Efficiency of Peppermint Oil Fumigant on Controlling *Callosobruchus maculatus* F. Infesting Cowpea Seeds

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Abstract: Fumigation tests were carried out to evaluate the efficiency of peppermint oil on controlling *C. maculatus* by studying its effect on survival of developmental stages, mating and oviposition behavior. Also, the effect of peppermint oil on the antennal segments and their sensilla was studied by using scanning electron microscope. The obtained data reveal the susceptibility of all stages of *C. maculatus* to fumigation with peppermint oil. The egg stage was the most susceptible stage. Mating frequency, fecundity and survivorship of the next generation progenies were significantly decreased by oil treatment. The effects of peppermint oil were always greater on treated male pairs than on treated female pairs. Fumigation of early pupae with 5 µl peppermint oil resulted in malformation and disorientation in the antennae and their associated sensilla in the emerged adults.

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1. Introduction

Cowpea (*Vigna unguiculata* (L.) Walp. is one of the most nutritious grain legumes (**Ehlers and Hall, 1997**) where it is valuable as a source of dietary protein as well as vitamins and minerals (**Singh *et al*., 2003**). Loss of seed yield in legumes crops during storage due to bruchid beetles is a very serious problem for farmers and traders (**Ress, 2004**). Cowpea weevil, *Callosobruchus maculatus* is one of the most destructive bruchid species to several legumes including cowpea. Their larvae being internal feeders are hard to control with insecticides. It was also not advisable to mix insecticides with food grains. Fumigation was being the most effective method for controlling stored grains insects. Many researches conducted managing cowpea weevil by fumigation with various essential oils (**Shaaya *et al*., 1997; Ketia *et al*., 2001; Braga *et al*., 2007 and Manzoomi *et al*., 2010**). Oil of *Mintha piperita* L. (peppermint oil), a widely used essential oil was evaluated for its insecticidal activity against several stored grain insects. **Klingauf *et al*. (1983)** concluded that the exposure to 6µl/liter, through fumigation with essential oil of *M. piperita* for 3 hours, has led to 100% mortality in *Sitotroga cerealella* and 50% in adults of *Acanthoscelides obtectus*. Also fumigation with essential oil of *M. piperita* in a concentration of 15 µ l/liter for a 3 hours-period, has caused mortality above 75% in adults of *Tribolium castaneum, Rhyzopertha dominica*, *Oryzaephilus surinamensis* and *Sitophilus oryzae* (**Shaaya *et al*.,1991**).

It has become evident that the antenna is a major channel of sensory input, including receptors for volatile odors and pheromones, contact chemoreceptors, sound perception and touch (**Ehmer and Gronenberg, 1997 and Renthal *et al*., 2003**). Moreover, antennal sensilla can play an important role in the insect pest control. **Leal (2005**) reported that the odorant substances including sex pheromones and host plant volatiles diffuse through the wall of the pore of antennal sensilla into the sensillar lymph and transferred to olfactory receptors on the dendrites of olfactory sensillae and odorant binding proteins. Volatile oil can disrupt communication in mating behavior of cowpea weevil by blocking the function of antennal sensilla and unsuccessful mating could lead to a lower fecundity and ultimately lower the population of insect pest (**Ahmed *et al*., 2001**).

The objective of this study is to evaluate the efficiency of peppermint oil on controlling *C. maculatus* by studying its effect on the survival of different developmental stages, and on mating and oviposition behavior of *C. maculatus* reared under laboratory conditions. The Scanning Electron Microscopy was also used to detect the effect of peppermint oil on the morphology of adults' antennae and their associated sensilla when early pupae were treated.

2. Material and Methods

Insect culture:

Cowpea weevil, *Callosobruchus maculatus* F. was reared for several generations on cowpea seeds, *Vigna unguiculata* L. under fluctuating relative humidity of 70± 5% and temperature of 27± 3ºC. All the experiments were carried out under these conditions in the Department of Entomology, Faculty of Science, Ain Shams University.

Volatile Oil:

Peppermint oil was purchased as pure oil (Branded in Egypt) from Katue Aromatic Company. The oil was extracted by steam distillation from the leaves of aromatic plant *Mintha piperita* L.

