THE EFFECT OF PLANT QUALITY ON SURVIVAL OF *LYMANTRIA DISPAR* (LEPIDOPTERA: LYMANTRIIDAE) LARVAE INFECTED BY *BACILLUS THURINGIENSIS* BERLINER SUBSP. *KURSTAKI*

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(Received 3rd May 2016; accepted 8th Mar 2017)

Abstract. In this study the influence of plant secondary compounds on the survival rate of *Lymantria dispar* (Linnaeus, 1758) which was infected with *Bacillus thuringiensis* Berliner 1915 subsp. *kurstaki* was investigated 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 was seen on the larvae which fed on the *E. rhammoides* that had the highest protein rate. The highest mortality rate was seen on the larvae which fed on the *C. monogyna* that had the lowest protein rate. Maximum deaths of the larvae infected by each food set occurred on the second day. These deaths were observed in larvae that fed on the lowest protein amount plant. We have discovered that survival rate correlated with gallotanen amounts. The survival rate of the larvae infected fed on *E. rhammoides* that have the highest gallotanen amount were higher than other diets. Our results showed that in the larvae which were treated with *Bacillus thuringiensis* subsp. *kurstaki* the survival rate positively correlated with proantosiyanidin (condensed tannin) and total phenolic content.
Keywords: *secondary compounds, entomopathogen, herbivor, plant-insect interaction, phytophagy*

Introduction

Bacillus thuringiensis (Berliner), which is the most important biopesticide of the world, is a spore-forming, ubiquitous gram-positive bacterium (Zhang et al., 2013). The crystal proteins, which are also called Cry proteins, are parasporal inclusion proteins from *B. thuringiensis* which show toxic effect to a target organism that can be proven through experiments (van Staden, 2015). Infection by pathogens imposes significant fitness costs on hosts, diminishing survival and/or the rate of reproductive output (Moore, 2002). Apart from their direct effects on hosts, pathogen infections are considered to impose significant resource costs related with the maintenance and activation of resistance mechanisms, which may conflict with other life-history traits (Zuk and Stoehr, 2002; Rolff and Siva-Jothy, 2003; Schmid-Hempel, 2005). The ability of the host to fight and withstand infection is related with its nutritional state (Coop and Kyriazakis, 2001).

Lymantria dispar, which is also called the gypsy moth, is among the most harmful phytophagous pest insects of the northern hemisphere. It has a vast capacity to feed on a

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1503_837847

total of more than 500 plant species, including various trees and shrubs (Barbosa et al., 1971). In order to defend against *L. dispar* L., a great number of secondary metabolites of host plants may show strong prooxidative effects on midgut tissue and may cause oxidative stress (Bi and Felton, 1995; Perić-Mataruga et al., 1997; Ilijin et al., 2014; Perić-Mataruga et al., 2014).

Plant-insect interaction shows an endless variation and change which makes this interaction a dynamic system (Mrdaković et al., 2011). Phytophagous insects can use many different host plants across their geographic distribution, but within their environments they usually use a small number of plant species (Ruiz-Montoya et al., 2003). Plants play a significant role in the evolution of insect-pathogen relationships. Host plants can alter the interactions between insect herbivores and their pathogens. Inter- and intra-specific differences in plant chemistry and structure may change the susceptibility of insects to infection and the production and environmental persistence of pathogens (Cory and Hoover, 2006). Host plant quality defines the components of the host plant (e.g., the levels of nitrogen, carbon, trace elements and defensive compounds) that in a positive or negative way have an effect on the performance of herbivorous insects (Awmack and Leather, 2002). A lot of secondary plant metabolites are regarded as strong defense of plants against herbivores and pathogens (Hwang et al., 2008). Reduced insect performance due to poor plant quality can increase the susceptibility of an insect to disease while these same phytochemicals can also diminish the effectiveness of entomopathogens in killing the host (Cory and Hoover, 2006).

This study aims to put forward whether there could be an effect of protein, phenolic and tannin content (hydrolysable and condensed tannin) in plants on the survival of *L. dispar* larvae, which are infected by *Bacillus thuringiensis* subsp. *kurstaki (Btk)*, when they feed on different plants.

Materials and methods

The process of 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. The eggs were treated with 10 % of sodium hypochlorite and disinfected. Then, they were washed with pure water and put into the refrigerator at a temperature of 5°C. About 6 months later, the eggs were taken out of the refrigerator and put into the climate cabin that was adjusted to the temperature of 22°C and 70 % rate 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 5cmx10cmx2cm) so that there could be 50 larvae in each food group and they were fed with 4 plants that were indicated in the study until the 4th larval period.

