Influence of Green Leaf Herbivory by *Manduca sexta* on Floral Volatile Emission by *Nicotiana suaveolens*[1][W]

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Plants have to cope with various abiotic and biotic impacts as a consequence of changing environments, which can impair their ability to sexually reproduce. The main objective of this study was to investigate whether green leaf herbivory, having one of the most hazardous biotic impacts, would have any direct effect on the production and emission of floral volatiles because volatiles are known to play a crucial role in pollination. *Nicotiana suaveolens* plants were challenged with *Manduca sexta* feeding on leaves, and alterations in the quality and quantity of the floral blend, shifts in emission patterns, and changes in expression patterns of the floral benzoic/salicylic acid carboxyl-methyltransferase were monitored in noninfested and infested plants. Leaves responded to larval feeding by herbivory-induced diurnal emission of semiochemicals, whereas the emission of floral volatiles remained unchanged in comparison to the noninfested control. Neither the volatile composition nor the quantity of components or the nocturnal emission patterns was altered. The mRNA and protein levels of the benzoic/salicylic acid carboxyl-methyltransferase, as well as its enzyme activity, also did not show any significant differences. These results indicate that metabolism in flowers at and postanthesis is an autonomous process and is independent of metabolic changes in green leaves. By this sustaining mechanism, *N. suaveolens* plants ensure sexual reproduction even under unfavorable conditions.

The emission of volatile organic compounds (VOCs) is a characteristic trait of flowers of most seed plants. Floral VOCs are part of pollination syndromes and thus represent a very crucial factor to ensure sexual reproduction (Pellmyr et al., 1991; Dobson, 1994, 2006; Pichersky and Gershenzon, 2002). However, plants are challenged by constantly changing abiotic and biotic environmental conditions that might affect a plant's capacity to invest in sexual reproduction (Euler and Baldwin, 1996; Carroll et al., 2001; García and Ehrlén, 2002). Besides direct impacts, like floral oviposition (Dufaÿ and Anstett, 2003) or floral herbivory (Leege and Wolfe, 2002; Röse and Tumlinson, 2004; Theis, 2006; Sanchez-Lafuente, 2007), flowers are mostly exposed to a changing environment as part of an entirely affected plant (Sampson and Cane, 1999; Carroll et al., 2001). One of the most deleterious biotic influences on plants is green leaf herbivory. Infested leaves respond with a complex defense strategy, including an immediate damage-induced de novo production and emission of volatiles that repels herbivores or attracts host-seeking parasites as well as prey-searching natural enemies of the plant plaguer (De Moraes et al., 1998; Röse et al., 1998; Paré and Tumlinson, 1999; Pichersky and Gershenzon, 2002; van Poecke and Dicke, 2004). Intraplant and interplant signaling mediated by herbivore-induced volatiles also triggers a defense response in noninfested parts of the plant or noninfested neighboring plants (Mattiacci et al., 2001; Baldwin et al., 2002; Röse and Tumlinson, 2005; Heil and Bueno, 2007). The most severe consequence of green leaf herbivory is the loss of photosynthetic capacity. Possible impacts on floral traits, like flower size (Steets and Ashman, 2004; Cole and Ashman, 2005; Ivey and Carr, 2005), flower number (Cresswell et al., 2001; Canto et al., 2004), morphology, and nectar production (Ornelas et al., 2004), a change in pollinator service (Steets and Ashman, 2004; Ivey and Carr, 2005), fruit and seed production (Ornelas et al., 2004), and progeny (García and Ehrlén, 2002), are rather well documented. However, little attention has been paid so far to the influence of green leaf herbivory on floral VOC production and emission.