Effect of peppermint oil on *C. maculatus* developmental stages:

The efficacy of peppermint oil in controlling *C. maculatus* was tested by fumigation on different developmental stages. One- day old adults were placed on clean cowpea seeds for egg laying. After few hours, the adults were removed from the seeds and the number of eggs was standardized to one egg /seed. Insects were treated when they were 0-1 day old (eggs), 13-14 days old (third larval instars), 18-19 days old (early pupae), and 20-22 days old (late pupae).

All fumigation tests were carried out in small glass vials (6 ml long, 2.5 ml in diameter). Amounts of peppermint oil (1, 3 and 5µl) were spread on 2 cm diameter filter paper discs. Seeds containing each insect stage were put in the vials which then were covered with nylon mesh clothes. Treated filter paper discs were put on the nylon clothes under the caps of the vials which then were screwed tightly onto the vials. So, the oil vapors saturated the atmosphere of the vials around the seeds containing the insect stage. Untreated filter paper discs were used in the controls. Five replicates of each treatment and control were set up. Ten seeds were used for each replicate of the egg, larval and pupal tests. The vials were kept under laboratory conditions till adult emergence and the mortality of eggs, larvae and pupae were calculated. Any morphological changes resulted from oil treatment during the development of different stages were observed and photographed.

Effect of peppermint oil on mating and oviposition behavior of *C. maculatus*:

To ensure the virginity of each adult, few days prior to the emergence, each seed containing single developing insect was placed in an individual 1.5 ml eppendroff tube. The emerged unmated males and females were differentiated according to criteria given by **South gate (1958)**. The unmated males and females were exposed separately to 5µl peppermint oil for different exposure periods (100, 150 and 200 minutes) by fumigation test as mentioned above. The effect of peppermint oil on mating behavior was tested at different combinations (T♂ X T♀, T♂ X N ♀, N♂ X T♀ and N♂ X N ♀). The mating was observed in 10 pairs of adults for each combination and 5 minutes observation of mating behavior was carried out for each pair and the numbers of mating pairs were counted. To study the oviposition behavior of the treated females, fifty fresh cowpea seeds were put in vials containing each combination pair and covered with fine nylon cloth then left for oviposition and adult emergence. Five replicated pairs were made for each combination and the number of eggs deposited by each female was counted, also the emerged adults were counted daily and the emergence rate was estimated for each combination.

Preliminary tests were carried out after which the amount of oil used and the testing times were chosen.

The relation between data was examined by analysis of variance (ANOVA).

Examination with the Scanning Electron Microscopy (SEM):

Fresh specimens of males and females from a colony maintained in the laboratory (untreated) and adult males and females resulted from treatment of early pupae with 5 µl peppermint oil were used. They were dried in the chamber of the scanning electron microscope, SEM (Jeol – JSM – 5600 LV in SEM Unit, Egypt) in the low vacuum mode, and then the micrographs were taken. Identification of antennal sensilla was carried out according to **Hu *et al*. (2009)**.

3. Results and Discussion

Effect of peppermint oil on developmental stages of *C. maculatus*:

Results of the present study reveal the susceptibility of all stages of *C. maculatus* to fumigation with peppermint oil. Adult emergence decreased significantly as peppermint oil amount increased (Table 1). Statistical analysis showed that the numbers of adults that emerged from treated eggs, larvae and pupae (early and late) were significantly lower than the control (Fig. 1). The most susceptible stage of *C. maculatus* was the youngest stage, 0-1 day old eggs. After exposure of this stage to 1µl oil, adult emergence decreased by 37.66% from control, and complete inhibition (100% reduction) was recorded after exposure to 3 and 5µl oil (Table 1). The ovicidal effect of peppermint oil may be due to infiltration of oil particles under the egg cover that may block respiration or disrupt the water balance of eggs and developing embryo (**Credland, 1992**). The effect of oil on the instars developing inside the seeds showed that at 5µl oil, percentage adult emergence was 20% compared with 84% in the control (Table 1). When early pupae were treated with 5µl oil, only 4% developed inside the seeds till adult emergence proving to be highly susceptible compared to 24% of the late pupae which were more tolerant. These results agree well with **Arti and Sujoita (2009)** who stated that some of the phytochemical substances act as general toxicant which generally kills the different life stages of the insect or interfere with growth. The results obtained are also in confirmation with the works of (**Eman and Abass, 2010**) who reported that the essential oils proved to be the most effective in reducing the population of *C. maculatus*. **Mbata *et al*. (2000)** reported that higher oxygen uptake was found in early pharate adults of *C. subinnotatus*, so that saturation of the atmosphere surrounding infested cowpea seeds with peppermint oil fumigant may cause suffocation and inhibition of various biosynthesis processes of the insect.

