

Preliminary Studies on the Humoral Immune Response of Mulberry Silkworm, *Bombyx mori* Larvae Fed on Different Mulberry Varieties

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Abstract: Diseases infecting silkworms cause 30-40% cocoon crop loss. The silkworm, *Bombyx mori* L. defense against pathogens is provided by open circulatory system through humoral and cellular response. Three mulberry varieties, *Morus alba* var. Rosa, *M. alba* var. Morittiana and *M. alba* var. Laevigata, were used to study the effect of host plant and bacterial contagious infection on the humoral immune of *B. mori* larvae. The specific activity of related enzymes in haemolymph of fifth instar larvae was estimated. Results indicated that mulberry varieties and bacterial contagious infection had significant effects on enzymes activity values in larval haemolymph. Moreover, *M. alba* var. laevigata was superior in the humoral immune response.

Keywords: *Bombyx mori* L., Antimicrobial proteins, humoral immunity, Mulberry varieties

INTRODUCTION

One of the yield limiting factors in silkworm crop are diseases. Insects have powerful defense mechanisms to fight infections by possessing an efficient innate immune system. The silkworms, *B. mori*, defense against pathogens divided to the primary defense which prevent the infection by body structural barriers and the secondary defense that provided by insects open circulatory system, haemolymph, through cellular and humoral response (Lemaitre and Hoffmann, 2007; Montano *et al.*, 2011).

Changes in the haemolymph biochemical composition reflect the biochemical modification in the insect body in response to infections. Thus antimicrobial proteins play major roles in humoral immunity, whereas melanization is involved in both humoral and cellular immune responses (Rosales, 2017; Yang *et al.*, 2018).

Variation in immune defenses caused by nutritional contents in host plants were studied in different insects such as the cabbage looper, *Trichoplusia ni* (Shikano *et al.*, 2010), *Spodoptera litura* (Vengateswari *et al.*, 2020), the European grape berry moth, *Eupoecilia ambiguella* (Vogelweith *et al.*, 2015) and Grasshopper *Schistocerca gregaria* (Emad *et al.*, 2016).

From many years ago till now, many studies focused on mulberry varieties and their effects on economic traits and growth of silkworms, on the other hand, many efforts had been done by genetic breeding to find out a diseases resistance hybrids of *B. mori* (Alipanah *et al.*, 2020; Hema *et al.*, 2011; Hou *et al.*, 2013; Kumar and Naik, 2011). But no investigations were done on the effect of mulberry varieties on immune response or disease tolerance. Thus current study aims to study the direct effect of the sole host plant, mulberry varieties, on the larvae immune responses and bacterial contagious infection tolerance.

MATERIALS AND METHODS

Silkworm, *Bombyx mori* larvae rearing and treatment:

Silkworm eggs, the four-way hybrid (KK × Hesa 1) × (Vesletz 2 × Gergana 2), imported from

Sericulture and Agriculture Experiment Station, Vratza, Bulgaria were used. After hatching, larval instars fed on clean washed mulberry leaves four times daily which collected from un-infested plants at the Suez Canal University Farm, Ismailia, Egypt. Removal of fecal matter and bed cleaning was done consistently. The silkworm rearing was conducted in the laboratory following the method described by Mahmoud (2017).

Mulberry leaves of *Morus alba* var. Morittiana, *M. alba* var. Rosa and *M. alba* var. Laevigata (grafted on *M. alba* var. Morittiana origin) were introduced to feed larvae from hatching till cocooning. All experimental treatments were triple replicated, each replicate contains 100 larvae.

Depending on bacterial contagious symptoms, Joshi (2013) and previous obtained results of identification of two pathogenic bacterial strains, *Bacillus megaterium* and *Bacillus circulans*, isolated from silkworm larvae, Abou El-Ela (2016), ten naturally bacterial infected and non-infected larvae from each replicate were randomly picked then punctured their proabdominal leg to collect haemolymph samples separately in glass tubes as pooling samples, deep frozen at -20°C as stock and used whenever required.

Enzyme haemolymph preparation and analysis:

The haemolymph was collected from the larvae of *B. mori* in a chilled tube coated with ice then centrifuged for 10 min at 2500 r.p.m. to remove the blood cells.

Phenoloxidase

(PO) The dopa chrome formation was determined spectrophotometrically at O.D. 490 nm, 30 min later.

One enzyme activity unit was defined as the amount of enzyme which causes a change of 0.001 OD490 per minute. Specific activity was calculated in terms of units/mg total plasma protein determined by Bradford assay. Phenoloxidase activity was determined according to a modification of Ishaaya (1971).

