Article

2-Isobutyl-3-methoxypyrazine as a Putative Male-Specific Aggregation Pheromone in *Labidostomis lusitanica* (Germar) (Coleoptera: Chrysomelidae)

Sergio López [1*], Sara Rodrigo-Gómez [2], Enrique Fernández-Carrillo [2], Cláudia Corbella-Martorell [1], and Carmen Quero [1*]

[1] Department of Biological Chemistry, Institute for Advanced Chemistry of Catalonia (IQAC-CSIC), Jordi Girona 18-26, 08034 Barcelona, Spain; claudia.martorell@iqac.csic.es
[2] Instituto Regional de Investigación y Desarrollo Agroalimentario y Forestal (IRIAF)-Centro de Investigación Agroambiental "El Chaparrillo", Ciudad Real, Spain; srodrigog@jccm.es, efernandezc@jccm.es.
* Correspondence: sergio.lopez@iqac.csic.es (S.L.), carme.quero@iqac.csic.es (C.Q.)

Simple Summary: The leaf beetle *Labidostomis lusitanica* (Germar) (Coleoptera: Chrysomelidae) is a voracious defoliator beetle regarded as a threat for pistachio (*Pistacia vera*) crops in Spain. During late April-early June, field aggregates containing both sexes are commonly found in pistachio leaves. Males collected from these aggregates release a sex-specific compound, namely 2-isobutyl-3-methoxypyrazine, that elicits both strong electrophysiological response and positive chemotactic behavior in males and females. Altogether, our findings suggest that 2-isobutyl-3-methoxypyrazine may act as an aggregative cue during the early season of the crop, although further field assays are required to address this question.

Abstract: In spite of its incidence on pistachio trees, the chemical ecology of *Labidostomis lusitanica* (Germar) (Coleoptera: Chrysomelidae) has been neglected so far. In this work we provide the first evidence of a biologically active male-specific compound that may be promoting field aggregation. Headspace collections by solid-phase microextraction from feral males and females reported the presence of 2-isobutyl-3-methoxypyrazine exclusively on males. Electroantennographic recordings revealed that males and females responded in a dose-dependent manner to increasing stimuli of 2-isobutyl-3-methoxypyrazine, with females overall displaying a higher response than males. In dual-choice tests, both males and females showed a significant preference for the compound in comparison to a pure air stimulus. In the light of these results, the possible role of 2-isobutyl-3-methoxypyrazine as an aggregation cue in *L. lusitanica* is discussed.

Keywords: *Labidostomis lusitanica*; Chrysomelidae; *Pistacia vera*; 2-isobutyl-3-methoxypyrazine; electroantennography; behavior.

1. Introduction

Pistachio (*Pistacia vera* L.) is a native species to central and southwestern Asia that is cultivated in some Mediterranean countries (Spain, Italy, Greece, Turkey, Tunisia), the Middle East (Iran, Syria), the United States (California and Arizona) and Australia [1–3]. Due to the quality of the edible nut, it is a species with a high commercial value worldwide. By the year 2003, pistachio nut production ranked in the sixth place in world tree nut production behind almond, walnut, cashew, hazelnut and chestnut [4], and currently only almond and walnut production surpass that of pistachio, with United States (47%) as the major producer country, followed by Turkey (30%) and Iran (19%) [5]. Such relevance of *P. vera* as a highly exploited resource stresses the need of gaining an exhaustive knowledge and awareness about those biotic agents (*i.e.*, pathogens and arthropod pests) that may affect cultivated plantations.

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Diverse works have been focused on describing the arthropods pests of pistachio in different countries [6–10]. Among the most damaging species are found, for instance, the pistachio seed wasps *Eurytoma plotnikovi* Nikol'skaya (Hymenoptera: Eurytomidae) and *Megastigmus pistaciae* Walker (Hymenoptera: Torymidae) [11,12], the bark beetle *Chaetoptelius vestitus* (Mulsant & Rey) (Coleoptera: Curculionidae) [13], the pistachio psylla, *Agonoscena pistaciae* Burckhardt & Lauterer (Hemiptera: Psyllidae) [14], or the lepidopteran species *Amyelois transitiella* (Walker) (Lepidoptera: Pyralidae) and *Kermania pistaciella* Amsel (Lepidoptera: Tineidae) [7,15].