Evaluation of the morphological abnormalities in *C. maculatus* after fumigation of the developmental stages with 5µl peppermint oil:

In the present study treated eggs were observed to be dry while treated cowpea seed with larvae inside showed pores with various sizes on the surface of the seeds (Figs. 3 b, c and d). The adults that were formed from treated early and late pupae with 5 µl peppermint oil also showed various abnormalities like malformation on the morphology of the antennal segments, absence of melanization of the antennae or incomplete melanization with dark parts on the antennal segments, incomplete development and collapse of legs with attached exuvia and enlarged abdomen (Fig. 3f, g and h). Similar results were recorded by **Aly *et al.* (2010).** They found that extracts of the wild plant, *Fagonia bruguieri* caused adult deformities of *Schistocerca gregaria*. **Lee *et al*. (2002)** concluded that the insecticidal constituents of many plant extracts and essential oils are mainly monoterpenoides. Monoterpenoides are typically volatile and rather lipophilic compounds that can penetrate into insects rapidly and interfere with their physiological functions.

Peppermint oil is one of the monoterpenes that can be toxic via penetration of the insect cuticle or the respiratory system (**Prates *et al*., 1998**). Application of peppermint oil probably disrupted the delicate balance of timing and concentrations of hormones in the intact insect, resulting in the formation of abnormal forms. Hence, peppermint oil shows effective Insect Growth Regulators (IGR) activity and exhibits great promise in suppression of population of insects. **Mesbah *et al.* (2006)** reported that all the efficiently tested essential and/or volatile oils acted principally as Insect Growth Inhibitors (IGIs) rather than antifeedants causing disruption of the insect development, abnormal larvae, pupae and adults that were lead finally to death. Also peppermint oil may interfere in ecdysis and resulted in formation of malformed individuals. Peppermint oil may cause inactivation or inhibition of the tyrosinase which result in incomplete cuticle hardening and darkening. The aromatic compounds act as inhibitors of tyrosinase, interact with the binuclear copper active site of enzyme, so disrupt the tertiary structure of the enzyme **(Kubo, 1997)**. Tyrosinase is the key enzyme involved in the biosynthesis of melanin. It catalyzes the rate limiting step, the oxidation of the aromatic amino acid tyrosine to 3, 4 dihydroxy phenyl alanine (DOPA) and subsequently to DOPA –quinone which converted by multi step reactions to melanine pigments (**Kramer and Hopkins, 1987**).

Effect of peppermint oil on mating and oviposition behavior of *C. maculatus*:

Results presented in Figure (2) and statistical analysis (Anova) reveal that oil treatment had a significant effect on the mating of males and females *C. maculatus.* In the control pair, more than 90% of cowpea weevils were mated within five minutes, while the insects in all treated combinations (T♂ X T♀, T♂ X N ♀ and N♂ X T♀) completely failed to mate during the five minutes after all the exposure time of treatments (100, 150 and 200 minutes). **Ahmed *et al*. (2001)** came to the same conclusion after treatment of *C. chinensis* with neem oil. They reported that mating success of unmated pairs depend on the chemical activity of females (production of sex pheromones) and the physical behavior activity of males (response to sex pheromones). In the present study, adult males' failure to perform normal mating may be attributed to the binding of odor molecules of peppermint oil to the olfactory receptors on the antennae. The vapor of the oil may chemically hamper the pheromone produced by the females and cause confusion to males; therefore, they may fail to recognize the pheromones. **Leal (2005)** reported that the odorant substances including sex pheromones and host plant volatiles diffuse through the wall of the pore of antennal sensilla into the sensillar lymph and transferred to olfactory receptors on the dendrites of olfactory proteins and odorant binding proteins.

Also fumigation of unmated females with peppermint oil disrupt communication in mating behavior, the vapor of the oil may prevent the female from perception of sex pheromones, so this disrupt the intraspecific communication between males and females. This is in accordance with **Chapman (1972)**.