The plants to be used during feeding experiment

In this study, we examined whether the plant quality had any effect on the survival of *L. dispar* larvae after they were infected by the bacteria. Thus, 4 different plant species, *E. rhamnoides, Q. cerris, C. maxima, C. monogyna* were used. These plants were collected daily and the larvae fed on them.

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Infection with the bacteria

In order to infect the larvae with the bacteria, the *Btk* suspension of 600 nm wavelength (OD_{600}) and with the optical density of 0.189 was obtained from the Department of Biology in Karadeniz Technical University. *Btk* was isolated and identified by Sevim et al. (2012). Each of the plant sample taken from the 4 plants used in feeding process was treated with 1 ml of *Btk* suspension. After each leaf in control group was cleansed with 50% of ethyl alcohol, they were treated with 1 ml of pure/distilled water and put into the containers for the experiment.

Feeding experiment

The larvae of the 4th period were put into the plastic containers (sized 5cmx10cmx2cm) so that there could be 45 larvae in each experimental group. Therefore, 60 larvae in total, 15 of which were control groups and others infected by *Btk*, were put in each food group in the experiment. During the feeding experiment, as there were 4 plants, 240 larvae in total were put into the containers. The plastic containers had 5 holes so that the larvae could get air. The control group and the larvae that were infected by the bacteria were fed for 10 days in different incubators that had the same temperature and humidity. During the feeding experiment, each day, a new food was given after it was scaled in assay balance that was sensitive to 0,001 gram and after the remaining food was dried in incubator, their dry weights were scaled.

The process of drying and grinding of leaves

The leaves were collected from the plant the larvae were fed in order to determine the amount of phenolic, nitrogen, gallotannin and proanthocyanidin in total and for feeding. Then, they were wrapped inside the folio and were dried for 2 months under laboratory conditions and 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 contents of the leaf samples were measured by semi-mikro 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 plant sample (Monk, 1987). The total phenolic contents of the samples were determined by a method originally used by Swain and Hillis (1959). The method used 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 described by Bate-Smith (1975).

The growth of Btk in nutrient agar with different concentrations of tannic acid

5 nutrient agars, one of which was for control, containing 1, 3, 7, 10 % of tannic acid concentration were prepared. *Btk* was inoculated in nutrient agar prepared beforehand. Then, 14 hours of reproduction rates were calculated.

Statistical analyses

The comparison of the amounts of protein, total phenolic, gallotannin and proanthocyanidin in plants was carried out by using ANOVA Duncan Test. Recording

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to 4 different plants; Kaplan-Meier Survival Analysis Test was used to determine the relationship between the survival rates of the larvae that were infected by the bacteria and the larvae of the control group. The consumption amounts of the larvae in the control group and the larvae infected by *Btk* were compared by two sample T test. In this comparison, the data of the survivors was used. In order to compare the effect of the amounts of protein, total phenolic, gallotannin and proanthocyanidin in plants on survival 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*. According to these results, the highest total protein content was obtained in the leaves of *E. rhamnoides;* while *C. monogyna* leaves produced the lowest protein content. Protein content of host plant species was found to be different significantly (ANOVA, F= 1577.5, p<0.001).

The gallotannin content of the plant samples observed in the present study was 5.2 % for *E. rhamnoides*, 2.6 % for *Q. cerris*, 4.3 % for *C. maxima*and 1.8 % for *C. monogyna* (ANOVA, F= 1508, p<0.001). These results showed that *E. rhamnoides* had much higher gallotannin content in leaves than that of other plant leaves. Total phenolic content of the leaves of *E. rhamnoides, Q. cerris, C. maxima* and *C. monogyna* was 10.6, 7.9, 9.9 and 6.2 % respectively. Results from statistical data analysis revealed that host plant species were different significantly in their total phenolic content (ANOVA, F= 3208.7, p<0.001). There were significant differences in the proanthocyanidin contents of the leaf samples (ANOVA, F= 11542.5, p<0.001). The proanthocyanidin contents of the leaves from *E. rhamnoides, Q. cerris, C. maxima* and *C. monogyna* were 3.9, 7.6, 11.5 and 7.2 % respectively.

The growth graphic of Btk in nutrient agar with tannic acid

In 5 different nutrient agars, by adding an amount of 1, 3, 7 and 10 % of tannic acid and control, the reproduction of bacteria was observed in every other hour and it was found that there was a significant decrease in nutrient agars where tannic acid concentration was 3 % and above. The reproduction graphic according to tannic acid concentration is shown in *Figure 1*.