In Spain, the European country with most pistachio crop area, there is still a lack of knowledge on the insect pests that may threaten pistachio production [8,16]. Recently, a comprehensive study conducted by Gómez and coworkers has pointed out the leaf beetle *Labidostomis lusitanica* (Germar) (Coleoptera: Chrysomelidae) as a potential serious threat for pistachio production in the Iberian Peninsula [17]. *Labidostomis lusitanica* is a polyphagous leaf beetle that feeds not only on pistachio, but also on *Quercus* L., *Salix* L. and *Populus* L., and on the herbaceous genera *Rumex* L. and *Polygonum* L.. In pistachio plantations, it is considered as a voracious species that may defoliate young trees in few hours [17]. The species is found mostly in the eastern and southern part of the Iberian Peninsula, overlapping with pistachio crops [17]. During late April-early June, evenly distributed aggregates containing both sexes are commonly found, in which they feed, mate and lay eggs for a period of 4-5 weeks, and subsequently the individuals disperse across the crop. This behavior led us to suspect the existence of a possible aggregation pheromone in the species.

Hence, the aim of current work was to isolate and identify the intraspecific chemical cues mediating this aggregation. For this purpose, we a) characterized the volatile profile of both aggregative males and females, b) measured the peripheral olfaction to a male-specific compound by means of electroantennographic recordings, and finally c) assessed the behavioral response of both sexes to the compound under laboratory conditions. Determining those chemical cues involved in the field aggregation of the insect would provide a valuable knowledge for further development of pheromone-based monitoring or mass trapping tools, in order to prevent the noxious effect of *L. lusitanica* on pistachio crops.

2. Materials and Methods

2.1. Insects

Feral aggregated *L. lusitanica* males and females were collected from 6th to 24th May 2021 in an organic farming-based pistachio orchard (0.36 ha) located in El Chaparrillo (39.0040, -3.9629; Ciudad Real, Spain). Immediately after collection, they were segregated by sex and sent to the facilities of the Institute for Advanced Chemistry of Catalonia, where each sex was kept separately inside plastic cubic cages (Bugdorm®, 30 x 30 x 30 cm, Entomopraxis, Spain) at 25 ± 1 °C, 55 ± 5% RH and 16:8 L:D. Insects were feed on fresh pistachio leaves (var. Kerman), and leaves were renewed every two days.

2.2. Chemicals

2-isobutyl-3-methoxypyrazine (97%) was obtained from Tokyo Chemical Industry-Europe (Zwijndrecht, Belgium). For preparing the dilutions to be tested in electroantennographic and behavioral assays, n-hexane (GC purity, SupraSolv®, Merck, Darmstadt, Germany) was used as solvent.

2.3. Headspace Solid-Phase Microextraction (HS-SPME)

The volatile profile of *L. lusitanica* males and females was analyzed separately by solid-phase microextraction (SPME), using with a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) coated fiber (50/30 μm; Supelco, Merck-Sigma Aldrich, Madrid, Spain). In each volatile collection, 20 individuals, either males or females, were exposed for 6 h to the SPME fiber inside a 40 mL screwed-cap vial (Supelco, Merck Sigma

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Aldrich). All collections (n=10 for both sexes) were conducted from 10:00 am to 18:00 pm at room temperature and 24-72 h after the arrival of the insects.

Samples were further analyzed in a Thermo Finnigan Trace 2000 gas chromatograph system coupled to a Trace MS quadrupole mass spectrometer (Thermo Fisher Scientific, Madrid, Spain). The injection port temperature was set at 270 °C, and samples were run in splitless mode (5 min). The following temperature program was set up: an initial temperature of 50 °C hold for 5 min, followed by an increase of 5 °C/min to 150 °C, and finally raised to 310 °C at 15 °C/min, with a hold time of 10 min. The MS system operated in electron impact mode (70 eV), and scan mode (40-500 amu), at 1.0 scan/s. Compound identification was achieved by comparison of the experimental mass spectrum with those of the synthetic standard and a commercial mass spectral library (NIST Registry of Mass Spectral Data, 2005).