In case of oviposition behavior, results in table (2) and statistical analysis (Anova) reveal that females in treated groups (T♂ X T♀, T♂ X N ♀ and N♂ X T♀) deposited a significantly lower number of eggs than females in untreated groups (N♂ X N ♀). The present findings get support from the earlier works of **Kamakshi *et al*. (2000)** who reported a significant reduction in the number of eggs laid by *C.*

maculatus when treated with *Mentha arvensis* and *Ocimum sanctum* as compared with control. **Raja *et al.* (2001)** also concluded that egg laying by *C. maculatus* was significantly influenced by treatments with volatile oils. Similar results were reported by **Kétoh *et al*. (2000)** after treatment of *C. maculatus* with essential oils. This reduction of egg laying could be attributed to the early death of adults of *C. maculatus* due to the effect of the oil vapors. Similarly, **Schmidt *et al*. (1991)** and **Mazibur and Gerhard (1999)** showed that the effect of essential oils of *Acorus calamus* on *Callosobruchus phaseoli* could involve ovarian changes similar to those caused by the chemosterilants by blocking females egg laying. This assumption was also put into view by **Aboua *et al*. (2010)** who studied the effect of three aromatic plants, *Ageratum conyzoides* L., *Citrus aurantifolia* and *Melaleuca quinquenervia* L. on *C. maculatus.*

Results also reveal that fecundity was always significantly lower in males treated pairs than in females treated pairs (Table 2). This tendency was also found in the adult emergence rate. These results suggest that an insufficient number of sperms were transferred to females, this may be due to short copulation period, or the oil may have some spermicidal effect on males so; treated males may produce lower number of sperms (**Ahmed *et al*., 2001**).

Data in table (2) show that, generally, the effect of oil on number of eggs laid and emergence of adults increased with increasing exposure time.

General description of antennae of *C. maculatus* :

According to **Hu *et al*. (2009)** both female and male has serrated flagellar antennae. The antennae consist of scape (Sc), pedicle (Pe) and nine flagellomeres. All flagellomeres, except for the first one, have acute angled wedge-shaped ventral extensions.

As shown in Figs. (4a and 5a, b and 6a), there are one type of Bohm bristles (BB), two types of sensilla trichoid (ST1,ST2), one type of sensilla chaetica (SC), two types of sensilla basiconica (SB1,SB2) and one type of grooved pegs (GP).

Bohm bristles (BB)

Each sensillum is a triangular peg-like structure inserted into wide sockets. The BB sensilla occur on the base of the scape and pedicel, at the joints between the scape and the head and between the scape and the pedicle.

The location of the BB on the scape and pedicel only suggests that these might be mechanoreceptors (**Schneider, 1964; Zacharuk, 1985**). Concentration of Bohm bristles at the intersegmental joints between the scape and the head as well as between the scape and the pedicel, in many insects, indicates that these sensilla probably perceive the antennal position and movements (**Merivee *et al*., 2002**). Absence of these sensilla or reduction in its number resulted in disorientation of the antennae (Fig. 4b).

Sensilla trichoid 1 (ST1)

The ST1 sensilla are sharp-tipped hairs with strong longitudinal grooves and are nearly straight or slightly curved toward the antennal shaft (Figs. 5a, b and 6a). The ST1 is the most abundant sensilla type on the whole antenna; it might indicate that they have an olfactory function (**Keil, 1999).**

Sensilla trichoid 2 (ST2)

The ST2 sensilla are blunt-tipped straight hairs with cuticle wall (Figs. 5a and b). With the increasing of antennal antennomere, the number of sinsilla is also increasing. They probably function as sex pheromone receptors (**Merivee *et al*., 1999**).

Sensilla chaetica (SC)

The SC occurs on each antennomere of the antennae. This type of sensilla is characterized by grooved surface and straight hairs with blunt tip. They are inserted into a wide socket (Figs. 4a, 5a and 6a). The (SC) sensilla are believed to have a dual function of mechanoreception and contact chemoreception (**Jourdan *et al*., 1995**).

Sensilla basiconica (SB1)

The SB1 are characterized by smooth cuticle and a straight blunt tip (Figs. 5a, b and 6a). They are distributed on the flagellomeres of the antennae, with the exception of the first and the second segments (**Hu, *et al*., 2009**). Most of the SB1 are located on the lateral side of the apex of the above flagellomeres.