The lysozymes:

The lysozyme activity was determined using the turbidity method according to De Azambuja *et al.*

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(1991) with slight modifications. One unit of enzyme activity is defined as the amount of enzyme that decrease the absorbance reading at rate of 0.001 absorbance units per min per *ul* sample in one ml of the reaction described.

Peroxidase:

Peroxidase (POD) specific activity was determined according to Vetter *et al.* (1958) and expressed as (units $\times 10^3$ /min/ mg protein. One unit was defined as an increase in absorbance of 0.001 per min and the enzyme activity was expressed as units of enzyme/mg protein.

Nonspecific esterases:

Alpha esterases (α -esterases) and beta esterases (β -esterases) were estimated according to Van Asperen (1962) using α -naphthyl acetate or β -naphthyl acetate as substrates, respectively.

Glutathione S-transferase (GST):

Glutathione S-transferase (GST) catalyzes the conjugation of reduced glutathione (GSH). The conjugate, S-(2,4-dinitro-phenyl)-L-glutathione could be detected as described by the method of Habig *et al.* (1974). One unit of enzyme activity is defined as the amount of enzyme that decrease the absorbance reading at rate of 0.001 absorbance units per min per *ul* sample in one ml of the reaction described.

Statistical analysis

Data were analyzed using SPSS program version 22.0 (SPSS 2008), which used independent samples t-test to compare means of enzymes units in infected and non-infected larvae and one way ANOVA. Statistical significance was assigned as ($p \leq 0.05$).

RESULTS

Effect of mulberry varieties on enzymes activity in haemolymph of *Bombyx mori* larvae:

Data in Table (1) and Figure (1) show the enzymes specific activity in larvae haemolymph fed on the tested mulberry varieties.

Phenoloxidases (PO) specific activity:

PO specific activity were 307.67, 384.00 and 340.0 (units /mg protein) in non-infected larvae fed on *M. alba* var. Rosa, *M. alba* var. Morittiana and *M. alba* var. Laevigata leaves, respectively. On the other hand, (PO) sp. activity was 208.67, 346.67 and 444.67 (units/mg protein) in infected larvae fed on *M. alba* var. Rosa, *M. alba* var. Morittiana and *M. alba* var. Laevigata, respectively.

Lysozymes specific activity:

Data indicated that there were no significant differences between lysosomes sp. activity values in non-infected larvae fed on all tested mulberry varieties, they were 29.03, 27.57 and 28.87 (Unit /mg protein) in larvae fed on *M. alba* var. Rosa, *M. alba* var. Morittiana and *M. alba* var. Laevigata leaves, respectively. While lysosomes values in infected larvae fed on *M. alba* var. Rosa, *M. alba* var. Morittiana and

M. alba var. Laevigata, were 20.80, 27.33 and 33.700 (units/mg protein), respectively.

Specific activity of Peroxidases (POD):

Peroxidases activity had similar trend in non-infected and infected larvae which recorded the significant highest and lower values in larvae fed on *M. alba* var. Laevigata and *M. alba* var. Rosa leaves, respectively. In non-infected larvae (POD) sp. activity registered 43.33, 65.67 and 78.67 while sp. activity registered 42.00, 62.00 and 67.00 (units/mg protein) in haemolymph of infected larvae fed on *M. alba* var. Rosa, *M. alba* var. Morittiana and *M. alba* var. Laevigata leaves, respectively.

Nonspecific esterases:

Nonspecific esterases sp. activity values registered different trend in larvae fed on the different mulberry varieties, which were 2930.00, 3204.00 and 2297.00 (unit/mg protein) in non-infected while sp. activity were 3149.33, 4164.33 and 7141.00 (units/mg protein) in infected larvae fed on *M. alba* var. Rosa, *M. alba* var. Morittiana and *M. alba* var. Laevigata leaves, respectively. A significant increasing in esterases values were estimated in haemolymph of larvae fed on all mulberry varieties except in haemolymph of larvae fed on *M. alba* var. Rosa.

Glutathione S-transferase (GST):

GST sp. activity were 333.67, 389.00 and 462.67 (units /mg protein) and 500.33, 494.33 and 683.67 (units /mg protein) in non-infected and infected larvae fed on *M. alba* var. Rosa, *M. alba* var. Morittiana and *M. alba* var. Laevigata leaves, respectively.

Effect of bacterial contagious infection on enzymes activity in haemolymph of *Bombyx mori* larvae fed on different mulberry varieties:

Data in Table (2) and Figure (1) compare (PO) sp. activity in infected and non-infected larvae, (PO) activity was low in infected larvae fed on *M. alba* var. Morittiana and *M. alba* var. Rosa leaves, on the opposite, PO activity was significantly higher in infected than in non-infected larvae fed on *M. alba* var. Laevigata leaves.