2.4. Electroantennographic (EAG) Response

The olfactory response of *L. lusitanica* males (n=8-10) and females (n=8-10) in response to increasing quantities of 2-isobutyl-3-methoxypyrazine (1, 10, and 100 µg) was determined by electrophysiological recordings from severed antennae. Briefly, one antenna of a cold-anesthetized adult was excised with the aid of a microscapel, and once excised, last two antennomeres were removed. The antenna was then fixed to a forked microelectrode holder (Syntech, Kirchzarten, Germany) with a drop of conductive gel (Spectra 360, Parker Lab. Inc., Hellendoorn, The Netherlands), placing the distal part of the antenna on the recording microelectrode, and the proximal part on the reference microelectrode. The holder was connected to an EAG Combi-Probe (Syntech) connected to a MP-5 micromanipulator (Syntech). Stimuli were delivered to insect antennae by applying pure air puffs (*ca.* 300 mL/min) for 100 ms on a disposable glass Pasteur pipette (150 mm long) which contained a filter paper disc (2.5 cm diameter, Whatman®, Merck-Sigma Aldrich) loaded with 10 µL of the corresponding hexane dilution of the test compound (0.1, 1.0, or 10 µg/µL). Two puffs per each amount of 2-isobutyl-3-methoxypyrazine were applied to each antennal preparation in increasing order of magnitude, with an interval of 60 s between puffs. Control puffs with 10 µL of hexane were intercalated between two consecutive 2-isobutyl-3-methoxypyrazine stimuli to determine the baseline depolarization of the antenna. A continuous humidified pure air flow (*ca.* 650 mL/min) passed over the antenna through the open end of a T-shaped glass tube (7 cm long × 5 mm diameter) positioned 1 cm over the sample, in order to prolong the life of the antennal preparation. Net EAG response evoked by each amount 2-isobutyl-3-methoxypyrazine was calculated by subtracting the mean EAG amplitudes of the hexane puffs before and after the test compound. The EAG signals were filtered (DC to 1 kHz) with the aid of an IDAC-2 interface (Syntech), digitized on a PC, and further analyzed with the EAG Pro software (version 2.0, Syntech).

2.5. Behavioral Bioassays

A vertically placed dual-choice glass olfactometer (main arm 10 cm long × 18 mm I.D., arms 8 cm long × 18 mm I.D., angle between arms 90º) was used to determine the walking preference of *L. lusitanica* males (n = 31-40) and females (n = 36-49) in response to 2-isobutyl-3-methoxypyrazine (0.1, 1, and 10 µg). A Whatman® filter paper disc loaded with 10 µL of the corresponding hexane-dilution of 2-isobutyl-3-methoxypyrazine was placed in one of the olfactometer arms, whereas the control arm held a filter paper loaded with 10 µL of hexane, as control. Filter papers were replaced every five insects, and the position of the arms were also switched to avoid any directionality. An incoming charcoal-filtered air flow of at 350 ml/min was set for both arms. All the system was surrounded by a white filter paper screen (45 cm height), to avoid the interference of visual stimuli [18]. Homogenous illumination (light intensity *ca.* 500 lux) was provided with a bulb placed 30 cm above the junction of the olfactometer. A Y-shaped copper wire was introduced to facilitate the insect movement along the olfactometer. Prior to the beginning of each trial, beetles were individually isolated in polystyrene cell culture dishes (Corning

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Inc, New York, United States), and left for 1 h to acclimate to room conditions. A positive response was considered if the insect entered any arm at least 2 cm beyond the arm junction. Beetles that did not make a choice were designated as non responders, and therefore, discarded for further statistical analysis. All the behavioral assays were conducted from 10:00 am to 18:00 pm at room temperature.

2.6. Statistical Analysis

Prior to any statistical analysis, the EAG net amplitudes in response to 2-isobutyl-3-methoxypyrazine were subjected to Saphiro-Wilk and Levene tests, and when neccesary, data were log-transformed to fulfill the asumptions of normality and/or homogeneity of variance. Differences in absolute EAG responses within a sex were analyzed by one-way analysis of variance (ANOVA). A subsequent Tukey HSD post-hoc test was applied for pairwise comparisons when ANOVA was significant. Differences between sexes to a concrete amount of stimulus were analyzed with Student´s t-test. Walking response in the double-choice olfactometer was subjected to a chi-square goodness-of-fit test to adresss if the arm preference displayed by each sex differed from a 50:50 distribution. All the statistical tests were performed using SPSS Statistics 17.0 software (SPSS, Chicago, IL, USA), at a significance level of α=0.05.

3. Results

3.1. HS-SPME collections

All the volatile collections from males revealed the presence of a sex-specific compound, whereas no traces were detected in females (Figure 1a). Further comparison of the naturally-occurring mass spectrum with those of the commercial library and the synthetic standard corroborated the identification of the eluting compound as 2-isobutyl-3-methoxypyrazine (Figure 1b).