Sensilla basiconica (SB2)

The SB2 are characterized by a blunt tip which curved at the distal end. They are inserted into wide sockets (Fig.5a). (SB1) and (SB2) sensilla may have a sex-pheromone receptor role and olfactory function.

Grooved pegs (GP)

This type of sensilla is characterized by grooved surface and straight pegs with blunt tip (Figs.5a and 6a). GP are bulb- like structures projecting from a depression in the center of raised area of cuticle. Grooved pegs are situated on the posterior sides of the dorsal extensions of the flagellomeres and near the distal margins, often close together. They can also be found on the front side of the terminal flagellomeres. The probable function of these sensilla is chemo or thermoreception (**Zacharuk, 1985**).

In the present study, treatment of early pupae with 5 μl peppermint oil caused a reduction in the number of BB bristle in the emerged adults, malformation and disorientation in the direction of antennal sensilla, fusion of sensilla trichodea (ST1, ST2).

Sticky malformed mouth parts with attached pupal skin, enlarged membranous joint and malformed swollen between the antennal segments were also observed after treatment (Figs. 4b, 5c and 6b).

The present results reveal that peppermint oil caused abnormalities in the shape of antennal sensilla especially trichoid sensilla which are specific for the female sex pheromones. The results obtained are in confirmation with the works of **Reda *et al.* (2010)** who reported that peppermint oil caused malformation and disorientation in the antennal structures and their associated sensilla in the museum pests. Similarly, **Soryia, *et al.* (2009)** observed morphogenic defects on antennae due to treatments of adults of *C. maculatus* with lufenuron.

The undesirable effects of peppermint oil on the structure of antennae and their associated sensilla may cause failure of treated males to mate with females and ultimately lower the population of insect pest.

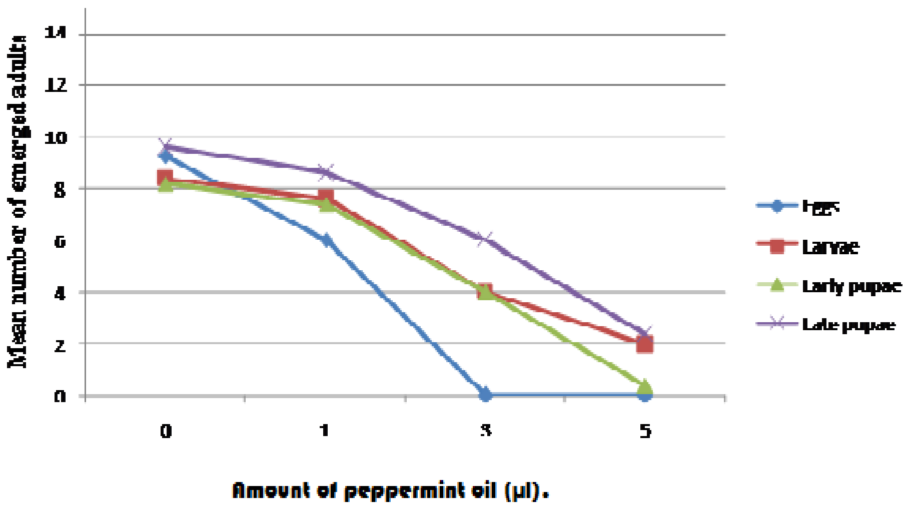

Fig. 1: Susceptibility of different developmental stages of *C. maculatus* to peppermint oil fumigation (estimated from the number of emerged adults).

