison of the consumption amount of virus-infected larvae with those in control group in regard to plants.

Plants	N	Groups	Mean ± SE	t	Р
	27	Infected	395.3 ± 1.6	- 30.0	< 0,001
Elaeagnus rhamnoides	45	Control	440,8 ± 0,7	- 30.0	
Quercus cerris	12	Infected	302.7 ± 5.2	- 45.7	< 0,001
	39	Control	414.3 ± 0.8	- 45.7	
Corylus maxima	12	Infected	265.8 ± 2.3	- 64.1	< 0,001
	42	Control	391,3 ± 5,2	- 04. I	
Crataegus monogyna	9	Infected	297.0 ± 2.1	- 54.4	< 0,001
	36	Control	412.5 ± 1.0	- 54.4	< 0,001





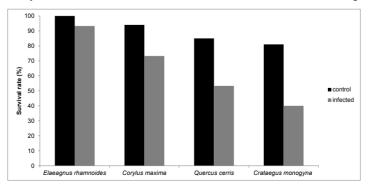


Fig. 2. The survival rates of virus-infected larvae and those in control group in regard to food.

The highest survival rate, 93.3%, was observed in larvae feeding on *E. rhamnoides*. The lowest survival rate, 40%, was found in larvae feeding on *C. monogyna*. While the survival rate was 53.3% in the larvae feeding on *Q. cerris*, it was 73.3% in those feeding on *C. maxima*. The most extraordinary result observed in virus-infected larvae was that some larvae in all food groups were pupae. Four of the larvae feeding on *E. rhamnoides*, *Q. cerris* and *C. maxima* and three of the larvae feeding on *C. monogyna* became pupae (Table 3).

Plants	N	Died larvae	AI	ived	Percent of alive (%)	
Fiants	N	Dieu lai vae	Larvae	Pupated	Fercent of allve (76)	
Elaeagnus rhamnoides	45	3	30	12	93.3	
Quercus cerris	45 21		12	12	53.3	
Corylus maxima	45	12	21	12	73.3	
Crataegus monogyna	45	27	9 9		40.0	
Total	180	63	117		65.0	

Table 3. The survival rates of virus-infected larvae.

It was found that there was a difference between the survival rates of virus-infected larvae in regard to plants. The survival rate of the larvae feeding on *E. rhamnoides* was different from that of the larvae feeding on *Q. cerris* and *C. monogyna* (Table 4, P<0.05). The survival rate of the larvae feeding on *C. maxima* was different in critical value from that of the larvae feeding on *C. monogyna* (Table 4, P=0,087).

Table 4. The comparison of the survival rates of the larvae feeding on the plants infected by virus in regard to plants with Log Rank test.

Plants	Elaeagnus rhamnoides		Quercus cerris		Corylus maxima		Crataegus monogyna	
Plants	Chi-Square	Р	Chi-Square	Р	Chi-Square	Р	Chi-Square	Р
Elaeagnus rhamnoides			6,285	,012	2,102	,147	9,009	,003
Quercus cerris	6,285	,012			1,359	,244	,263	,608
Corylus maxima	2,102	,147	1,359	,244			2,921	,087
Crataegus monogyna	9,009	,003	,263	,608	2,921	,087		

Pupae analysis

The larvae of the control group which were fed on four species of plants had much more pupal protein amount when compared to infected-larvae. The highest pupal protein amount among larvae, which were both in control groups and were infected, has been observed in the larvae feeding on *E. rhamnoides*. The protein amount of the larvae feeding on *C. monogyna* was the lowest among the larvae which were both in control groups and were infected. The protein amount of the infected-pupae was 9-17% less than control groups (P<0.05) (Fig. 3).

The effect of protein and secondary compounds on the survival of infected larvae

Cox-Regression analysis results have shown that total protein, proanthocyanidin and total phenolic contents have a positive effect on the survival of the infected larvae.

However, virus infection increased the mortality 12 fold. The results of the analysis are shown in Table 5.

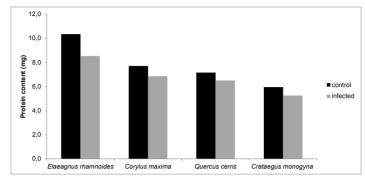


Fig. 3. The protein content of virus-infected larvae and those in control group in regard to food.

Table 5. The comparison of the effect of virus infection	, protein and secondary substances on survival
by Cox-Regression analysis.	

	в	SE	Wald	df	Sig.	Exp (B)
Infection with virus	2,503	,865	8,372	1	,004	12,225
Protein	-,939	,319	8,664	1	,003	,391
Phenolic	-,177	,026	46,030	1	,000	,838
Gallotannin	-,302	,057	27,987	1	,000	,740
Proanthocyanidin	-,466	,155	9,064	1	,003	,628

B: Coefficient of regression, SE: Standard error, Wald: Significance of the regression coefficients, df: Degree of freedom, Sig.: Significant, Exp (B): Hazard proportion.