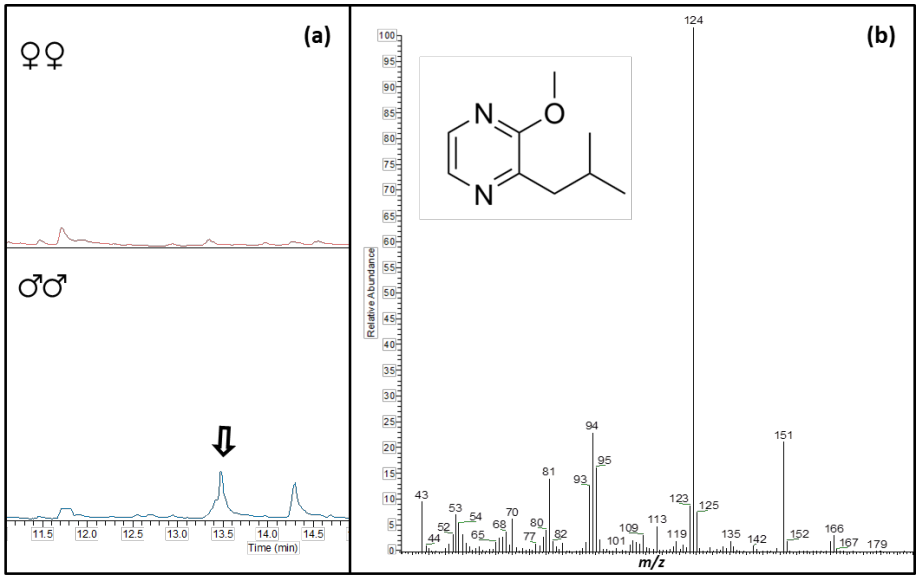

Figure 1. Identification of 2-isobutyl-3-methoxypyrazine from HS-SPME collections of feral *L. lusitanica*. **(a)** Zoomed-in region of the elution time of the male-specific peak (black arrow); **(b)** Mass spectrum of male-released 2-isobutyl-3-methoxypyrazine.

3.2. EAG Response

The EAG response of both sexes followed a dose-dependent response pattern (males, $F_{2,24} = 5.304$, $p = 0.012$; females, $F_{2,23} = 10.249$, $p = 0.001$) (Figure 2). In terms of differences between sexes, overall females showed a higher sensitivity to increasing amounts of 2-isobutyl-3-methoxypyrazine than males (Figure 2). A stimulus of 10 µg elicited a

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significantly higher EAG response in females than in males (t = -3.571, df = 11.526, p = 0.04), while the response to 100 µg was close to significance (t = -2.023, df = 14, p = 0.063) (Figure 2).

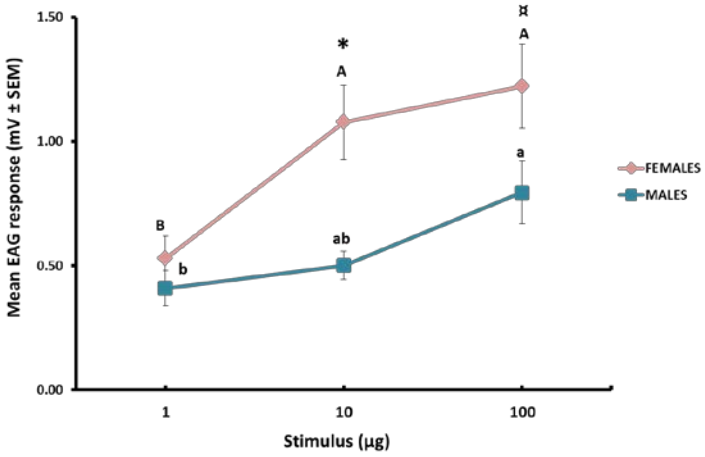

Figure 2. Mean EAG response (mV ± SEM) from excised antennae of feral *L. lusitanica* males (n = 8-10) and females (n=8-10) to puffed stimuli of 2-isobutyl-3-methoxypyrazine (1, 10 and 100 µg). Different letters within each sex denote significant differences in the EAG response among quantities (one-way ANOVA followed by Tukey HSD post hoc test, at α = 0.05). Asterisk indicates significant differences between sexes to a concrete amount of 2-isobutyl-3-methoxypyrazine (Student´s t-test, at α=0.05; ¤, p =0.067).

