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ORIGINAL ARTICLE

Study of the Toxicity of Azadirachtinon Larval Mortalityand Fertility of Carob Moth's Female*Ectomyeloisceratoniae* (Lepidoptera, Pyralidae) Under Controlled Conditions

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M.S. Mehaoua, A. Hadjeb, M. Lagha, M.K. Bensalah and M.L.Ouakid: Study of the Toxicity of Azadirachtinon Larval Mortalityand Fertility of Carob Moth's Female*Ectomyeloisceratoniae* (Lepidoptera, Pyralidae) Under Controlled Conditions

ABSTRACT

Carob moth *Ectomyeloisceratoniae* Zeller is the most dangerous pests of dates in Algeria. This species is polyphagia, its wide distribution in space and on various hosts make chemical control the most using in palm grove without success. Under these conditions, only the biological control may be able to limit the damage of this pest and reduce the negative effect of chemicals on the environment and fauna life in palm grove. The study of azadirachtin effect on the mortality on the first larval stage revealed that the doses used were significantly and positively correlated with mortality corrected for different durations of exposure of larvae L1 to biopesticide (24 hours: $r^2 = 0.872$; 48 hours: $r^2 = 0.906$; 72 hours: $r^2 = 0.905$; 96 hours:$r^2 = 0.880$ and 120 hours: $r^2 = 0.779$). The higher LD50 (437.59 ppm) was recorded for a lethal time of 24 hours however the lowest LD50 (76.86 ppm) was observed for a lethal time of 120hours. We have also recorded a decrease in female's fertility issue from larvae's survived to the treatment withazadirachtin;with an average low lying eggs of54.67±19.41 induced by the doseof 384 ppmand low hatching rate of eggs with 17.51±2.94 % occasion by the dose of 192 ppm, compared at thecontrolwith 159.67 ± 35.04 eggs laid and hatching rate with 94.96±2.53 %. Also thedosesusedwere significantly andnegativelycorrelated with thenumber of laidand hatchedeggs($r^2 = 0.755$ and $r^2 = 0.712$ respectively).

Key words: Carob moth, biological control, azadirachtin, larva, LD50,fertility.

Introduction

Carob moth*Ectomyeloisceratoniae* Zeller is among the most dangerous pests of palm grove in Algeria. It affects both awaiting production and stored (Jarraya and Vinson, 1980 and Dhouibi, 1989). It can cause considerable damage that can reach 30% of the production of dates (Fatni, 2011). Eggs laid by this small butterfly turnintolarvae are entering into dates and depreciate considerably the quality and the commercial value of it (Jouveet*al*., 2006). The damage caused by infestation dates prevents any fresh consummation and conservation of every opportunity. The polyphagia of this specie, its wide distribution in space and on various hosts makes it difficult to give a correct andefficient chemical lute.

During these last years the palm date farmers turned massively to chemicals as a mean of struggle. These chemicals interventions used up to date have failed to bring an efficient protection for the production in reason to the biology and feeding behavior of the carob moth; larvae's feeds on and develop in the date, where they are protected (LebdiGrissa, 2010 and Peyrovi, 2011). In addition, the misuse of pesticides and ignorance of their danger by farmers have adverse effects on human health, animals and the environment causes the depletion and destruction of beneficial fauna (Oueld EL Hadj *et al.*, 2003 Ben Saad, 2009and Bisaadet*al*., 2011).Synthetic pesticides are highly toxic fornon-target fauna, birds, mammals, amphibians, they cause the concentration of residues in the food chain, pollution, development of resistance by pests after exposure of several generations (Musabyimana and Bélanger, 2005; Abdullah, 2009 and Richard, 2010).

The main shortcoming of these new synthetic molecules is not to be destroyed or degraded, such as natural organic and minerals substances by microorganisms (Escoubet, 2011).

It is therefore urgent to turn to other means of lute, and in particular to see what substances are available in the plant world that have a potential preventive and curative and are able to control insect population's pests and

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reduce the risk of environmental contamination caused by synthetic molecules and non-biodegradable (Philogene, 1991; Mossini and Kemmelmeier, 2005).

