OLFACTORY RESPONSES OF *CHAETOPTELIUS VESTITUS* (COLEOPTERA: CURCULIONIDAE) TO HOSTS AND CONSPECIFIC ODORS

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ABSTRACT: The twig borer, *Chaetoptelius vestitus* (Coleoptera: Curculionidae) is one of the most important pests of pistachio trees, *Pistacia vera* L. (Anacardiacae). Laboratory studies were undertaken to identify host plant semiochemicals that can be exploited and utilized to insect control. Pistachio tree is the natural host of this bark beetle suggesting that volatile compounds from odor of different parts of the plant may be attractive to this insect. The main goal of this research was to determine if host or conspecific odors can act as attractants to pistachio bark beetle. The olfactory responses of the pistachio bark beetle were tested with a Y-tube olfactometer. The odors included green and cut and / or dried buds, fresh or dried twigs, infested twigs and pistachio bark beetle and their feces. Stimuli were tested singly and in combinations. Using still-air bioassays, *C. vestitus* males proved to be the most attracted to fresh buds compared to cut and / or dried pistachio twigs suggesting the presence of host kairomones. The infested twigs were also found attractive for beetles in dual-choice tests. Males and females bark beetle feces attracted both male and female, indicating the existence of aggregation pheromones or allelochemicals (undigested plant compounds) in the feces. Further studies should be made to conclude the identities of these substances. Identification and synthesis of the semiochemicals that attract the pistachio bark beetle would allow developing new strategies for monitoring and controlling this pest.

KEY WORDS: *Pistacia vera* L., pistachio bark beetle, behavior, host plant volatiles, olfactometer, attractant

The pistachio tree (*Pistacia vera* L., Anacardiacae) is a fruit tree commonly grows in many countries of the world (Chebouti-Meziou et al., 2006). One of the principal limiting factors for pistachio fruit production is the damage caused by insect pests such as the pistachio bark beetle, *Chaetoptelius vestitus* Mulsant & Rey, 1960 (Coleoptera: Curculionidae) (Rizk et al., 1981a,b,c). This insect is one of the serious monophagous pest limiting productivity of pistachio plantations. In recent years, severe incidence of pistachio bark beetle has been reported from different parts of Tunisia and it is becoming very serious in southern Tunisia particularly in Gafsa. The adult *C. vestitus* cause severe damage to the plant. According to these authors, the species has a feeding phase and a reproduction phase. The feeding period begins in March where adults feed upon tree buds and boring galleries along the twigs just beneath the buds. The species always attacks healthy trees for feeding during the spring, summer and autumn destroying either fruits or vegetative buds. The beetles stay inside the tunnel until early October then they begin to abandon their feeding location seeking oviposition sites. Females choose to oviposit in pruned pistachio branches or twigs, the damaged trunks or unhealthy branches or trees, inside or outside the pistachio orchard to reproduce. The larvae tunnel the larvae move perpendicularly to the maternal gallery, undergo five instars, and after pupation the newly emerging adults exit the branches towards the buds and young twigs for their feeding phase. Larval lesions can also cause the branches to dry out and consequently to reduce production. This pest can damage up to 80% of the branches in untreated orchards. The reproduction period covers the whole year except the summer period owing to the rapid desiccation of branches and twigs. (Braham & Jardak, 2012).Currently, *C vestitus* in Tunisia is managed mainly by contact insecticides (Fenitrothion 50% EC: organophosphate family) and by the removal and incineration of infested branches (Braham & Jardak, 2012). Unfortunately, insecticides have non-target effects and can be harmful to human health and the environment. In fact, it can contaminate soil, water, and other vegetation. In addition to killing insects or weeds, insecticides can be toxic to a host of other organisms including birds, fish, beneficial insects, and non-target plants (Steinemann, 2004; Aktar et al., 2009). Moreover, these chemicals are too expensive and may not be effective against the pest because all its development is subcortical. In addition, the application of insecticides on infested plants and orchards must be carried out at the same time to prevent further infestation of neighboring farms where measures to phytosanitation control have not been taken into consideration. The aim of the IPM is to combine all the available methods or tools in order to minimize the usage of chemical insecticides and thus to maintain the ecosystem free from pollutants (Howse, 1996). Semiochemicals baited-traps have been used successfully in pest control of many insect. Curculionids are usually controlled or monitored using traps baited with host-plant allelochemicals, pheromones ((Bernays & Chapman, 1994) or combinations of pheromones and allelochemicals (Vanderbilt et al., 1998; Bruce et al., 2005). Traps baited with semiochemicals used to monitor and control populations of *C. vestitus* in pistachio orchards would be of great importance to improve pest management practices for this insect pest. The behavioral approach identifies the reactions of an insect exposed to an olfactory stimulus. This approach is based on the use of an olfactometer which is a device allowing the study of the behavior of an organism (insect) put in the presence of varied stimuli, odors for example. Different olfactometric devices have been designed for the study of behavior: olfactometers in Y, binary or four-way choice, or even a flight tunnel (Barth, 1970; Descoins, 2009).

