

Quantitative Variations in the Essential Oil of *Minthostachys mollis* (Kunth.) Griseb. in Response to Insects with Different Feeding Habits

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Plants display a diverse array of inducible changes in secondary metabolites following insect herbivory. Herbivores differ in their feeding behavior, physiology, and mode of attachment to the leaf surface, and such variations might be reflected in the induced responses of damaged plants. Induced changes were analyzed for *Minthostachys mollis*, a Lamiaceae with medicinal and aromatic uses, and four species of folivore insects with different feeding habits (chewing, scraping, sap-sucking, and puncturing). In *M. mollis* leaves experimentally exposed to the insects, levels of the two dominant monoterpenes pulegone and menthone were assessed 24 and 48 h after wounding. Menthone content generally decreased in the essential oil of damaged leaves, whereas pulegone concentration increased in all treatments. These changes occurred also in the adjacent undamaged leaves, suggesting a systemic response. The relatively uniform response to different kinds of damage could be attributable to the presence of such a strongly active compound as pulegone in the essential oil of *M. mollis*. The effects of wounding on essential oil concentration may be significant from a commercial point of view.

KEYWORDS: Phytochemical induction; aromatic plants; monoterpene; pulegone; menthone; *Minthostachys mollis*

INTRODUCTION

Induction of resistance has been widely observed in plants following damage by insect herbivores (1–3). Plant defensive compounds may show a broad range of toxicity mechanisms including membrane disruption, inhibition of transport or signal transduction, metabolic alterations, and even disruption of hormonal control of developmental processes (1, 4, 5). These defenses can be either direct, making the plant more resistant to further herbivory, or indirect, facilitating “top down” control of herbivore populations by aiding pathogens, predators, and parasitoids in host or prey location (2, 6). The nature and intensity of induced defenses can be related to the kind of damage suffered by the plant, because plants seem to be able to generate differential responses to different types of herbivory (7–10).

Monoterpenes, the major constituent found in aromatic plant essential oils, are colorless, lipophilic, volatile substances representing some of the best studied examples of plant defenses. There is a general consensus that these compounds constitute a basic armament in the defensive potential of plants against biotic

attack, with reported antiherbivore, antibacterial, antifungal, and allelopathic functions (11–16).

Nowadays, plants of key agricultural and trade significance are not restricted to traditional food, forage, and fiber crops but increasingly include species with secondary metabolites valued for their aromatic or therapeutic attributes or as main natural inputs to the proliferating perfume and chemical industries. Also, some specific oil constituents are used as chiral auxiliaries in synthetic organic chemistry and microbial transformation of common structures to give highly functionalized substances of enhanced economic value (17).

The yield of essential oil bearing plants has been shown to be strongly influenced by environmental factors such as temperature, photoperiod, light intensity, nutrition, genotype, stage of development, moisture, and salinity (17). However, studies involving the effects of insect herbivory on essential oil constitution and production have received little attention (18–21). In the present paper we analyze the effects of herbivore insects with different feeding strategies on the essential oil of *Minthostachys mollis* (Kunth.) Griseb., a Lamiaceae native to central Argentina with medicinal and aromatic uses in the region. The major constituents of *M. mollis* essential oil are terpenoids, compounds that have been shown to increase following herbivory in various plant species (1, 18, 22, 23).

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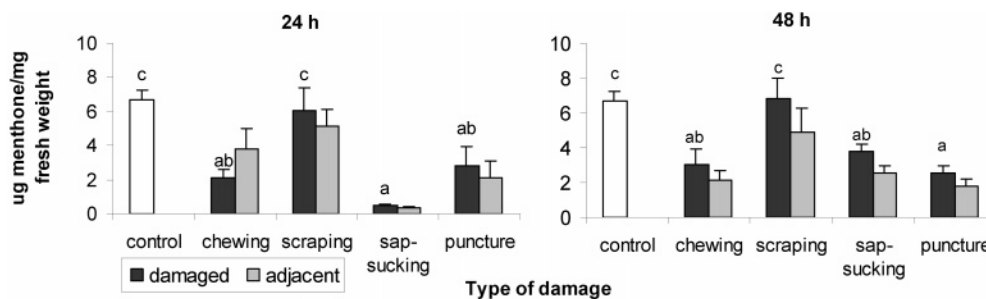