This work was carried out in order to study the toxicity of azadirachtin, biopesticides of plant origin, against the larvae of the carob moth *Ectomyeloisceratoniae* and its effect on female fertility and eggs under controlled conditions to reduce the harmful effects of its products on the environment and beneficial fauna useful in our palm groves.

Materials and Methods

Mass-Rearing of carob moth:

Our breeding was conducted with a strain of *Ectomyeloisceratoniae* dates from shady year (2011) the palm grove ofBiskra.We have put infested dates in the breeding cage in acontrolled room (temperature 27 ± 2 ° C and a relative humidity of 65 ± 10% and photoperiod of 16 hours light and 8 hours darkness) (Al-izziet*al*., 1987). With the emergence,the adults of *Ectomyeloisceratoniae* are captured using a test tube, then they are put inside the coupling jarswithout sexing.

After mating, female's will lay eggs inside the jars, they are discharged through a fine mesh tulle in the feeding location (flour dates) in plastic boxes. After some days, the eggs hatch and we get larvae's of first stage for biopesticide treatment. Other larvae will complete their larval development in feedinglocation until the last larval stage (L4 - L5) in which we can make difference between males and females.At this stage the larvae's males and females each are separately placed in a test tube with a piece of corrugated, closed with a cotton plug to permit the passage of larval to pupae stage, the test tubescontain larvae's of each sex are grouped and maintained by elastics and placed in plastic boxes (Dridiet*al*., 2001).

Study of the toxicity of azadirachtin on larvae:

In Petridish containing the feeding, we applied a treatment of five doses of the azadirachtin (24 ppm, 48 ppm, 96 ppm, 192 ppm and 384 ppm) with a control; the all with threerepetitions,then we've filed 20 larvae's offirst stage per box. The observations were made every 24 h for counting dead larvae.

Study of the effect of azadirachtin on female fertility and eggs:

Larvae that have escaped the toxic effect of biopesticide are placed in a breeding location to reach the adult stage. At adult emergence, we placed 30 couples issue from larvae's treated with azadirachtin (6 couples used for each dose) and 6 couples control; each couple in a Petri dish or counting eggs laid. Then there are eggs hatches after incubation.

Statistical Analysis:

In the case of azadirachtin measured variable corresponds to the rate of larval mortality. The mortality rate is corrected by Abbot's formula (1925) which allows knowing the real toxicity of the insecticide. Different rates of mortality undergo an angular transformation according to the tables established by Bliss (Fisher and Yates, 1975). The transformed data are subject ofanalysis variance (ANOVA) with a single classification criterion.The calculation of the least significant difference (LSD) allows the classification of different concentrations used.

In order to characterize the power of insecticidal molecules used, we determined the 50% lethal dose (LD 50). The rates corrected mortality obtained are transformed into probitwhichpermit to establish a regression line based on the logarithms decimal of the doses used. With the help of the curve wedetermine all doses remarkable mathematical processes according to Finney (1971). The method of Swaroop *et al*. (1966), allows the calculation of the confidence interval for the LD50.

Abbott'sformula: $\text{Corrected percentage of mortality} = 1 - \frac{n\text{in T after treatment}}{n\text{in C after treatment}}$ X100

Where n = number of larvae, T = treated, C = control.

Comparison of means is performed by parametric tests. The calculations were realized by the program XLSTAT.

Results:

Study of mortality of E. ceratoniaelarvae exposed to Azadirachtin:

The analysis of variance of the corrected mortality of larval of first stage after 24h, 48h, 72h, 96h and 120h of exposure to azadirachtinshows differences between very highly significant five doses used P <0.001, 0.001, 0.004, 0.006 and 0.000 respectively (Table 1).

Table 1: Corrected mortality ratesof first stage larvae of *E.ceratoniae* treated with the azadirachtin.