The main objective of this study was to investigate whether green leaf herbivory treatment of *Nicotiana suaveolens* would interfere with its floral VOC production and emission. *Manduca sexta* (Sphingidae), as one of the most intensively investigated model insects, served as the herbivore. *N. suaveolens* is native to coastal areas of Southeastern Australia (Japan Tobacco Inc., 1994). Floral VOCs are primarily nocturnally emitted (Loughrin et al., 1990; Raguso et al., 2003). Up to 40 compounds were identified. One of the main constituents of the floral blend is methyl benzoate. It derives from L-Phe and is most likely synthesized via β-oxidation of (*E*)-cinnamic acid (Ribnicky et al., 1998; Dudareva and Pichersky, 2006) and *O*-methylation by an *S*-adenosyl-L-Met:benzoic/salicylic acid carboxyl-methyltransferase (BSMT; AJ628349). This BSMT is specifically expressed in flowers (Pott et al., 2004) and, in

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this study, represented a proxy to monitor potential alterations of the floral volatile production at the molecular level. Our interest was focused on (1) an immediate response within a few hours as known from the green leaf response; and (2) a delayed response within a few days during ongoing herbivory. Subsequently, two different approaches were pursued. (1) VOC emission and floral BSMT expression were followed according to the flowering period of individual flowers. Parameters were compared by examination of two consecutive flowering periods of noninfested plants and noninfested and infested plants. (2) BSMT enzyme expression was followed during the progression of larval damage using flowers of the same age. Effects should be observed in alterations of the quality and/or quantity of floral VOCs emitted, in shifts in the emission pattern of certain VOCs, or in alterations of the characteristics of BSMT expression. To obtain an exact description regarding the response of *N. suaveolens* upon green leaf herbivory by *M. sexta*, the herbivore-induced leaf volatile emission was analyzed as well.

RESULTS

Manifestation of Damage Due to Larval Feeding

Larvae of *M. sexta* were reared on leaves of young *N. suaveolens* plants. Larvae hatched after approximately 4 d post egg positioning. The transfer to the plants used for analysis did not impair these young first instar larvae. Already a few hours after transfer, they resumed their activity and first signs of feeding were obvious. The larvae preferably stayed on the abaxial surface of the leaves. After 5 d, distinct feeding signs at the leaf margin and hole injuries could be observed. Every single plant of the batch was affected. Ten days of herbivory caused severe damage (Fig. 1).

Larvae-Infested Green Leaves Emit Herbivore-Induced Volatiles

Leaves of noninfested *N. suaveolens* plants consistently emitted fatty acid derivatives at very low concentrations (data not shown), whereas infested leaves (Fig. 2A) emitted at least seven new VOCs as a reaction to larval herbivory (Supplemental Table S1). The main compounds identified were (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, α-bergamotene, and α-farnesene. Traces of β-farnesene, (*Z*)-3-hexenyl butanoate, and β-linalool were also detected. The terpenoids (*E*)-β-ocimene, α-bergamotene, and α-farnesene appeared on day 1 of herbivory and reached maximal emission on day 2 (Fig. 2B). The emission of these terpenoids showed diurnal oscillation, with a maximum observed during the day between 6 AM and 6 PM. The major component in the blend of herbivore-induced VOCs was the sesquiterpene α-farnesene (Fig. 2C). (*Z*)-3-hexenyl acetate could be found within the first 6 h after the application of larvae. It was emitted at low concentrations. These results demonstrate the susceptibility of *N. suaveolens* to foliar herbivory of *M. sexta* larvae and the system was used for investigating possible alterations in floral VOC emission upon green leaf herbivory.

Figure 1. Damage to *M. sexta*-infested *N. suaveolens* plants after 10 d of larval herbivory.

Fresh Weight of Flowers Does Not Change during Green Leaf Herbivory

Under the chosen conditions of plant cultivation, the flowering period of an individual flower of *N. suaveolens* lasted, on average, for 6 d. The weight on the day of anthesis ranged between 70 and 80 mg per flower (Supplemental Fig. S1). Postanthesis, the flower weight increased during flower development from day 3 on, and flowers weighed, on average, 120 mg, but could reach, in a few cases, a weight of 160 to 170 mg per flower. The weight did not significantly differ in two consecutive flowering periods regardless of whether plants were noninfested or infested.

Similar results were obtained when the flower weight was analyzed during ongoing larval feeding (Supplemental Fig. S2). Noninfested plants produced 2-d-old flowers that constantly weighed between 100 and 120 mg, which was not significantly influenced by the introduction of larvae and subsequent green leaf herbivory up to 10 d.