The consumption amounts of experimental groups in regard to the plants

It was found that there was a decrease in the consumption amounts of the larvae infected by the bacteria with regard to the control group. In all 4 plants, there is a significant difference between the larvae in the control group and the larvae infected by the bacteria in terms of consumption amounts. Moreover, the highest consumption amount in control group and in the groups infected by the bacteria was obtained from the larvae feeding on *E. rhamnoides.* The comparison of the consumption amount of the larvae infected by the bacteria with those in the control groups in terms of food is shown in *Table 1*.

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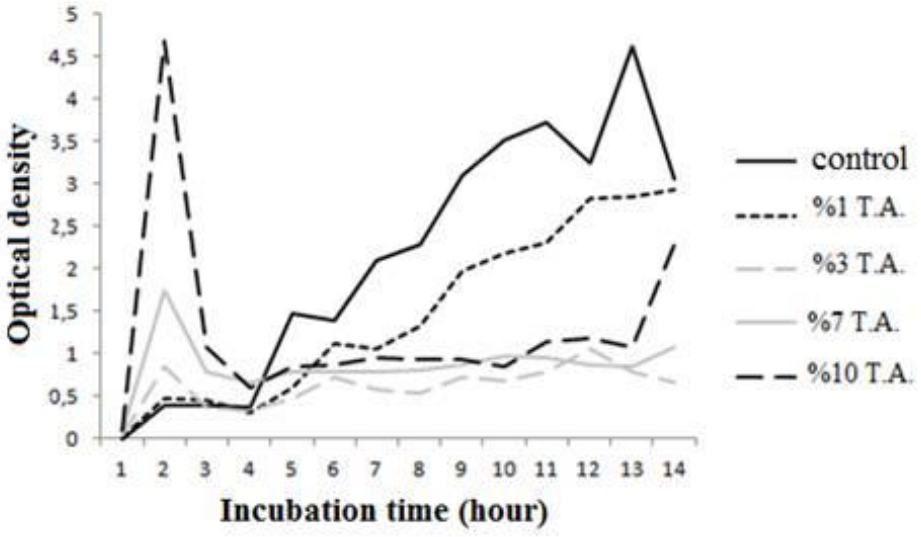

Figure 1. *The effect of tannic acid in different concentrations (1, 3, 7, 10 %) in nutrient agar on the growth of Btk*

Table 1. *The comparison of the consumption amount of the larvae infected by the bacteria and the larvae in the control groups in terms of food*

Plants	N	Groups	Mean±SE	t	*p*
H. rhamnoides	30	infected	405.4±1.5	-24.0	<0.001
	15	control	442.8±0.8		
Q. cerris	6	infected	311.7±0.8	-45.7	<0.001
	13	control	417.8±0.9		
C. maxima	12	infected	265.8±2.3	-64.1	<0.001
	14	control	394.3±8.9		
C. monogyna	3	infected	300.8		
	12	control	416.4±1.0		

The survival analysis of the larvae infected by Btk with regard to food

It was found that there is an important difference (p<0.001) between the survival rates of the larvae infected by the bacteria with regard to control group. It was found that while the survival rate of the larvae infected by the bacteria is 28.3 %, the survival rate of the control group is 90 %. The highest survival rate was 66.7 % and it was observed in the larvae feeding on *E. rhamnoides*. The lowest survival rate was 6.7 % and it was observed in the larvae feeding on *C. monogyna*. This rate was confirmed as 13.3 % in oak *Q. cerris* plant and as 26.7 % in hazelnut *C. maxima* plant (*Fig. 2*).

It was found that there is a difference between the survival rates of the larvae infected by the bacteria with regard to plants they fed on. The survival rate of the larvae feeding on *E. rhamnoides* is different from the larvae feeding on *Q. cerris* and *C. monogyna* (p<0.05) and is different in critical value from the larvae feeding on *C. maxima* (*Table 2*).

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DOI: http://dx.doi.org/10.15666/aeer/1503_837847