Exposure time	24ppm	48ppm	96ppm	192ppm	384ppm	d.f.	F	P
24 hours	21,67±2,89	30,00±8,66	26,67±2,89	43,33±7,64	50,00±5,00	4	11,700	0,001
48 hours	23,68±5,49	32,02±9,82	30,44±4,36	47,46±2,50	54,21±5,18	4	12,438	0,001
72 hours	31,05±10,57	37,81±6,76	37,89±1,82	53,42±5,44	55,26±4,56	4	7,644	0,004
96 hours	36,14±8,57	41,23±7,60	41,40±1,22	60,44±11,53	63,95±7,75	4	7,063	0,006
120 hours	35,07±7,60	41,81±8,34	43,75±4,38	75,29±8,78	68,73±8,89	4	14,547	0,000

Our results showed that the application of five doses of azadirachtin between 24 ppm and 384 ppm on the first stage larvae of the carob moth caused mortality which varies between 21.67 and 75.29% for different duration's exposure of larvae to the product.

The data summarized in Table 1 showed that the toxic effect of azadirachtin has increased more than the increase concentrations used. The results also indicated that the insecticide effect of the azadirachtin increases more than the duration of exposure of larvae to biopesticide increase.

The two highest concentrations of azadirachtin (192 ppm and 384 ppm) resulted in the most significant mortality of larvae *E. ceratoniae* 75.29 and 68.73% respectively in lethal time long enough (120 h). While the lowest dose used (24 ppm) induced in 24 h and 48 h the lowest mortality rate with 21.67% and 23.68% respectively (Table 1).

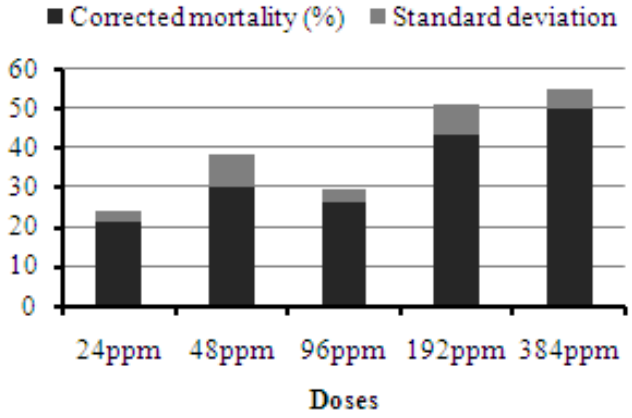

Fig. 1: Corrected mortality of larvae *Ectomyelois ceratoniae* after 24 hours of exposure to five doses of azadirachtin

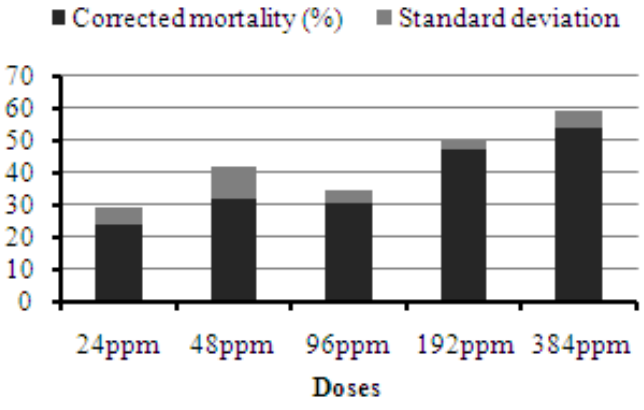

Fig. 2: Corrected mortality of larvae *Ectomyelois ceratoniae* after 48 hours of exposure to five doses of azadirachtin

Exposure of larvae's of carob moth during 24h and 48h to five doses of azadirachtin showed that the corrected mortality has reached or exceeded 50% only in larvae's treated with the dose of 384 ppm (Fig. 1 and 2).

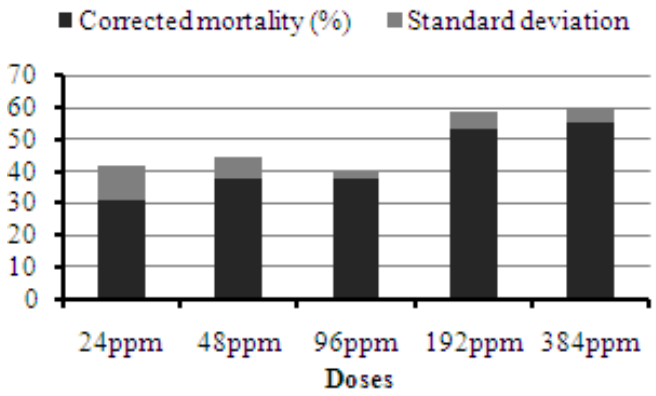

Fig. 3: Corrected mortality of larvae *Ectomyelois ceratoniae* after 72 hours of exposure to five doses of azadirachtin