DISCUSSION

The results showed that the protein amount in the plant on which the insects infected with the virus feed had a positive effect on its survival. The highest survival rate in the larvae infected by LdNPV has been obtained from the larvae feeding on *E. rhamnoides* containing the highest protein amount. The highest mortality has been found in the larvae feeding on *C. monogyna* containing the lowest protein amount. Protein is a vital substance to produce the immunological components used in resisting to viral infections (Washburn *et al.*, 1996; Trudeau *et al.*, 2001). The results show that when the protein amount in the plant which the larvae infected by LdNPV feed and survival rate were increased. This result showed that protein had astrong important role in *L. dispar*'s immune system. Moreover, the protein amounts of the pupae of the infected larvae in all food groups are less than the pupae of their own control groups.

Host's ability to defend and to stand out against infection is related to its diet (Chandra, 1996; Lochmiller and Deerenberg, 2000; Coop and Kyriazakis, 2001).

Lee *et al.* (2006) have put forward that the larvae feeding on artificial food containing low protein-high carbohydrate amount lose performance three fold more than food containing high protein-low carbohydrate amount. It has been found that the survival rate is lower in the ones feeding on artificial food containing low protein-carbohydrate amount. The fact that when a virus-infected insect is fed on food containing more protein, the survival rate increases have been shown in the previous studies of the field (Thompson and Redak, 2000; Lee *et al.*, 2002; Simpson *et al.*, 2004).

The most interesting result of this study is that some of the larvae infected by virus became pupae in all food groups. The previous studies in the field have put forward that the development period of the larvae infected by NPV prolongs either indirectly by resisting the infection or directly by lethal virus infection (Rothman and Myers, 1996; Cooper *et al.*, 2003; Cory and Myers, 2003).The results of this study emphasize that *L. dispar* larvae infected by NPV reduce the development period.

In this study, the maximum deaths of the larvae infected by virus occurred on the second day. These deaths were observed in the larvae feeding especially on *C. monogyna* containing low protein amount. Previous studies have emphasized that the resistance to NPV, encapsulation, melanization with phenoloxidase, and the process of dismissing viruses out of haemolymph happened 2-3 days after the infection and the protein was especially chosen (Washburn *et al.*, 1996; Trudeau *et al.*, 2001; Lee *et al.*, 2006). The results of this study have shown that when the protein amount decreases, the pathogen resistance stated above also decreases.

Biologically activated phytochemicals can be bound to the structure (Oclusion Body) in midgut of the larvae and can reduce the infection of virus on host insect (Felton and Duffey, 1990). This interaction can be regulated by digestion methods of the host insect (Glare *et al.*, 2003). The effect of gallotannin on survival rate has importance in critical value in the larvae infected by the virus. In a previous study, it was found that when the host plant was treated by the virus which is consumed by the larvae; the mortality changed with the effect of virus and this effect was related to amount of hydrolysable tannin (polyphenol) (Keating *et al.*, 1988).

This study has pointed out that in the virus-infected larvae; the increase in total phenolic amount has a positive effect on the survival rate. It was stated in a previous study that the chemicals obtained from a plant were bound to midgut or they changed the host's physiology by effecting normal immune system sufficiently, or such chemicals gave starting signals for stages that would decrease number of cells that are vulnerable to infection (Lee *et al.*, 2006). The results of this study show that there is a positive correlation between total phenolic amount of the diet consumed and the survival rate.

Washburn *et al.* (1998) have found out that when *Trichoplusia ni* and *Heliothis virescens* larvae infected by *Autographa californica* nucleopolyhedrovirus fed on artificial food free from secondary compounds, the mortality rate increased. The results of this study show that there is a positive correlation between the survival rate of the larvae and condensed tannins.

Even though the viruses as entomopathogens have a negative effect on the survival of insects, the resistance of herbivores against entomopathogens can differ

depending on the plants they feed on. Secondary compounds that are regarded as defensive substances against herbivores seem to change the negative effects of entomopathogens. Moreover, peritrophic membrane in insects has an important role in changing these effects. Although this study focuses on protein and the effect of secondary compounds in the plant on the resistance of insects against entomopathogens, it can be seen that this relation is complicated. It will be better to pay attention to the secondary compounds in the plants along with protein and the digestive system of the host.

ACKNOWLEDGEMENTS

This study was supported by the Ondokuz Mayıs University Research Foundation (PYO.FEN.1904.11.027).

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Received: December 18, 2015

Accepted: August 02, 2016