Figure 1. Changes in menthone concentration (means \pm SE) in the essential oil of *M. mollis* leaves, as a result of different types of insect feeding damage, at different postwounding times. Different letters indicate statistical difference among damage treatments ($p < 0.05$, Kruskal–Wallis).

MATERIALS AND METHODS

Bioassays. Healthy, pestfree, 6-month-old plants (average 18 leaves) of *M. mollis*, grown in a glasshouse without supplementary lighting, were used. Plants were exposed to insects representing the following feeding habits: (a) leaf chewing, adults of *Systema* sp. (Coleoptera: Chrysomelidae), surface feeders with chewing mouthparts, ingest whole pieces of the leaf lamina, leaving holes or notches; (b) leaf scraping, nymphs and adults of “thrips”, *Tubulifera* sp. (Thysanoptera), remove the contents of leaf epidermal or parenchymal cells, producing a characteristic mottling of the affected surfaces (24); (c) sap-sucking, adults of *Halticus* sp. (Hemiptera: Miridae) insert their feeding stylets a short distance into the host tissue, leaving small, discolored spots where the spongy mesophyll cells are broken and emptied, causing local necrosis (24); (d) leaf puncturing, adult females of *Liriomyza huidobrensis* (Diptera: Agromyzidae) pierce the leaf tissues with their ovipositor and suck up the exuded cellular contents. The insects were collected from field *M. mollis* plants, either cultivated or growing spontaneously near Córdoba city, in central Argentina. Adults of *L. huidobrensis* were reared from leafmining larvae collected on *Vicia faba* crops in the same location.

In each test, a plant was exposed for 4–6 h in a cage (wood, glass, and gauze, 30 cm on each side) to at least five insects from one of the feeding types above-mentioned. All experiments were made under similar conditions of light, temperature (22 ± 2 °C), and relative humidity ($\sim 70\%$). At least five leaves (of approximately the same age and size) from different nodes were offered to the insects; the leaf opposite to each of the former was covered with a thin plastic film to avoid insects feeding on them. Care was taken that the whole amount of physical damage to the plant was not larger than 30–40% of the leaf area. All insects were then removed, and the plants were placed in an insect-free environment. After 24 and 48 h, damaged leaves and their adjacent (undamaged) ones were cut off to assess chemical induction and translocation effects. Five leaves, similar in size and age to the damaged ones, from an undamaged plant were used as controls. Film-covered leaves from undamaged plants were used as controls for the damage-adjacent leaves. The essential oil composition of control film covered leaves did not significantly differ from that of the uncovered leaves on the same plant (Wilcoxon signed ranks test, $p > 0.05$), thus dismissing possible effects of the film cover. Different plants were used for each treatment (damage type and response time); the youngest and oldest pairs of leaves were never included in the analyses. All leaves were frozen until the chemical analysis was carried out. Between 5 and 10 different plants (replications) were used for each treatment.

Essential Oil Extraction. After weighing, the plant material was submitted to hydrodistillation in a micro-Clevenger-like apparatus for 40 min, and the volatile fraction was collected in dichloromethane. An internal standard was added (12 μ g of thymol in 2 μ L of dichloromethane). Essential oils of *M. mollis* contain 50 different compounds, with 2 monoterpenes accounting for $\sim 70\%$ of their volume: pulegone = cyclohexanone, 5-methyl-2-(1-methylethylidene); and menthone = cyclohexanone, 5-methyl-2-(1-methylethyl) (18, 25). These two compounds were used for the present study. The monoterpenes were quantified with respect to thymol. The FID response factors for each compound generate an equivalent area with a negligible error ($< 5\%$) (unpublished data).