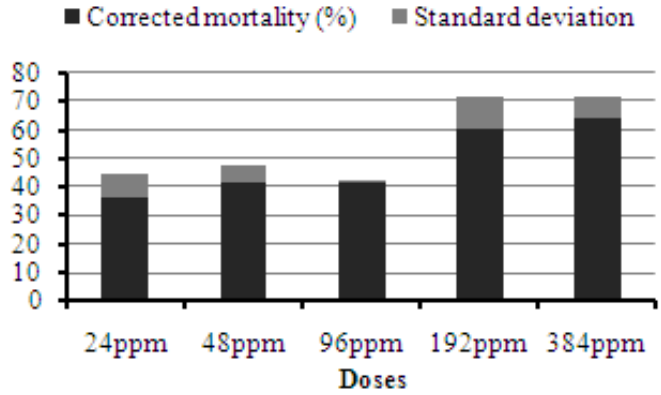

Fig. 4: Corrected mortality of larvae *Ectomyelois ceratoniae* after 96 hours of exposure to five doses of azadirachtin

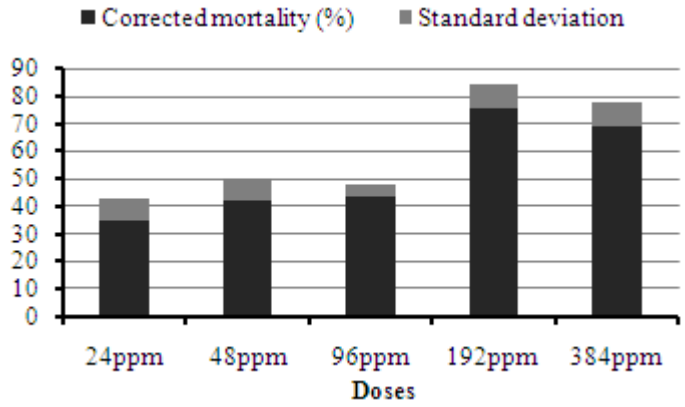

Fig. 5: Corrected mortality of larvae *Ectomyelois ceratoniae* after 120 hours of exposure to five doses of azadirachtin

By against larvae's exposed to azadirachtinduring 72 h, 96 h and 120 h showed a mortality does not reach 50% for doses of 24 ppm, 48 ppm and 96 ppm, whereas it exceeded 75% and 68% respectively for dosages of 192 ppm and 384 ppm (Fig. 3, 4 and 5).

The toxicological study of azadirachtinshows that the LD50 and LD90 the highest (437.59 ppm and 44652.24 ppm respectively) were recorded for an exposure time of 24 h with $r^2 = 0.872$ and a regression line $y = 0.638 x + 3.315$ with a Slope of 36.21, while the LD50 and LD90 the lowest (76.86 ppm and 2320.54 ppm respectively) were obtained for a duration of exposure of 120 hours with $r^2 = 0.779$, a regression line $y = 0.866 x + 3.367$ and the Slope is 14.07(Table 2).

Table 2: Toxicological parameters of azadirachtin after 24h, 48h, 72h, 96h and 120h of Exposure

Exposure time	Regressionequation	r²	DL16	DL50	DL84	DL90	Slope
24 hours	y = 0,638x + 3,315	0,872	12,09	437,59	15843,21	44652,24	36,21
48 hours	y = 0,678x + 3,326	0,906	10,05	294,46	8626,62	22871,36	29,30
72 hours	y = 0,547x + 3,736	0,905	3,11	204,54	13455,03	45055,49	65,78
96 hours	y = 0,628x + 3,720	0,880	2,85	109,20	4186,15	11994,47	38,34
120 hours	y = 0,866x + 3,367	0,779	5,46	76,86	1081,59	2320,54	14,07

The results shown in Table 2, show that the LD50 of azadirachtin calculated for a long time of death (120h) is about 6 times lower than that recorded for an exposure time of 24 h. Thus, larvae of *E. ceratoniae* exposed to doses of 24 ppm, 48 ppm, 96 ppm, 192 ppm, 384 ppm of azadirachtinpresents LD50 which are negatively correlated to different lethal time (24h, 48h, 72h, 96h and 120h).