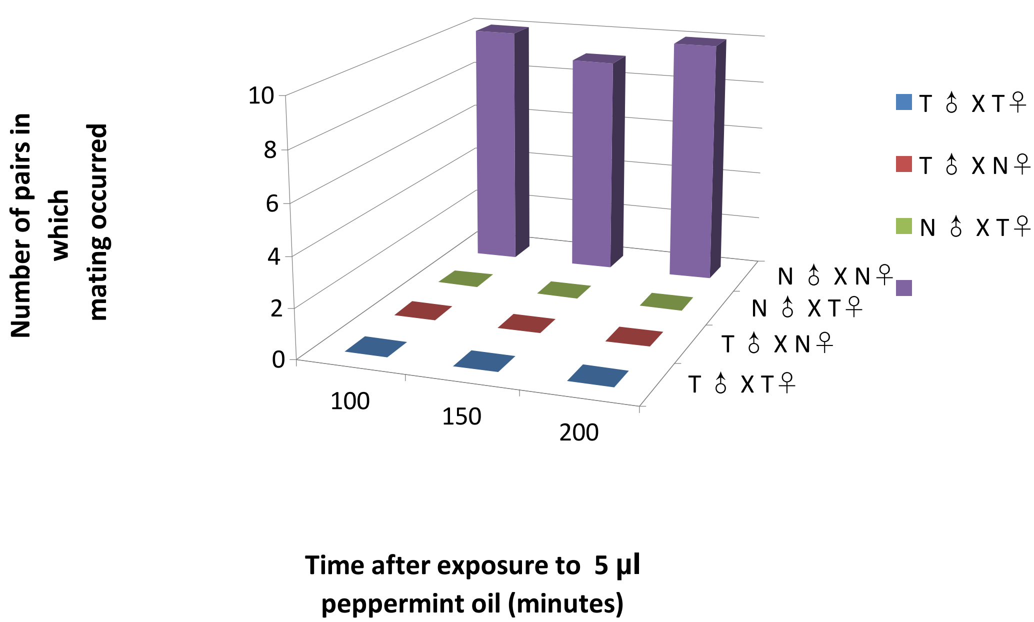

Fig. 2: Effect of peppermint oil on mating behavior for different combinations of pairs (observations within five minutes) after different exposure times (T: treated insects; N: untreated insects).

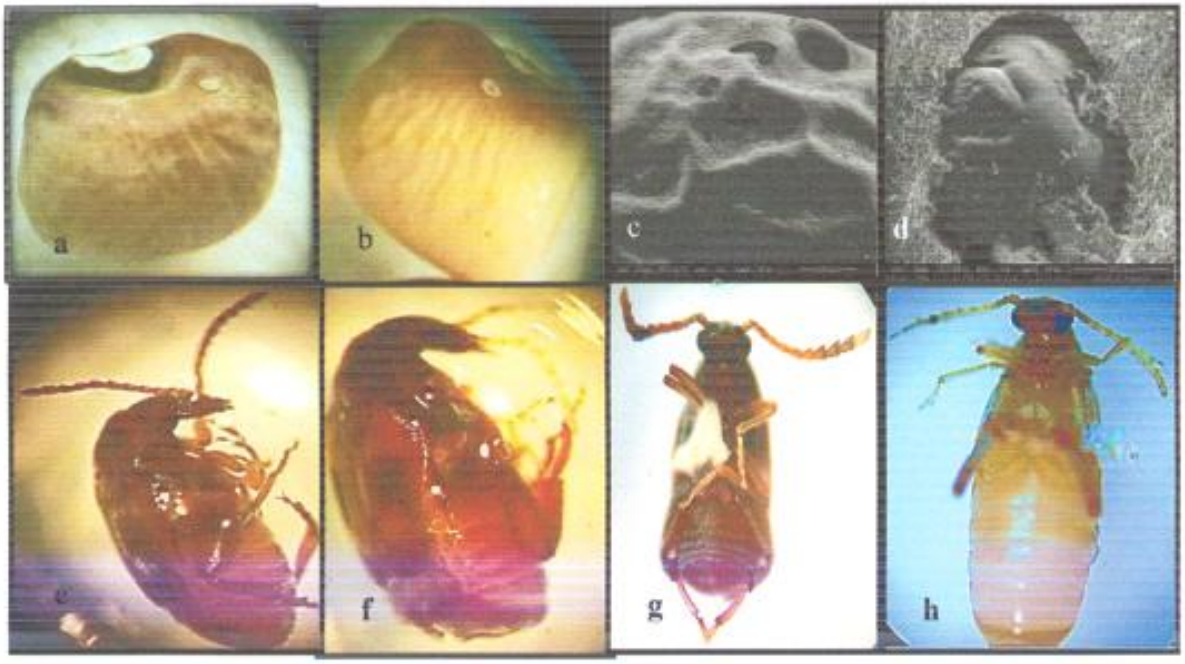

Fig. (3): Morphological changes induced on developmental stages of *C. maculatus* due to fumigation with peppermint oil.