However, no previous works have been conducted about chemical communication of this bark beetle. Finding semiochemicals involved in insect-plant interactions requires studies of behavioral responses to olfactory stimuli. The purpose of this study was to elucidate laboratory data on response of the bark beetle to insect and host-plant volatiles.

MATERIAL AND METHODS

Insects. *Chaetoptelius vestitus* adults emerged under laboratory conditions from naturally infested pistachio material collected between the end of October and December 2015 in Elguettar, (Gafsa governorate, Tunisia) were used. Virgin bark beetle were obtained at mid-February 2016 from the laboratory rearing carried out in a room maintained at 28 ± 2 °C, 70 ± 5% relative humidity and photoperiod of 16:8h (light/dark). Newly emerged adults were sexed in the laboratory (Ghrissi et al., 2018) and were maintained individually in Circular plastic boxes (4 cm in diameter and 2.5 cm high) with pieces of young twigs and pistachio buds as food. Before testing, the weevils were deprived of food for 24 hours in a room free from other odors.

Odor Source. Plant material and odor stimuli used in this study are listed in Table 1.The plant materials used as olfactory stimuli were collected the day before each test in an orchard in Elguettar Gafsa, and transported in insulated boxes to the laboratory. All plant organs from pistachio trees were healthy, without sign of damage or diseases. To reduce deterioration, plant material was maintained in a refrigerator at 4 ± 2 °C and 80 ± 5% relative humidity, until 30 minutes before the tests were initiated.

The beetles used as odor sources were collected in the field on the day before the bioassays and were maintained individually in plastic boxes without feeding under the conditions described above. The feces of the weevils were collected during 48 hours on filter paper (4 cm in diameter) placed on the bottom of the plastic box.

Extracts of the different parts of the host plant used as food substrates (twigs of the year, buds) were made using two types of solvents of the same polarity: pentane and hexane (apolar solvent). These solvents make it possible to extract predominantly the apolar compounds. Fifty grams of each substrate are cut into small pieces and immersed in 100 ml of solvent. The extract is stirred constantly for 60 minutes, and then filtered on glass wool in order to remove all the impurities.

Experimental Protocol. A dual-choice olfactometer was devised for use in these experiments, which is similar to a model used in another study made with bark beetles (Ruiz-Montiel et al., 2003). The olfactometer consisted of a Y-tube. Each arm of the Y-tube was 15 cm long with 1.5 cm internal diameter. Odors in the olfactometer were driven by a compressor, with an airflow rate of 0.1L/min. The air was purified with an activated charcoal filter and a one-liter flask that contained 500 ml of distilled water. The air was bubbled through the distilled water to clean and humidify the airstream. The extremities of the arms of the olfactometer were connected to two-liter glass containers that held the olfactory stimulant or clean air (control).The olfactory tests took place in an obscure and silent room maintained at 28 ± 2 °C, 70 ± 5% relative humidity lit by a red lamp with low power in order to observe the behavior of the individuals. All exogenous factors that can alter the results have been eliminated (other odors, light, noise, ...).The tests were performed from 9:00 to 14:00 h. Each day, 10 adults of each sex per treatment were tested. The tests were continued in successive days until 50 individuals per treatment were tested. Beetles were tested individually and used only once. Only virgin adults were used in the tests. After the odor sources were placed in the glass chambers, the air compressor was turned on and the first insect placed in the main tube of the olfactometer after the air flow had stabilized at 0.1 L/min. The choice made by each beetle and the time to reach the extremity of one of the branch tubes were recorded. Based on the results of a similar experiment made with another beetle species, the maximum duration of each test was established as five minutes (Ruiz- Montiel et al., 2003). Fresh and dried buds and twigs were collected each day for testing the following day.