The effect of bacterial contagious infection on lysosomes values show that infected larvae have lower enzyme sp. activity value than in non-infected larvae fed on *M. alba* var. Rosa, while no changes found in larvae fed on *M. alba* var. Morittiana leaves. On contrary, lysosomes activity was higher in infected larvae than in non-infected larvae fed on *M. alba* var. Laevigata leaves. Bacterial contagious infection has a reduction effect in peroxidases activity in larvae fed on all the three mulberry varieties. This reduction was significant only in larvae fed on *M. alba* var. Laevigata leaves.

There were no significant differences in GST levels between non-infected and infected larvae fed on all mulberry varieties, while GST values were the highest in haemolymph of infected larvae fed on *M. alba* var. Laevigata leaves.

Table (1): Mean of enzymes sp. activities of non-infected and infected *Bombyx mori* larvae fed on different varieties of mulberry leaves.

Mulberry varieties	Mean of enzymes specific activity									
	Phenoloxidase		Lysosomes		Peroxidase		Nonspecific Esterases		GST	
	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected
<i>Morus alba</i> var. Rosa	307.67 ± 15.70	208.67 ± 21.60	29.03 ± 1.12	20.80 ± 1.18	43.33 ± 5.86	42.00 ± 4.36	2930.00 ± 238.14	3149.33 ± 523.61	333.67 ± 57.83	500.33 ± 88.10
<i>Morus alba</i> var. Morittiana	384.00 ± 23.52	346.67 ± 25.58	27.57 ± 2.00	27.33 ± 1060	65.67 ± 7.77	62.00 ± 7.00	3204.00 ± 207.59	4164.33 ± 514.71	389.00 ± 60.67	494.33 ± 26.86
<i>Morus alba</i> var. Laevigata	340.0 ± 5.51	444.67 ± 47.25	28.87 ± 2.76	33.70 ± 4.50	78.67 ± 26.29	67.00 ± 4.58	2297.00 ± 316.07	7141.00 ± 281.26	462.67 ± 39.37	683.67 ± 51.19
Sig.**	.016	0.00	0.657	0.004	0.002	0.003	0.013	0.00	0.067	0.014

Mean ± standard deviation

ns Means no significant differences between treatments

**Means significance between the same column

Table (2): Effect of the Bacterial contagious infection on enzymes specific activity in haemolymph of *Bombyx mori* larvae fed on different mulberry varieties leaves

Mulberry varieties	Mean of enzymes specific activity				
	Phenoloxidase	Lysosomes	Peroxidase	Nonspecific Esterases	GST
<i>Morus alba</i> var. Rosa	0.003*	0.001*	0.768 ns	0.545 ns	0.052 *
<i>Morus alba</i> var. Morittiana	0.136 ns	0.882 ns	0.576 ns	0.040 ns	0.051 *
<i>Morus alba</i> var. Laevigata	0.028 *	0.038 *	0.048 *	0.000 *	0.004 *

* Means statistical significantly different ($P \leq 0.05$).