Green Leaf Herbivory Does Not Alter the Composition of the Floral VOC Blend

Flowers of noninfested *N. suaveolens* plants emitted a complex VOC mixture, which was dominated by the benzenoids benzyl benzoate, benzyl salicylate, methyl benzoate, and benzyl alcohol (Table I). Cinnamic acid derivatives, as well as benzenoids like methyl salicylate, β-phenylethyl alcohol, *p*-cresol, and β-phenylethyl benzoate, terpenoids like 1,8-cineole and α-farnesene, and nitrogenous compounds like indole and methyl nicotinate contributed to the group of 15 main components. The mixture was completed with compounds that could only be found in traces and could not always be identified. Overall, 50 different VOCs were found (Supplemental Table S2). The composition quality of the floral blend was not altered

during green leaf herbivory. Compounds found in the floral blend of noninfested plants could also be found in the floral blend of herbivore-infested plants and vice versa.

Green Leaf Herbivory Does Not Alter the Amount and Emission Pattern of Floral VOCs

Besides an influence on the quality of the floral blend, VOC amounts and emission patterns could be altered by green leaf herbivory. To address this presumption, the amounts of the main 15 components of the floral blend were quantified to compare (1) the total amount of these VOCs emitted over the whole flowering period of an individual flower; (2) the relative amounts of a single VOC within the floral VOC mixture; and (3) the emission pattern of major constituents.

In two consecutive flowering periods, identical individuals of *N. suaveolens* plants yielded a similar total amount of the 15 main volatiles regardless of whether plants were noninfested or larvae infested (Fig. 3), which corresponded with independent control experiments comparing two consecutive flowering periods of identical noninfested plants (Supplemental Fig. S3). The relative percentage of these components within the floral VOC mixture also did not vary significantly (Fig. 4). The dominant component by far was benzyl benzoate, which represented 57% of the total amount in noninfested and 63% in larvae-infested plants. All other main components were equally consistently emitted during both flowering periods. Significant differences were only observed for the cinnamic acid derivatives. Larvae-infested plants emitted significantly less cinamaldehyde and cinnamyl alcohol compared to noninfested plants (Fig. 4, inset). However, these differences could also be observed in independent control experiments using noninfested plants in two consecutive flowering periods (Supplemental Fig. S4). Regardless of larval infestation, flowers of some *N. suaveolens* batches investigated did not emit cinnamic acid derivatives at all during one or the other flowering periods or emitted them at undetectable levels, which may explain this phenomenon (see also Supplemental Table S3).

The emission patterns of the four major floral VOCs benzyl benzoate, methyl benzoate, benzyl salicylate, and benzyl alcohol for noninfested plants and infested plants are shown in Figure 5. Both patterns were similar. The emission of these four volatiles peaked between midnight and 6 AM. Flowers already released considerable amounts of benzoic acid esters on the first day postanthesis, but maximal emission was reached between days 2 and 4 postanthesis. The emission of benzyl alcohol gradually increased up to its maximum on day 4. On day 5 postanthesis, the emission of all four volatiles declined, and on day 6 only very small amounts were detectable. Noninfested independent control plants exhibited almost identical oscillation characteristics in two consecutive flowering periods (Supplemental Fig. S5), leading to the conclusion that the emission pattern of floral VOCs also remained uninfluenced by green leaf herbivory.

Table I. *Main headspace volatiles emitted from flowers of noninfested and M. sexta-infested N. suaveolens plants*

AS, Identification based on comparison with authentic standards; NP, identification based on comparison with an essential oil (natural product); RI, identification based on retention index; MS, identification based on comparison of mass spectra. $n = 6$.

No.	Compound	RI	Identification
Major components			
1	Benzyl alcohol	1,046	AS, RI, MS
2	Methyl benzoate	1,109	AS, RI, MS
3	Benzyl benzoate	1,825	AS, RI, MS
4	Benzyl salicylate	1,953	MS
Minor components			
5	1,8-Cineole	1,051	AS, RI, MS
6	*p*-Cresol	1,109	RI, MS
7	2-Phenylethyl alcohol	1,129	RI, MS
8	Methyl nicotinate	1,155	RI, MS
9	Methyl salicylate	1,213	AS, RI, MS
10	(*E*)-Cinnamaldehyde	1,295	RI, MS
11	1H-Indole	1,318	RI, MS
12	Cinnamyl alcohol	1,326	RI, MS
13	Methyl cinnamate	1,406	MS
14	α-Farnesene	1,516	NP, RI, MS
15	β-Phenylethyl benzoate	1,937	MS