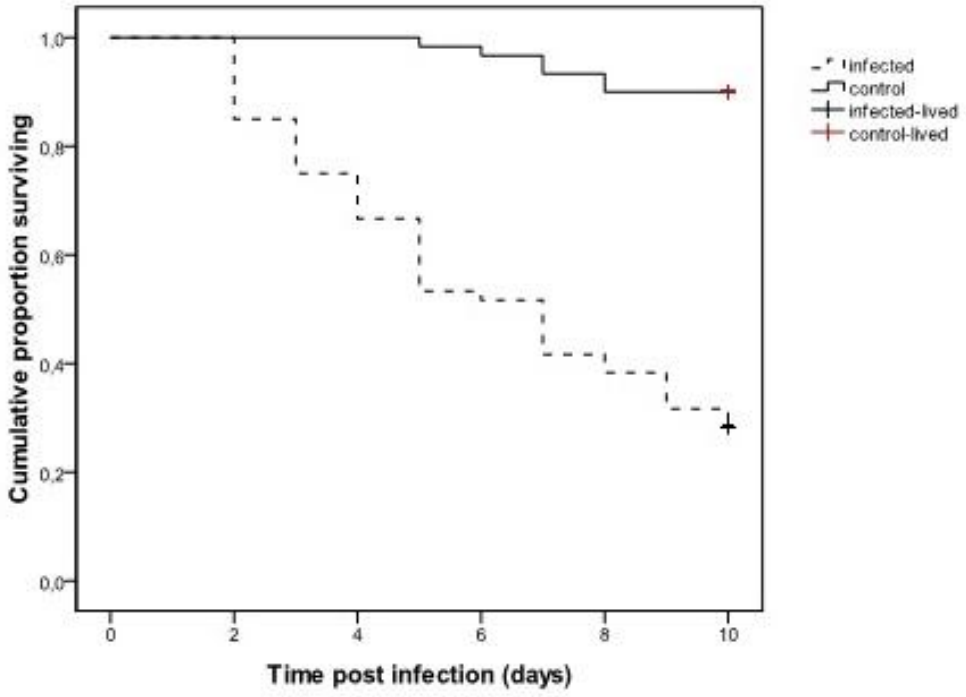

Figure 2. *The possibility of cumulative survival of the larvae in the control group and the larvae infected by Btk*

Table 2. *The comparison of the survival rates of the larvae feeding on the plants infected by the bacteria with regard to plants with Log Rank test*

Plants	*H. rhamnoides*		*Q. cerris*		*C. maxima*		*C. monogyna*	
	Chi-Square	*P*	Chi-Square	*P*	Chi-Square	*P*	Chi-Square	*P*
H. rhamnoides			8.104	0.004	3.672	0.055	9.716	0.002
Q. cerris	8.104	0.004			0.952	0.329	0.185	0.667
C. maxima	3.672	0.055	0.952	0.329			1.771	0.183
C. monogyna	9.716	0.002	0.185	0.667	1.771	0.183		

The Effect of Protein and Secondary Compounds on the Survival of the Larvae Infected by Btk

According to Cox-Regression analysis results, protein, proanthocyanidin and total phenolic have a positive effect. Infection by the bacteria increases death risk 200-fold. Cox-Regression analysis results are shown in *Table 3*.

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DOI: http://dx.doi.org/10.15666/aeer/1503_837847

Table 3. *The comparison of the effect of protein and secondary compounds on the survival of the larvae infected by Btk with Cox-Regression analysis*

	B	SE	Wald	*df*	*P*	Exp(B)
Infected by *Btk*	5.303	1.156	21.048	1	.000	200.849
Protein	-1.339	0.322	17.243	1	.000	0.262
Total phenolics	-0.239	0.036	43.988	1	.000	0.787
Gallotannin	-0.351	0.065	29.184	1	.000	0.704
Proanthocyanidin	-0.643	0.155	17.250	1	.000	0.526

Discussion

The results show that the protein amount in the plant, which the insect infected by the bacteria feeds on, has a positive effect on its survival. The highest survival rate in the larvae infected by *Btk* 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. These results put forward that protein has an important role in *L. dispar'*s immune system to be strong. Kleiner et al. (1998) have indicated that when *L. dispar* larvae that are infected by *B. thuringiensis* are fed on hybrid *Populus* plants, the mortality of the larvae feeding on the plants with higher protein amounts decreases.

This insect feeds on a wide range of plants which contain allelochemicals, such as phenolics, tannins, and terpenoids and a great number of these allelochemicals are toxic to bacteria (Broderick et al., 2004; Dillon and Dillon, 2004). In a host, selection pressure on the microbial community may be caused by toxic compounds. The compounds that would otherwise be toxic to the insect are degraded and metabolized by the surviving bacteria (Broderick et al., 2004). When each food group is considered, it can be clearly seen that the consumption amount of the larvae in control group is higher than the infected larvae. The highest consumption amount among the infected larvae has been observed in the larvae feeding on *E. rhamnoides*. The protein amount of this plant is higher than the other plants in experimental group. This is also the plant that has the lowest mortality. Therefore, this consequence emphasizes the significance of protein for an infected insect.