3.3. Behavioral Bioassays

Both males and females responded positively to 2-isobutyl-3-methoxypyrazine when they were released individually in a double-choice olfactometer (Figure 3). Specifically, 68% of tested males made a choice for the arm containing 0.1 µg of the compound (X^2 = 4.235, df = 1, p = 0.04), while high amounts of 2-isobutyl-3-methoxypyrazine yielded non-significant attraction percentages. Indeed, a significant aversive effect was detected when males were exposed to 10 µg of the compound (X^2 = 3.846, df = 1, p = 0.05). A similar response pattern was observed in females, with 73% of these displaying a significant preference for 1 µg of the compound (X^2 = 5.538, df = 1, p = 0.019) (Figure 3). Conversely, no significant trend towards 2-isobutyl-3-methoxypyrazine was detected at neither 0.1 (X^2 = 1.485, df = 1, p = 0.223) nor 10 µg (X^2 = 0.059, df = 1, p = 0.808).

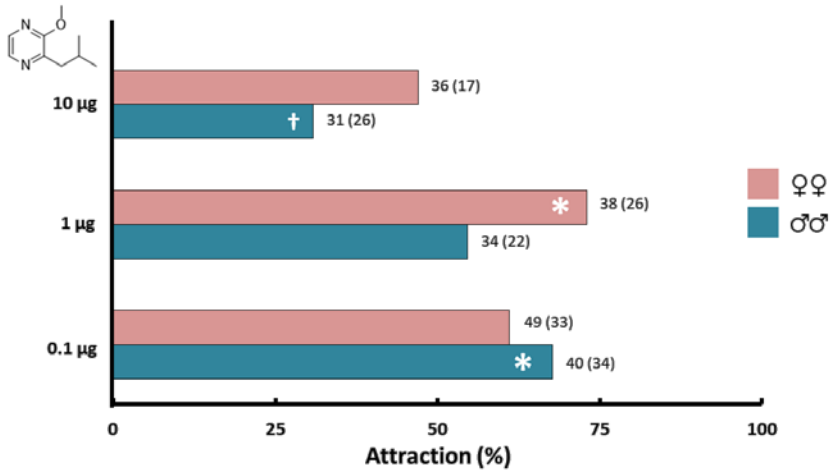

Figure 3. Behavioral response of feral *L. lusitanica* males and females to 2-isobutyl-3-methoxypyrazine (0.1, 1 and 10 µg) in a double-choice olfactometer. Numbers aside each bar indicate the total number of insects tested, and those which made a choice for either arm of the olfactometer (in

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parentheses). Asterisks denote a significant preference towards the compound, while the dagger (†) indicates an aversive effect of the compound (chi-square goodness-of-fit test, at $\alpha = 0.05$).

4. Discussion

Pyrazines are nitrogen-containing heterocyclic compounds with relevance in insect chemical signaling at different levels (*i.e.*, intraspecific and interspecific interactions). For instance, some plant-related pyrazines have been described as synomones [19–22]. Especially remarkable is the sexual deception displayed by some orchid species, which mimic the sex pheromone blend of female pollinator wasps (Hymenoptera: Thynnidae) [19,20]. Diverse 2-alkyl-methoxypyrazines released from the flowers of palm species of the genera *Acrocomia* and *Attalea* attract two florivorous scarab species (Coleoptera: Melolonthidae) [21], while the cones from the African cycad *Encephalartos villosus* Lem. emit 2-isopropyl-3-methoxypyrazine (hereafter referred to as IPMP), resulting attractive to pollinator beetles [22]. Additionally, 2-alkyl-methoxypyrazines act as allomones in aposematic insects of different Orders [23–26]. 2-Alkyl-methoxypyrazines are common warning cues with a distinctive odor, that along with additional signals (e.g. visual [27,28]), constitute a multimodal defense mechanism to avoid predation. To cite an example, the wood tiger moth *Arctia plantaginis* (L.) (Lepidoptera: Arctiidae) produces 2-isobutyl-3-methoxypyrazine (IBMP), and 2-*sec*-butyl-3-methoxypyrazine (SBMP), that deter birds and ants [29], although they are ineffective against spiders [30]. In *Oncopeltus fasciatus* (Dallas) (Hemiptera: Lygaeidae), its chemical defense mechanism relies on the expelling of a fluid that contains IBMP [26]. Particularly remarkable is the role of 2-alkyl-methoxypyrazines in some ladybugs species (Coleoptera: Coccinellidae), since they have been coadapted as allomones and aggregation pheromones in diapausing individuals [31,32].