(a): Photograph of normal untreated egg on cowpea seed (30x).
(b): Photograph of treated egg (30x).
(c): Scanning electron micrograph of treated cowpea seed with larvae inside showing pores on the surface of the seed. Bar 500 µm.
(d): Scanning electron micrograph showing treated larva making pores on the surface of a cowpea seed. Bar 300 µm.
(e): Photograph of normal adult *C. maculatus* showing normal structure of antennae and legs (30x).
(f): Photograph of adult *C. maculatus* resulted from treatment of early pupa with 5µl peppermint oil, showing disoriented unmelanized antennae and collapse of legs (30x).
(g): Photograph of adult *C. maculatus* resulted from treatment of late pupa with 5µl peppermint oil, showing different degrees of melanization of antennae, collapse of legs and attachment of exuvia to the legs (30x).
(h): Photograph of malformed *C. maculatus* resulted from treatment of early pupa with 5µl peppermint oil, showing some black color in antennae, collapse of legs with attached exuvia and enlarged abdomen (30x).

Table (1): Effect of fumigation with different amounts of peppermint oil on survival of developmental stages of *C. maculatus*.

Amount of oil (µl)	Developmental stages							
	Eggs		Larvae		Early pupae		Late pupae	
	% Adult emergence	*% Reduction	% Adult emergence	*% Reduction	% Adult emergence	*% Reduction	% Adult emergence	*% Reduction
0	96.25	-	84.00	-	82.00	-	96	-
1	60.00	37.66	76.00	9.52	74.00	9.76	86	10.42
3	0.00	100.00	40.00	52.38	40.00	51.22	60	37.50
5	0.00	100.00	20.00	76.19	4.00	95.12	24	75.00

***Reduction of adult emergence from control (0 µl)**

Table (2): Effect of fumigation with peppermint oil (5 µl) on fecundity and adult emergence of *C. maculates* after different exposure times.

Exposure time (minutes)	Crossing pairs	Mean no. of eggs ±S.D	% Reduction	Mean no. of emerged adults ±S.D	% Reduction
100	N♂ XN ♀	61.6±5.85		51.2±3.56	
	T♂X T♀	30.0±29.06	51.29	0.0±0.0	100.0
	T♂XN ♀	43.0±30.72	30.19	2.6±5.81	94.92
	N♂ X T♀	53.75±12.72	12.74	4.2±6.9	91.79
150	N♂ XN ♀	61.6±5.85		51.2±3.56	
	T♂X T♀	5.4±10.9	91.23	0.0±0.0	100.0
	T♂XN ♀	11.2±12.47	81.81	0.6±1.34	98.83
	N♂ X T♀	19.6±18.22	68.18	3.6±5.68	92.97
200	N♂ XN ♀	61.6±5.85		51.2±3.56	
	T♂X T♀	2.2±3.89	96.42	0.0±0.0	100.0
	T♂XN ♀	2.0±2.91	96.75	1.4±1.51	97.27
	N♂ X T♀	3.0±5.19	95.12	1.0±2.23	98.05

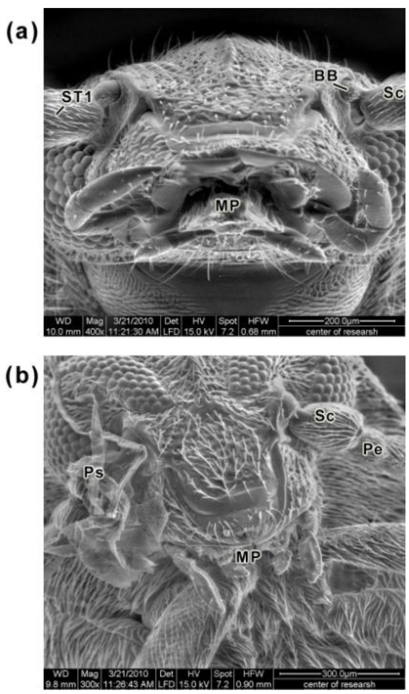

Fig.4(a):Scanning electron micrograph of frontal view of head of normal *C. maculatus* showing Bohm Bristles (BB) on joint between head and scape (Sc) Sensilla Trichoid (ST1) and normal structure of mouth parts (MP). Bar 200 µm.
(b) Scanning electron micrograph of frontal view of head capsule of adult resulted from treatment of early pupae with 5µl peppermint oil, showing absence of Bohm bristles (BB) on the base of the scape, malformation in the direction of antennal sensilla, fusion of sensilla and sticky malformed mouth parts (MP) with attached pupal skin (Ps). Bar 300 µm.