Appel and Schultz (1994) conclude that oak tannins reduce the effectiveness of this *B. thuringiensis* formulation (Thuricide), that stand composition may have a major effect on the effectiveness of microbial control measures, and that tannin inhibition may represent a useful target for formulation. In this study, the high survival rate of the larvae infected by *Btk* can be associated with the excess of hydrolysable tannin concentration. When tannic acid concentration which are added to *Btk*'s nutrient agar increases, the bacteria reproduction decreases, which supports the argument of this study. Unlike our findings, Gibson et al. (1995) found that tannic acid caused an increase in the efficacy of *B. thuringiensis* subsp. *kurstaki* against *Heliothis virescens* F. and *Trichoplusia ni* (Hübner) larvae.

Interactions between plants and herbivores are mediated by plant chemicals (Bednarek, 2012; Mithöfer and Boland, 2012). For instance, in defense against specialist insects, tannins are specifically important as digestibility-reducing compounds

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1503_837847

(Forkner et al., 2004; Müller-Schärer et al., 2004). Tanniferous plants which have relatively low levels of protein would be more deterrent to herbivores with the same levels (Barbehenn and Constabel, 2011). In this study, the highest survival rate of the larvae infected with *Btk* was obtained from the larvae feeding on *E. rhamnoides*. This plant has the highest amount of gallotannin. The larvae feeding on *C. maxima* have both a high survival rate and a high amount of gallotannin.

Different toxins have been proven to be active against a great number of insects; however, in general, each toxin is restricted to a few species within one insect order in terms of action (Raymond et al., 2007). The growth of various bacteria including *Listeria monocytogenes*, *Escherichia coli*, *Bacillus subtilis*, *Lactobacillus spp*., and *Staphylococcus aureus* are inhibited by phenolic acids (Cueva et al., 2010; Ramos-Nino et al., 1996; Sánchez-Maldonado et al., 2011; Wen et al., 2003). A strong negative correlation was found between larval performance of a specialist caterpillar (*Gadirtha inexacta*) and tannins content (Huang et al., 2010; Wang et al., 2012). On the contrary, results of this study show that the survival rate of the larvae infected by *Btk* is correlated positively with condensed tannins.

Appel and Schultz (1994) suggest that mortality was correlated negatively with the concentration of total phenolic, gallotannin and protein binding activity in leaves. It was found that total phenolic amount has a negative effect on the survival rate of the larvae infected by bacteria. This may be the result of high mortality in the larvae feeding on *C. maxima* which contains a high amount of phenolic. However, the survival rate is high in the larvae infected by bacteria feeding on *E. rhamnoides* containing the highest total phenolic amount when compared with the larvae feeding on other plants. In a previous study, it has been suggested that plant-derived chemicals cross the midgut or initiate signaling cascades, which alter host physiology sufficiently to impact normal immune function, or reduce cell permissiveness to infection (Lee et al., 2006). The results of this study suggest that the chemicals in the content of total phenolic can alter the answer.

Studies on entomopathogenic bacteria focused on finding their efficacy in terms of lethal concentration and killing speed (Deilamy and Abbasipour, 2013), their susceptibility of different stages of larval development to the pathogen (Erb et al., 2001; Gilliland et al., 2002) and the efficacy of sublethal effects on larval development (Mohan et al., 2008). Significant reductions were found in pupal weight, larval weight, normal pupation and prolonged larval period of *Heliothis armigera* Hübner (Lepidoptera: Noctuidae) larvae fed on sublethal doses of *B. thuringiensis* (Mohan et al., 2008). In this study, bacteria intensity was reduced by subtilizing 10-fold less than normal dose for infection. The role of *B. thuringiensis* spores eaten by the larvae can immensely alter in terms of bacteria intensity, bacteria type, the concentration of Cry toxins, different combinations and species (Hansen and Salamitou, 2000).

In conclusion, the negative impact of entomopathogens on insects is obvious. Insects aren't vulnerable to entomopathogens. In this study, even though the impact of secondary compounds and protein in plants on defense against entomopathogens is emphasized, this correlation is complicated. Broderick et al. (2006) reported that *B. thuringiensis* did not kill larvae of the gypsy moth in the absence of indigenous midgut bacteria. They suggested that the enteric bacteria might remove an immunological barrier, such as defensive enzymes or antimicrobial peptides that prevent growth of *B. thuringiensis*. Midgut bacteria affect the insect survival rate. Therefore, it may be much useful to think of a synergistic effect rather than the mere effect of secondary compounds and protein in plants. Further researches about this argument will set a much clear frame.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1503_837847

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

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