At the beginning of each test, the food or extract to be tested (the extract is deposited by a micro-syringe on a small filter paper 2 x 1 cm folded in a roof) is randomly placed at one or the other of the ends of the secondary branches. The other secondary branch is traversed only by a stream of pure air. The female to be tested is then placed at the end of the main branch. We note the number of females that go up the main branch and the choice made. Using a stopwatch, we note the "response time" which corresponds to the moment of introduction of the female until it reaches the intersection of the two secondary branches, it goes up in one branches; The choice of the insect and the time it takes to reach the odor source is also noted (course time). Each test is repeated 20 times.

Tests occurred during the same phenological stage of pistachio plants (flower bud to initial flowering stages). All of the stimuli were tested daily with beetles of both sexes, in random order. The order in which the insects of each sex were tested each day was randomized. A preliminary test was made of the apparatus with 50 beetles (without odor stimuli) to determine if the olfactometer or the room had some effect, no choice tendency was found (data not shown). After each test the position of odor sources in the olfactometer was switched and the Y-tube was replaced by a clean one.

Data analysis. The behavioral responses and differences of velocity of response of *C. vestitus* to plant odors and conspecifics volatiles were analyzed by ANOVA followed by Duncan's significant difference test ($\alpha < 0.05$). We also compared the time spent by females to respond to different stimuli.

A chi-squared test (Vaillant & Derridj, 1992) was used to analyze if there was a significant difference in the number of females and males who respond to each stimulus and to the control, assuming a null hypothesis of no preference. Insects that did not respond to odor sources (stimulus or control) were not included in the statistical analysis.

RESULTS

The olfactory response of *C. vestitus* shows that both sexes significantly preferred the volatiles emitted by plant odors and conspecific beetles when compared with fresh air. The preference was changed according to the stimulus and according to its state as is presented in table 2. Dried twigs and all stimuli together were not attractive for the Curculionids. Between plant stimuli, green buds significantly attracted male weevils (Table 2). On the other hand, females were not attracted by green buds ($P = 0.547$). In the tests made with pistachio bark beetle odors, both sexes were significantly attracted to feces odors, but not toward volatiles from conspecific (Table 1). In the same way, both sexes were more attracted to volatiles released by Feces of females than to females alone. However, neither sex showed any preference for volatiles from Males + Females or for Males alone. Males were not more attracted to volatiles from feces of males than to volatiles from feces of females. In contrast, females preferred the volatiles from feces of females over volatiles from feces of males (Table 3).

When we analyzed the time elapsed to respond to different stimuli in the olfactometer tests *C. vestitus* females responded significantly faster than males (148.84 ± 3.41 seconds and 158.43 ± 3.81 seconds, respectively, $F = 364.66$; df=1; $P < 0.0001$, Fig. 1).

When we analyzed the time elapsed to respond to different stimuli in the olfactometer tests *C. vestitus* females responded significantly faster than males (88.0 ± 2.35 seconds and 97.7 ± 2.68 seconds, respectively, $F = 7.35$; df =1; $P < 0.035$, Table 2). The velocity of the female response also varied significantly with odor source ($F = 3.28$; df =10; $P < 0.001$, Table 2) showing a shorter response time to feces odors.

The olfactometer tests (Fig. 3) show that extracts of green buds, fresh twigs and all stimuli together with pentane and hexane attract all the *c. vestitus* females. Extracts with pentane and hexane caused a significantly different attraction in *C. vestitus* females. The response time of females (Fig. 1A), is faster when attracted by the extract of the fresh buds and twigs and both together and longer when testing the hexane extracts of these same stimuli. On the other hand, females take almost ten times as much time to raise the olfactometer in the presence of extracts of the fresh buds with hexane than when using the pentane extracts. In the same context, the response (response time) of females to extracts of fresh twigs and the two stimuli together is faster with respect to odors of the pentane extract. Also, the rise towards the source of odor (Course time) (Fig. 1B) is faster in the presence of the pentane extracts and longer compared to the extracts of the hexane. The hexane extract of all stimuli together is the least attractive to females.