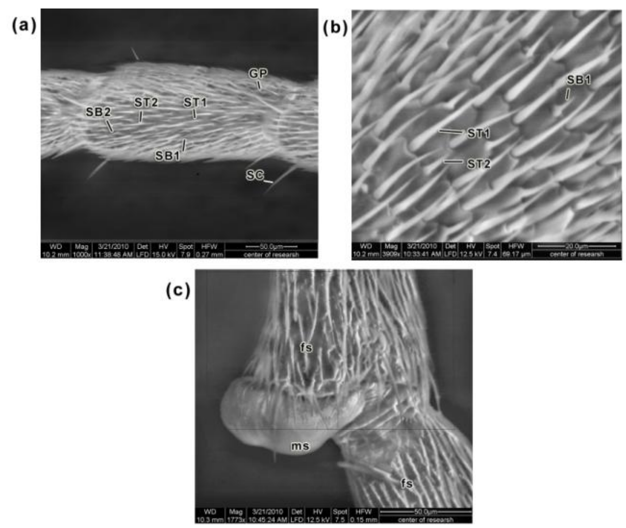

Fig. 5 (a): Scanning electron micrograph of dorsal view of antennal sensilla in FL_6 of normal *C. maculatus* showing Sensilla chaetica (SC), Sensilla trichoid1 (ST1), Sensilla trichoid2 (ST2), Sensilla basiconica1 (SB1), Sensilla basiconica2 (SB2) and grooved pegs (GP). Bar 50 µm.
(b): Scanning electron micrograph of dorsal view of antennal sensilla in FL_6 of normal *C. maculatus* showing sensilla trichoid1 (ST1), Sensilla trichoid2 (ST2) and Sensilla basiconica1 (SB1). Bar 20 µm.
(c): Scanning electron micrograph of joint between Fl_5 and fl_6 of treated *C. maculatus* showing malformed swollen (ms) and fused Sensilla (fs). Bar 50 µm.

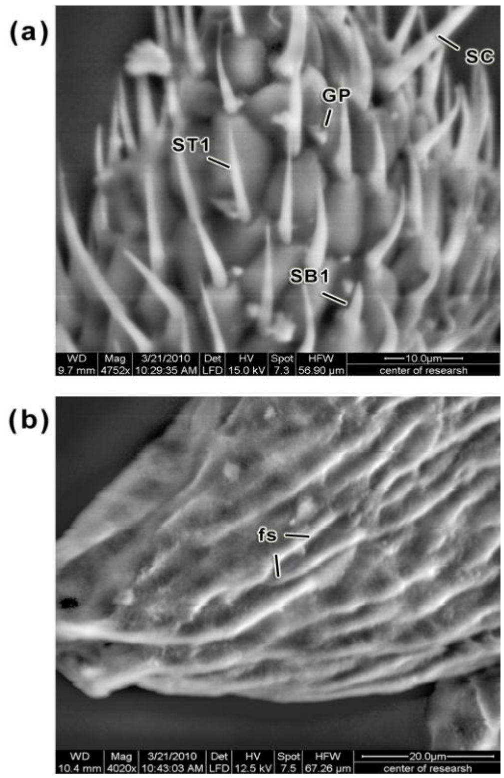

Fig. 6: Scanning electron micrograph of terminal antennal segment 9th of :
(a): untreated adult *C. maculatus* showing Sensilla chaetica (SC), Sensilla trichoid1 (ST1), Sensilla basiconica1 (SB1) and grooved pegs (GP). Bar 10 µm.
(b): adult resulting from treatment of early pupa with 5 µl peppermint oil showing fused sensillae (fs) Bar 20 µm.

This study shows that the peppermint volatile oil has an insecticidal activity; it may be toxic by penetrating the insect body via the respiratory system. The use of essential oils extracted from plants will have purely to be advised for the safeguarding of the environment and the health of the user. Essential oils could be used as biodegradable and natural bio protector for controlling stored product pests.

Results obtained in this study suggest an effect of the oil used on melanization of antennae. Further study on the effect of volatile oils on the amino acids involved is recommended.

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