Study of female fertility and eggs of E. ceratoniae:

The statisticanalysis (ANOVA) of the average number of eggs laid per female and the average number of eggs hatched from *E. ceratoniae*issue from lots handled by five doses (24 ppm, 48 ppm, 96 ppm, 192 ppm, 384 ppm) showed very highly significant difference with P <0.0001 (Table 3).

Table 3: Average number of eggs laid per female and percentage of hatched eggs

	Control	Doses					d.f.	F	P
		24 ppm	48 ppm	96 ppm	192 ppm	384 ppm			
Average number of eggs laid per female	159,67±28 ,27	117,67±35,04	107,33±27,48	95,5±53,87	66,67±19,22	54,67±19,4	5	7,96	0,0001
Average rate of hatched eggs (%)	94,96±2,53	73,37±6,84	51,75±7,98	60,94±5,91	17,51±2,94	19,28±2,34	5	202,53	0,0001

Figure 6 and Table 3 show that the highest number of eggs was recorded at the females control (without treatment), followed by females issue from treated larvae's with doses of 24 ppm, 48 ppm, 96 ppm and 192 ppm, by against the number of the lowest eggs is laid for females from larvae's treated with the dose of 384 ppm.

For the rate average hatching, we note that it is higher in eggs from control females with 94.96% and low in eggs of females issuefrom larvae treated with doses of 192 ppm and 384 ppm with 17.51% and 19.28% respectively(Table 3 and Fig. 7).

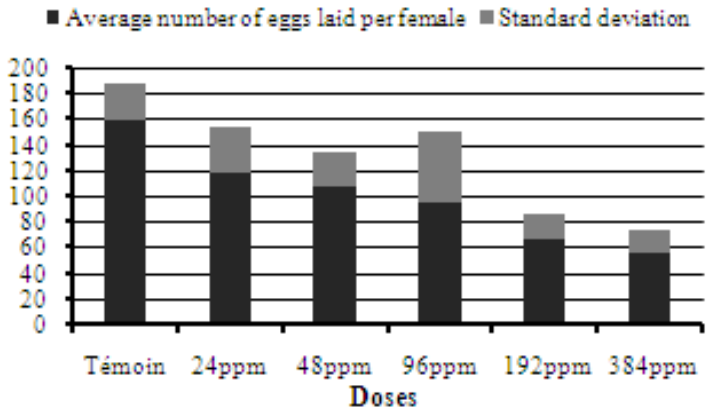

Fig. 6: Average fertility of females *Ectomyelois ceratoniae* after exposure to five doses of azadirachtin

Our results show that azadirachtin strongly reduced female and eggs fertility; we also note that the number of eggs laid by females and the number of hatched eggs are negatively correlated to the different doses used (r^2 = 0.755 and r² = 0,712 respectively).

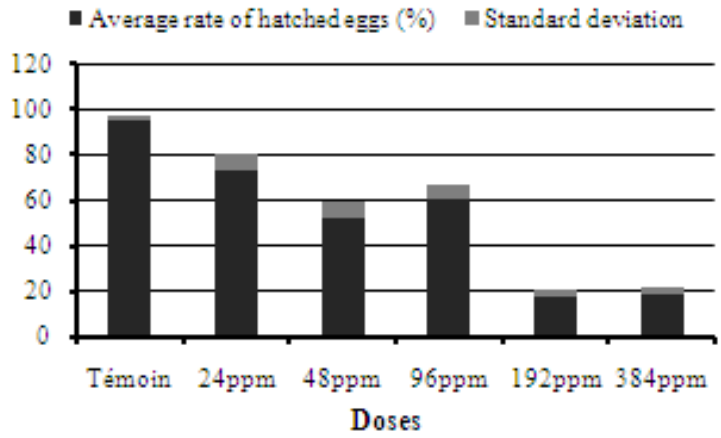

Fig. 7: Average fertility of eggs *Ectomyelois ceratoniae* after exposure to five doses of azadirachtin