DISCUSSION

Behavioral analysis identify the influence of one or more odorous molecules on the insect studied, and demonstrate the attractive or repulsive effect of a compound on an insect in order to know what are the nutrients and especially their odors that attract the individuals of this species. Semiochemicals or insect behavior modifying chemicals, which include pheromones, have been proved to provide better and selective pest control or management in the protection of plant and forests (Welter et al., 2005). The use of aggregation pheromones, which attract both male and female insects in association with high volatiles, has led to development of mass trapping as a control strategy for several weevil species (Weissling et al., 1994).

The olfactometer tests show clearly the attractive role of different odorous sources on both sexes of *Chaetoptelius vestitus*. However, when tested volatiles of females and males with their conspecifics, there was no significant response. Because odors from feces attracted males and females, we suggest that an aggregation rather than a sex pheromone is present in pistachio bark beetle feces. An alternative explanation to this attraction may be allelochemicals (undigested plant compounds presents in beetle feces). Certain insects can acquire host plant compounds and use them as attractants (Landolt & Phillips, 1997). Thus, semiochemicals in feces could give a reliable indication of the availability of host plants with suitable fruits for ovipositing females and availability of females for males searching mates. Insect pests are generally attracted to their hosts by specific parts of the plant. With respect to this result, there are reports of Curculionids that are attracted to specific organs of their host plants (Schlyter, 1986). Pistachio bark beetle males are probably attracted to the flower bud odors because this part of the plant is usually consumed. In field, when we collected beetles for the experiments we frequently saw males feeding on flower buds. It is also probable that volatiles that become more conspicuous in this part of the plant in the flowering beginning are also used by pistachio bark beetle males as a kairomonal cue to find the plant.

The difference in response time of females to feces odors may be explained by the hypothesis of Vanderbilt et al. (1998) who suggest a faster response to aggregation pheromone in order to increase reproductive success. So, the pheromone should both promote insect aggregation and maintain them competitive. Along this line, the approximation of *C. vestitus* beetles promoted by feces odors would be accompanied by increasing movement and consequently increased probability of finding sexual partners.

In the recent past, the use of pheromones as one of the eco-friendly tactics in the pest management program has assumed a greater dimension as a novel technique in monitoring and mass trapping insect pests (Peacock, 1973). The results help in chemical identification and planning the synthesis of pheromonal chemicals which could be used as trapping agents for the integrated pest management of pistachio bark beetle, *C. vestitus*.

In conclusion, we showed the first evidence of odors in green buds of pistachio plants and in weevil feces that attract males and either males or females of *C. vestitus*, respectively The attractiveness to both weevil sexes indicates that semiochemicals in the feces may be a prevalent component of chemical communication between pistachio bark beetle. The pistachio beetle odor of feces and green buds could be used as bait to capture adult *C. vestitus* in monitoring or control traps in the context of developing new pest management programs. Further studies should be performed to isolate and to characterize these substances and to determine if this attractants would be efficient enough to compete with natural sources of odors from the host and from the conspecific.

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Table 1. Plant material and odor sources.

Odor souce	Stimuli	
Plant Odors	Green buds	10g
	Infested buds	10g
	Dried buds	10g
	Fresh twig	10g
	Infested twigs	10g
	Dried twig	10g
	All stimuli	10g
Insect Odors	Males	20 Males
	Females	20 Females
	M+F	10 Males+10 Females
	Feces of Females	from 20 individuals for 48 hours
	Feces of Males	from 20 individuals for 48 hours

Table 2. Behavioral responses of both sexes of *Chaetoptelius vestitus* to volatiles from conspecifics and Pistacia in a Y-tube olfactometer.

Treatment (source 1 vs. source 2)	Sex	Response to source 1 vs. source 2	NR	*P* value
Green buds vs. clean air	Male	40 vs. 10	4	<0.05
	Female	26 vs. 24	12	0.547
Dried buds vs. clean air	Male	31 vs. 19	11	<0.0001
	Female	30 vs. 20	8	<0.01
Infested buds vs. clean air	Male	40 vs. 10	3	<0.0001
	Female	40 vs. 10	6	<0.0001
Fresh twigs vs. clean air	Male	32 vs. 18	10	<0.05
	Female	33 vs. 17	9	<0.001
Dried twigs vs. clean air	Male	22 vs. 28	15	0.114
	Female	21 vs. 29	11	0.523
Infested twigs vs. clean air	Male	29 vs. 21	11	0.685
	Female	28 vs. 22	8	0.839
Male vs. clean air	Male	25 vs. 25	9	0.344
	Female	22 vs. 28	7	0.114
Female vs. clean air	Male	24 vs. 26	8	0.547
	Female	25 vs. 25	9	0.344
Male+ Female vs. clean air	Male	27 vs. 23	10	0.060
	Female	30 vs. 20	7	<0.01
Feces of Females vs. clean air	Male	44 vs. 6	2	<0.0001
	Female	30 vs. 20	8	<0.01
Feces of males vs. Clean air	Male	26 vs. 24	11	1.000
	Female	42 vs. 8	1	<0.0001
All stimuli vs. clean air	Male	22 vs. 28	14	0.114
	Female	25 vs. 25	10	0.344