ns Means no significant differences between treatments

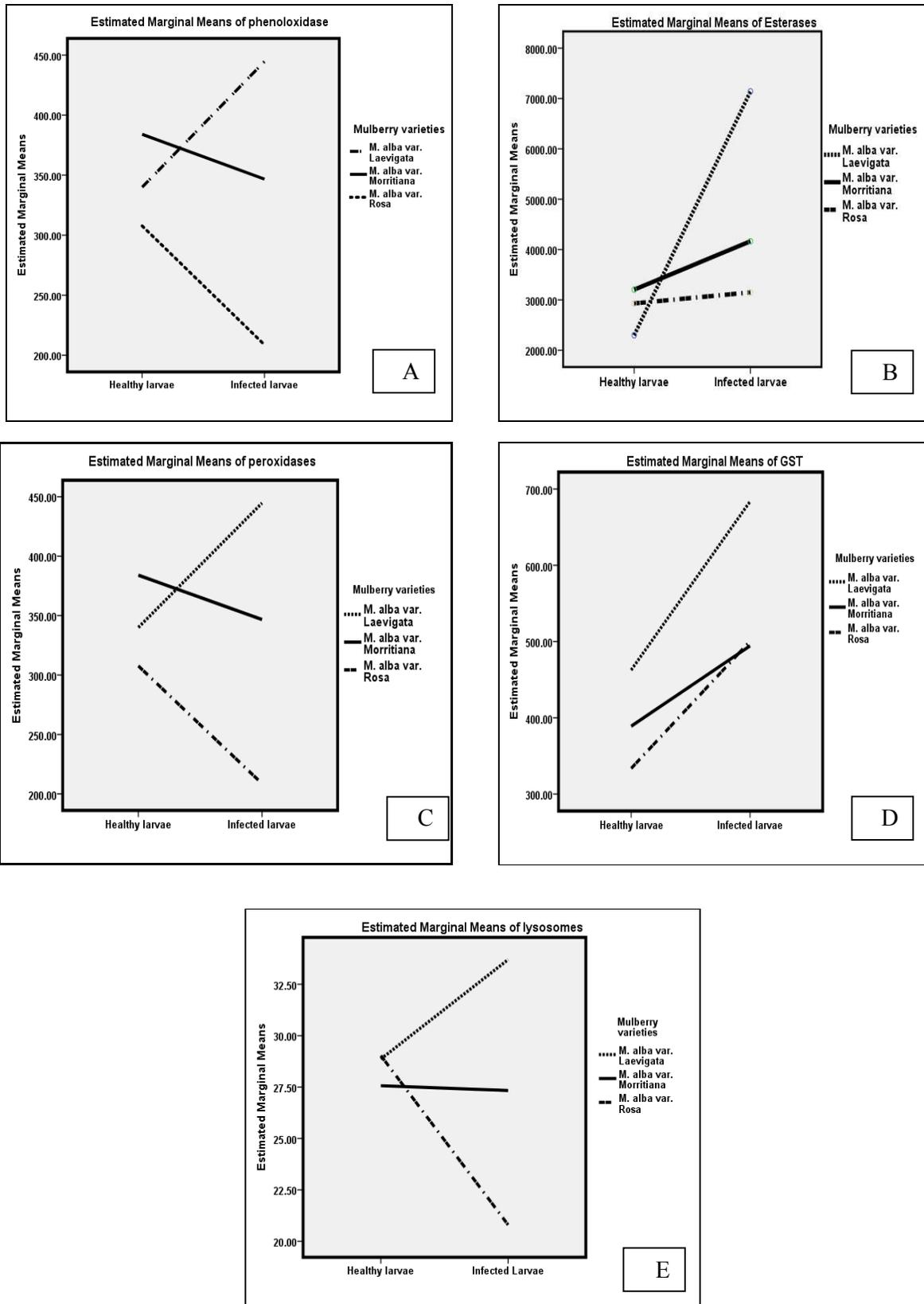


Figure (1): Mean of enzymes sp. activities of non-infected and infected *Bombyx mori* larvae fed on different varieties of mulberry leaves

The interaction effects between mulberry varieties and bacterial contagious infection on the studied enzymes activity in *Bombyx mori* larval haemolymph:

Table (3) show the significant effects of infection on GST and Esterases sp. activity (P= 0.000) while there were no significant effects of infection on other

observed enzymes sp. activity, (P>0.05). Mulberry varieties have significant effects on all the observed enzymes sp. activity, (P<0.05). While interaction between infection and mulberry varieties show that the both factors significantly affect on Esterases, lysosomes and phenoloxidase sp. activity (P<0.05).

Table (3): The interaction effects between mulberry varieties and bacterial contagious infection on the studied enzymes activity in *Bombyx mori* larval haemolymph

Source	GST	Esterases	Lysosomes	Peroxidases	Phenoloxidase
Infection	0.000 *	0.000*	0.322 ns	0.072 ns	0.446 ns
Mulberry	0.001*	0.000*	0.003*	0.000*	0.000*
Infection * mulberry	0.256 ns	0.000*	0.002*	0.325 ns	0.000*

ns: Means no significant differences between treatments

* Means statistical significantly different (P ≤ 0.05)

DISCUSSION

Since the host plants are important biotic factors with strong influences on the ecology and evolution of herbivores, and their specialist natural enemies such as pathogens and endoparasitoids, so that specialist herbivores insects may respond very differently to host plant defensive chemical composition and nutritive value, Lampert (2012). These varied responses may be apparent as coupling with important physiological functions linked to survival. Results concluded from our study on the significant variations of enzymes activity in *Bombyx mori* larval haemolymph after feeding on different mulberry varieties, adding to our observations about the bacterial contagious infection on these larvae, it is interesting to note that, host plant were shown to affect immunity of insects to natural bacterial contagious, susceptibility to the bacterial diseases and the expression of their immune defences. Similar results were found in many other insects (Emad *et al.*, 2016; Shikano *et al.*, 2010; Vogelweith *et al.*, 2015).