Discussion:

The results showed that the applied concentrations of azadirachtin to various on first instar larval of *Ectomyeloisceratoniae* inhibits the development and growth of larvae which causes their death (Mordue and Blackwell, 1993). From the observations recorded we note that mortality rates are positively correlated to the different doses used, regardless of the duration of exposure of larvae to azadirachtin. Thus, our results confirm the work of Rharrabeand*al*. (2008), which precise that the treatment of larval *Plodiainterpunctella*Hübner by azadirachtin, shows a positive correlation between dose and observed mortality rate (7% to 2 ppm and 4 ppm to 10%) for a lethal time of 96h. The azadirachtin exerts an effect dose-mortality, with a significant increase in larval mortality when the dose increases (Chougourou *et al*., 2012).

Our bioassay shows that the percentage of larvae killed by azadirachtin 120 h after treatment was significantly higher than that killed in 24 hours, reflecting a delayed toxicity of the tested product. The azadirachtin is toxic to the larvae of *E. ceratoniae*, but it seems that it requires a long period of biopesticide exposure to kill the larvae of this Lepidoptera. Thus, Martinez and van Emden (2001), reported that azadirachtin caused a significant increase in mortality of *Spodopteralittoralis*, which intensified during insect development. It induces a high response time (Chougourou *et al*., 2012).

However, bio-insecticide had a dose-mortality with an immediate anti-appetite leading to weight loss in larvae of *Anoplophoraglabripennis* and *Plectroderascalator* (Poland *et al*., 2006). The same authors reported that prolonged larvae's mortality was probably due to a combination of toxic effects and starvation accumulated during diet. Thangavelu and Singh (1998) have also observed that in the fly resulting Azadirachtin*Uzi blepharipa* delay and inhibition of the formation of the puparium, weight loss, the death of the pupa, the suppression of adult emergence and malformation of adult structures. So the azadirachtin repels insects, inhibits their diet,affects their hormonal balance and preventing their maturation (Sharma *et al*, 2004).

Rapid mortality observed in 24hours larvae's treated with different doses of azadirachtin (between 24 ppm and 384ppm), is may be due at the effect contact of larvae'swith the biopesticide. Thus, Mordue and Blackwell (1993), precise that the azadirachtinreact only by contact, ingestion effect is a phenomenon that is not necessarily because the insect mortality.

Larval mortality by different doses used is positively correlated with the duration of exposure of carob moth to the product. This is probably the result of the combined effect produced by contact and ingestion. Despite, prolonged exposure (120 hours) of larvae's to azadirachtin; the doses of 24 ppm, 48 ppm and 96 ppm could not inducea mortality of 50% compared to the dose of 192 ppm and 384 ppm.

Lethal doses calculated for 50% mortality (LD50) in different time's lethal, show that the azadirachtin is less toxic to the larvae of the *E. ceratoniae* for a short exposure time (24 hours), but it becomes more and more toxic than exposure to biopesticide larvae's is protracted. Our results are similar to those obtained by Martinez-Tomas and*al*. (2009), which proved that the toxicity of the azadirachtin is positively correlated with the duration of exposure of larvae's of *Culexquinquefasciatusau*to the product with an LD50 of 498, 316 and 262 ppm calculated during 48h, 72h and 96h after treatment respectively. Our results showed that this compound led to a dose-time-mortality since the LD50 value decreases in function of time (Tine and*al*, 2011).