Chemical Analysis. Analyses were accomplished with the use of a Perkin-Elmer Q-700 gas chromatograph equipped with a CBP-1 capillary column (30 m \times 0.25 mm, film thickness = 0.25 μ m) and a mass selective detector. Analytical conditions were as follows: injector and detector temperatures, 250 and 270 °C, respectively; oven temperature, programmed from 60 °C (3 min) to 240 °C at 4 °C/min; carrier gas, helium at a constant flow of 0.9 mL/min; source, 70 eV. The oil components were identified by a combination of mass spectral and retention time data, which were compared both with those of authentic compounds and with those published in Zygadlo et al. (25). GC analyses were performed with a Shimadzu GC-RIA gas chromatograph, fitted with a 30 m \times 0.25 mm fused silica capillary column coated with a Supelcowax 10 (film thickness = 0.25 μ m). The GC operating conditions were as follows: oven temperature, programmed from 60 °C (3 min) to 240 °C at 4 °C/min; injector and detector temperatures, 250 °C; detector, FID; carrier gas, nitrogen at a constant flow of 0.9 mL/min.

Statistical Analyses. Differences in menthone and pulegone content among treatments from independent samples were analyzed with the Kruskal–Wallis test, because data were not normally distributed. Comparisons between damaged and undamaged adjacent leaves were performed using the Wilcoxon signed ranks test (paired samples). The software used for all data analyses was INFOSAT.

RESULTS

Herbivory induced significant changes in the leaf chemistry of *M. mollis*. The concentration of menthone (Figure 1) in the essential oil decreased after 24 h with most types of damage, without significant differences among damage treatments and maintaining lower levels than control leaves after 48 h ($p < 0.05$, Kruskal–Wallis, $H_{24h} = 20.08$, $H_{48h} = 11.98$). The only exception to this pattern was a lack of response in scraped leaves, which kept menthone levels similar to those of controls. Pulegone concentration (Figure 2) increased significantly in all damage treatments ($p < 0.05$, Kruskal–Wallis, $H_{24h} = 19.38$, $H_{48h} = 29.87$). Again, scraped leaves were the exception to a generalized response, showing the highest pulegone level 24 h postwounding, whereas for all other damage types this compound was still increasing 48 h after damage.

Menthone concentration in undamaged leaves adjacent to wounded leaves did not differ from the latter (Figure 1), either 24 or 48 h after any type of damage ($p > 0.05$, Wilcoxon signed ranks test). Pulegone content (Figure 2) followed the same pattern ($p > 0.05$, Wilcoxon signed ranks test), although leaves adjacent to those damaged by scraping and sap-sucking insects showed a significant increase at 24 and 48 h after wounding, respectively ($p < 0.05$, Wilcoxon signed ranks test), thus magnifying the changes observed in the damaged leaves.

DISCUSSION

Insect feeding generally resulted in decreased contents of menthone in *M. mollis* leaves as compared to undamaged plants,

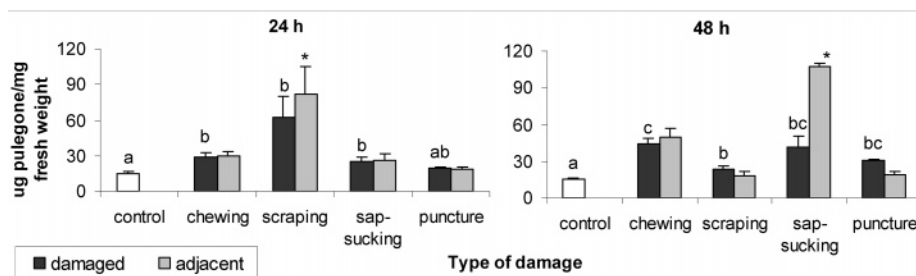


Figure 2. Changes in pulegone concentration (means \pm SE) in the essential oil of *M. mollis* leaves, as a result of different types of insect feeding damage, at different postwounding times. Different letters indicate statistical difference among control and/or damaged leaves ($p < 0.05$, Kruskal–Wallis). An asterisk (*) indicates significant differences between damage treatments ($p < 0.05$, Wilcoxon signed ranks test).