* Significantly different (* $P < 0.05$, ** $P < 0.01$, $\chi 2$ test).
N = 50 individuals.
NR, no response.

Table 3. Behavioral responses of *C. vestitus* Females to volatiles from conspecifics and Pistacia in a Y-tube olfactometer.

	Females responding to odors
Infested buds	40.667 a
Feces of Females	37.333 ab
Feces of Males	37.000 ab
Fresh twigs	33.333 bc
Green buds	30.667 dc
Dried buds	30.667 dc
Males+ Females	30.333 dc
Infested twigs	27.000 de
Females	24.000 fe
All stimuli	24.000 fe
Males	21.333 fe
Dried twigs	20.667 f

Means followed by the same letter are not significantly different (Duncan test, $P < 0.05$), N = 50 individuals.

Table 4. Behavioral responses of *C. vestitus* Males to volatiles from conspecifics and Pistacia in a Y-tube olfactometer.

	Males responding to odors
Feces of Females	44.333 a
Infested buds	40.667 b
Green buds	39.000 b
Fresh twigs	31.667 c
Dried buds	31.333 c
Infested twigs	28.000 d
Feces of Males	27.333 de
Males+ Females	26.667 def
Males	25.333 ef
Females	24.667 f
All stimuli	21.667 g
Dried twigs	21.667 g

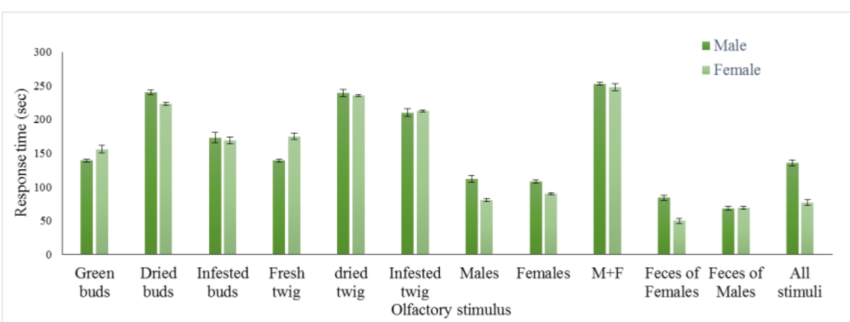

Figure 1. Time spent by both sexes of *Chaetoptelius vestitus* to respond to different volatiles from pistachio host plant and conspecific in a Y-tube olfactometer.

Figure 2. Time spent by both sexes of *Chaetoptelius vestitus* to reach different volatiles from pistachio host plant and conspecific in a Y-tube olfactometer.

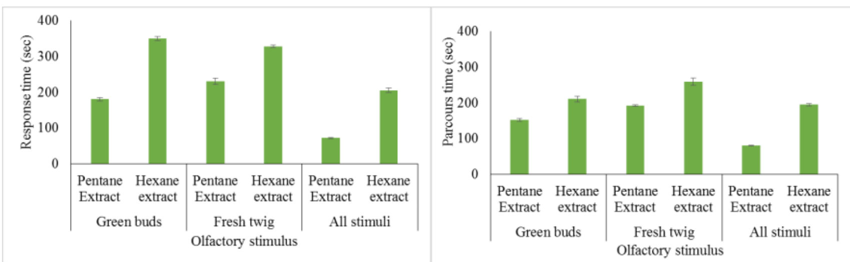

Figure 3. Time spent by *Chaetoptelius vestitus* female to respond (A) and to reach (B) different volatiles from pistachio host plant both pentane and hexane extract in a Y-tube olfactometer.