It seems that the azadirachtin is more toxic to the carob moth compared to adult aphids (LD50: 3782 ppm) for a lethal time of 48 hours, but with 96 hours of exposure, azadirachtin manifests a high toxicity among individuals of the same adult aphids (LD50: 30.37 ppm) and is much higher in aphid nymphs (41.91 and 3.99 ppm in 48 to 96 hours post-treatment) compared to larvae's*E. ceratoniae* (Tang and*al*., 2001). Also Khalequzzaman and Nahar (2008) have shown that the azadirachtinas a natural insecticide of plant origin are more toxic than conventional insecticides used against different aphid species with LC50 0.41 µg/cm^2 for

both*Myzuspersicae* and *Lipaphiserysimi*. As well, Alouanie*tal*. (2009) have observed high toxicity of azadirachtin on larvae and pupae of *Culexpipiens* with an LC50 of 0.35 and 0.42 mg / L respectively after 24 h exposure.

Insects of different orders have different behavior toazadirachtin. Lepidoptera are extremely sensitive to azadirachtin, this sensitivity is expressed by a strong inhibition of food, with effective doses causing 50% inhibition of feeding (ED50) of <1-50 ppm, depending on the species, whereas 100% inhibition of feeding in the Coleoptera, Hemiptera and Homopteraare achieved at doses of 100-600 ppm revealing so, theirweak response to theazadirachtin(Mordue and Nisbet, 2000). Poland and*al*. (2006) reported that two species *Anoplophoraglabripennis* and *Plectroderascalator* (Coleoptera: Cerambycidae) show different sensitivity in regard to azadirachtin with LC50 23.55 and 1.58 ppm respectively.

Our study showed that regardless of dose, the azadirachtin causes a decrease fertility of female's and eggs 65.76% and 81.56% respectively, compared to the control. Our results are similar to those obtained by Manal and Frantisek (2000),which indicate that the treatment of *Spodopteralittoralis* by azadirachtindose of 100 ppm induced a lying of 24 ± 8 eggs with a hatching rate of 0% whereas the dose of 10 ppm female fertility is 483 ± 16 eggs with a hatching rate of 85.9%. The same authors reported that insects treated with azadirachtin, which have escaped to the lethal effects, emerged adults often showed a reduced fertility. Therefore, the reduction of reproduction affected by azadirachtin is correlated with changes in protein metabolism in the female pupa (Huang *et al*., 2004). The azadirachtin blocks hormonal secretion and stops the morphogenetic development by altering cycle moult and blockage of the reproductive cycle of the insect (Mouffoket*al*., 2008). Also, Tine and*al*. (2011) have proved that treatment with azadirachtin on newly hatched females *Blattaorientalis* significantly reduces the number of oocytes. Schmutterer (1995), cited by Djenontinet*al*. (2012), indicates that Neem oil contains many active substances causing feeding inhibition, growth, ovipositionand having insecticidal activity. However, the work of Martinez-Villaret*al*. (2005) have revealed that the treatment of *Tetranychusurticae* with doses of 64 and 128 ppm of azadirachtin, affected the fertility and mortality, but had no effect on fertility and offspring development.

The Neem based products cause repulsion, anti-oviposition, sterility, reduced fertility, loss of flying ability, disruption of sexual communication and reduced intestinal motility (Mossini and Kemmelmeier, 2005). The same effects were described by Petit (2008), including stopping or slowing the development of eggs and larvae; blocking metamorphosis of larvae and nymphs disruption of pheromonal communication at the time of breeding adults; adults sterilization; poisoning; inhibition of feeding process and chewing; inhibition of chitin synthesis. It was also shown that azadirachtin may cause an effect on the reproductive processes of both sexes male and female (Mordue and Nisbet, 2000).

Conclusion:

For this work we have determined that azadirachtin has a larvicidal activity against the *Ectomyeloisceratoniae*. The observed mortality is positively correlated with the dose and duration of exposure of larvae's to azadirachtin. The calculated LD50 is positively correlated with the duration of exposure of larvae'sbiopesticide; it is low in a longer time-lethal and high for a short time-lethal. This proves that azadirachtin induced in time, a high toxicity to the larvae's of the carob moth.

We have also found that the treatment with azadirachtin on the larvae of the first stage significantly reduces fertility females and eggs even at very low doses. So the azadirachtinreact on the development and growth of carob moth.

The obtained results show that azadirachtin is promising as larvicidal against the *Ectomyeloisceratoniae*; it might be a good alternative to chemical pesticides, while preserving human health and the environment.

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