Present data cleared that phenoloxidase, and lysosomes enzymes decreased in haemolymph of infected larvae fed on *M. alba* var. Rosa and *M. alba* var. Morittiana while they increased in infected larvae fed on *M. alba* var. Laevigata, which mean that larvae fed on *M. alba* var. Laevigata leaves may have more diseases tolerance or immunity because of the high level of phenoloxidase which lead to high tolerance response against pathogenic bacteria (Reddy and Venkatappa, 2016). The lysosomes was the main antibacterial factor responsible for immunity of vaccinated insects thus upon pathogens infection, antibacterial proteins, lysozymes rapidly appear in the haemolymph after infection with bacteria (Reddy *et al.*, 2012)

Experiments with three lepidopteran larvae of *Galleria mellonella*, *Bombyx mori* and *Agrius convolvuli*, similarly, have showed increased lysozyme activity upon immune challenging by *Escherichia coli* (Yu *et al.*, 2002).

Peroxidase activity values significantly different between mulberry varieties, larvae fed on *M. alba* var. Laevigata leaves had the highest significant peroxidases value. Infection significantly reduced peroxidase activity values in haemolymph of larvae fed on all mulberry varieties leaves. Same results stated by Vengateswari *et al.* (2020) who found that (POX) activity in *Spodoptera litura* increased in 2–4 hours as a result of *Bacillus thuringiensis* infection of all treated groups, after that, there was decreased in all groups.

Nonspecific esterases and GSP sp. activity were increased in haemolymph of larvae fed on the three tested mulberry varieties after infection. In non-infected larvae, nonspecific esterases were the highest value when larvae fed on *M. alba* var. Morittiana while GSPs were the highest value in larvae fed on *M. alba* var. Laevigata. Similar results, GST activity increasing after infection, obtained by Vengateswari *et al.* (2020)

Esterases, degradation enzymes, may eliminate toxic molecules generated during microbial infection of the silkworm, *B. mori*. Therefore higher esterase activity was induced by the stimulation of bacterial infection (Shiotsuki and Kato, 1999). The Glutathione-S-transferases (GSTs) are play a central role in detoxification of endogenous and xenobiotic compounds also are involved in physiological processes such as protection against oxidative stress (Enayati *et al.*, 2005; Kostaropoulos *et al.*, 1996).

Kasmaeiand Mahesha (2012) found that the esterases may be used as marker molecules during the evolution of new breeds of silkworm *B. mori* with better economic characters. On the contrary, obtained present data declared that although nonspecific esterases in larvae fed on *M. alba* var. Laevigata was the lowest value in the non-infected larvae, they increased to be the highest value after infection which mean it is better to evaluate esterases in larvae challenging diseases which done by Somasundaram *et al.* (2013). They focused on the investigations of induced proteins in the haemolymph of silkworm

breeds challenged with pathogens and recommended to make them as biochemical markers for associating the immunity of the different silkworm races of the *B. mori*.

The interaction effects between mulberry varieties and infection on the different studied enzymes sp. activity indicated that mulberry varieties affect disease responses in larvae. Thus it is recommended to give an attention to host plant while evaluating infection response or breeding new tolerant or immune insect races or hybrids. This information could be very useful for incorporating biological control program with pathogens for insect pest and their host plant.

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الاختلافات في الاستجابة المناعية بين يرقات فراشة الحرير التوتية التي تتغذى على أصناف توت مختلفة

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إن الأمراض التي تصيب ديدان الحرير تؤدي إلى خسارة ما يقرب من ٣٠-٤٠% من محصول الشرائق سنوياً. إن دفاع دودة الحرير التوتية ضد الممرضات يمدها به الجهاز الدوري المفتوح في سائل الجسم (الهيموليمف) من خلال نوعي المناعة الخلوية والخلطية. تم تغذية ديدان الحرير التوتية على ثلاثة أصناف من التوت هي الصنف البلدي، الصنف الرومي والصنف العماني وذلك لدراسة تأثير العائل النباتي وكذلك حدوث الإصابة بالأمراض على المناعة. ذلك بتقدير نشاط عدد من الإنزيمات داخل هيموليمف يرقات العمر الخامس. وقد أشارت النتائج إلى أن العائل النباتي للحشرة وكذلك الإصابة بإحدى مسببات الأمراض أحدثت تأثير معنوي على قيم نشاط الإنزيمات التي تم تقديرها. كما أوضحت النتائج وجود تفاعل معنوي بين العائل وبين الإصابة يؤثر على قيمة نشاط الإنزيمات التي قدرت بهيموليمف الحشرات كما وجد أن صنف التوت العماني يكسب اليرقات نوعاً من المناعة المرتفعة نتيجة تغير نشاط الإنزيمات بهيموليمف الحشرات بعد التغذية عليه.