In this sense, pheromone-mediated aggregation is a common behavior in insects, including Chrysomelidae beetles [33], in which obviously chemical cues play a pivotal, although not exclusive, role. Indeed, the elucidation of aggregation pheromones has been widely documented in chrysomelid species, with males as the sex responsible of their emission [34–43]. Here, we present the first empirical evidence of IBMP as a male-specific volatile compound that may be mediating field aggregation in the leaf beetle *L. lusitanica*. This hypothesis is partially supported by our findings, with males being the sex responsible of the IBMP emission, as reported in other leaf beetle species, and both sexes showing a significant preference towards the compound. To the best of our knowledge, no pyrazine has been identified as pheromone in leaf beetles so far. Indeed, most of the research of the pyrazine motif as pheromones has been conducted in ants, in which they act as alarm or trial pheromones [44–46], in true fruit flies (Diptera: Tephritidae) [47–49], in thynnine wasps [19], and in ladybugs [31,32,50]. As stated above, in some coccinellid species an aggregative effect is induced by 2-alkyl-methoxypyrazines in diapausing individuals, apart from their roles as allomones. Al Abassi and coworkers reported that IPMP is attractive for both sexes of *Coccinella septempunctata* (L.) (Coleoptera: Coccinellidae) under laboratory conditions, inducing an attractive and arrestant effect attributable to the effect of an aggregation pheromone [31]. Similarly, IBMP alone or mixed with IPMP results attractive for diapausing individuals in *Adalia bipunctata* (L.) [50]. In other ladybug species, such us *Harmonia axyridis* Pallas and *Hippodamia convergens* Guérin-Méneville, IPMP, IBMP, and SBMP have been identified [51] and demonstrated to be behaviorally active. In the case of *H. convergens* adults, SBMP, and especially IBMP and the ternary blend at its natural ratio, induce significant aggregative effect in laboratory and field assays, whereas high amounts of IPMP and SBMP were showed as repellant [32]. Likewise, an aversive behavior on *L. lusitanica* males was evident when they were confronted to the highest amount of IBMP in olfactometer trials. Given the dual activity of 2-alkyl-methoxypyrazines in ladybugs, it seems probable that IBMP may be playing a similar role in *L. lusitanica*, not only promoting an aggregative behavior but also conferring a defense mechanism against predation.

According to field observations, *L. lusitanica* aggregates with high number of individuals (more than 50) are found only in few pistachio trees, while most of the trees harbor

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few number (less than 15) [17]. In a similar vein, the response of males and females of *Aphthona nigriscutis* Foudras to aggregation cues decreases according to the density of conspecifics on the plant [37]. In the latter, it is not acknowledged if this density-dependent response may be conditioned by deterrent plant volatile organic compounds released upon feeding by conspecifics [52–54]. For example, *Diorhabda carinulata* Desbrochers (Coleoptera: Chrysomelidae) avoids previously defoliated saltcedar plants, and this avoidance has been correlated to the release of 4-oxo-(*E*)-2-hexenal from the host, inducing a repellence effect on reproductive males and females [52]. Nonetheless, since *L. lusitanica* colonizes healthy and non-consumed pistachio leaves from young trees, this hypothesis should be *a priori* discarded. Alternatively, we suggest that chemical cues from pistachio trees may be signaling the suitability of the host to some extent , partially explaining therefore the patchy spatial pattern of aggregates of variable number of individuals across the crop. Indeed, plant volatiles are demonstrated to be relevant in the aggregation processes of Chrysomelidae, either by luring the pioneer individuals [42,55], or instead by enhancing/synergizing the effect of the aggregation pheromone [37,56–59], as it occurs in other coleopteran families [60,61]. Furthermore, being in contact with the host plant and/or feeding seem to be *sine qua non* conditions for pheromone release in leaf beetle species [40–43,55,62]. Examples highlighting this phenomenon are found in *Acalymma vittatum* (F.), whose feeding males are more attractive to conspecific than non-feeding males [42], in *Phyllotreta* spp. [40,43] and in *Oulema melanopus* (L.), in which the release of aggregation pheromone is tightly related to males feeding on the host plant [41]. In our case, IBMP was detected from males deprived of pistachio leaves during the volatile collection, although they were not subjected to starvation prior to the sampling. Whether the presence of plant volatiles and/or pistachio leaves consumption are crucial for the release of IBMP remains thus unanswered.

In summary, we have identified a male-specific compound that actively attracts both sexes under laboratory conditions. Future field research is ongoing to gain a broader understanding of the role of the compound in the chemical ecology of *L. lusitanica*. Determining whether its potential aggregative effect is restricted to communal feeding and mating during the early season of the crop, or in opposition it remains active during the whole life cycle, should be considered. Last, but not least, a thorough understanding of population dynamics would be also paramount for deciphering the biological implication of the compound.

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