with the only exception of scraping damage inflicted by thrips, which did not induce any significant changes. These responses contrast with the 6.5-fold menthone increase in mechanically damaged leaves of the same plant species (21). On the other hand, pulegone content significantly increased in all damage treatments, this response being most pronounced 48 h after wounding. Again, leaves facing scraping damage showed a different response, generally being most intense 24 h upon wounding and declining at 48 h. This response resembles the one reported for mechanical damage, where pulegone concentration increased dramatically 24 h after wounding and then dropped to control levels (21). The changes in essential oil concentration observed after damage suggest that biosynthetic processes are elevated during the postwounding period (22). From an ecological perspective, rapid chemical changes may be interpreted as an attempt to minimize additional feeding (26).

Furthermore, the results obtained in this study suggest a translocation of the response observed in the leaves damaged by insects. The content of menthone and pulegone in undamaged leaves adjacent to wounded ones was equivalent to that of the latter, thus mirroring or, in some cases, even magnifying the induced response. The systemic increase in pulegone content after damage observed here is consistent with several reports of increased terpene formation at damaged and unharmed sites within plants subjected to herbivory stress (4, 22). On the other hand, this result is clearly different from the response elicited by mechanical damage in the same plant species, where no translocation was observed (21). The fact that this phenomenon was not observed with mechanical damage suggests that an elicitor related with the insects may be required for a systemic response to be induced (22, 27).

In other systems, induction of phytochemical production has been shown to differ qualitatively and/or quantitatively depending on the insect species and feeding habits (7, 9, 28–30). Presumably, variations in induced responses to different damage types could be due to changes generated at the wound site. These signals may originate from the plant (e.g., cell wall fragments) or the insect (e.g., salivary factors) (27). The strength of an induced response may depend on the number of wounded cells in contact with healthy cells (31); other factors include the duration, persistence, and magnitude of a single damage event, the cell or tissue types affected by damage, and the kinds of forces acting on the leaf (2, 31, 32).

Although the herbivores here studied differ greatly in their feeding behaviors, remarkably similar induced changes in monoterpene concentration were observed in *M. mollis*. Such changes occurred whether insects removed pieces of leaf, punctured epidermal cells with the ovipositor, or penetrated the plant cuticle to feed from the mesophyll cells, injecting relatively large quantities of saliva or scraped into the parenchyma cells (24). The relatively uniform response observed in *M. mollis*

could be attributable to the possession of such a strongly active compound as pulegone, which in high concentrations might prove to be an insurmountable obstacle for most insect herbivores. Pulegone is probably the most potent inhibitor of acetylcholinesterase (AChE) present in the neuromuscular junction in invertebrates (23, 33); additionally, it has been proved to destroy symbionts of herbivores, thus creating a biochemical barrier for host plant utilization (23). Moreover, toxic (23, 33–37), growth-inhibitor (38), repellent, and oviposition-detering (23) properties have been demonstrated. In addition, the observed decrease in menthone after damage (Figure 1a) could magnify the toxic effect of pulegone, because the two compounds seem to be antagonistic (36). Whether plants containing one strong active compound tend to show more homogeneous responses to damage than plants with a more diversified defensive structure is a question that deserves further study.

It is likely that changes in oil concentration serve important ecological roles during the leaf postwounding period. In addition, from a commercial point of view the effects of wounding on leaf oil concentration may be significant. Given the economic importance of monoterpenes for the fragrance, flavor, and pharmaceutical industries, knowledge of the processes that affect monoterpene accumulation in plants can be of value in increasing the yields of these commercially valuable natural products.

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