Floral and Herbivore-Induced α-Farnesene Display an Opposite Emission Pattern

The sesquiterpene α-farnesene was emitted from flowers as a component of the floral blend and, additionally, from leaves as a component of the blend of herbivory-induced semiochemicals. Both floral and foliar α-farnesene emission showed oscillation; however, the emission patterns were found to be opposite (Fig. 6). Whereas the floral α-farnesene was nocturnally emitted between 6 PM and 6 AM, the foliar α-farnesene exhibited a diurnal rhythm with maximal emission between 6 AM and 6 PM. Whereas the emission of the foliar α-farnesene was obviously triggered by herbivory, the flower independently maintained the typical nocturnal α-farnesene emission. This indicates that the VOC emission in both tissues was independently regulated.

Green Leaf Herbivory Does Not Significantly Influence the Expression Characteristics of the Floral BSMT

In addition to monitoring the methyl benzoate emission (Fig. 5), a comparative analysis of the relative values of the *bsmt* transcript level, protein level of BSMT, and BSMT enzyme activity was performed to gain an overall insight into the BSMT expression status at the molecular level. Flowers of noninfested and larvae-infested plants displayed similar transcript, protein, and activity levels as well as expression patterns

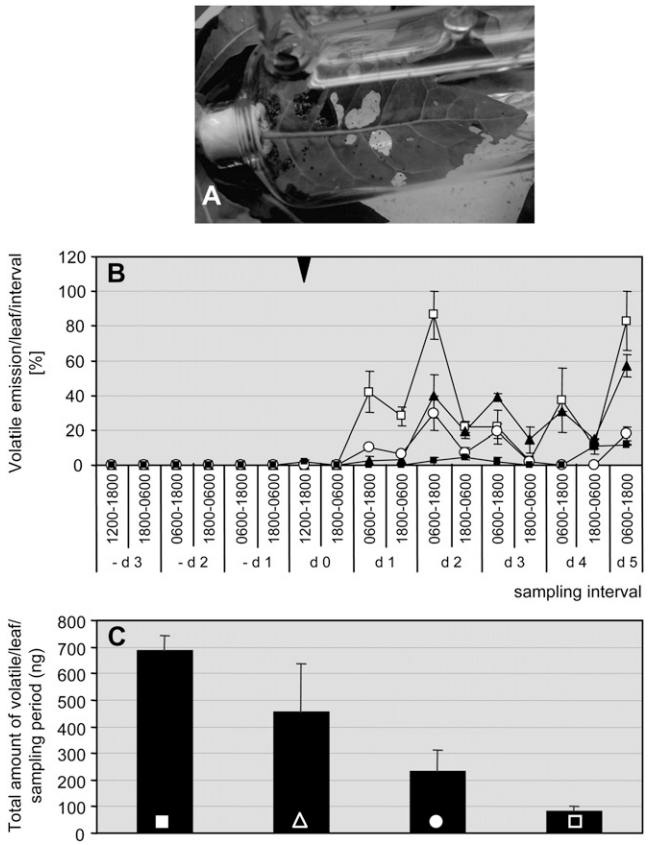

Figure 2. Larval damage and volatile emission from *M. sexta*-infested green leaves of *N. suaveolens*. A, Damage after 5 d of larval herbivory; B, emission pattern; C, total amounts of volatiles emitted during 5 d of sampling. □, α-Farnesene; ▲, α-bergamotene; ○, (*E*)-β-ocimene; ■, (*Z*)-3-hexenyl acetate. A sampling period of 3 d served as the noninfested control (−d 3 to −d 1). First instar larvae of *M. sexta* were introduced on day 0 at noon (▼). Volatiles were collected for the following 5 d. Time-controlled sampling allowed two collection intervals per day. $n = 2$; data = means ± difference.

(Fig. 7). High amounts of the *bsmt* transcript could only be found in young flowers. The maximal daily oscillation was reached around 6 PM (Fig. 7A). The BSMT protein levels did not exhibit pronounced oscillation (Fig. 7B), whereas the BSMT activity showed weak nocturnal oscillation (Fig. 7C). The protein level and enzyme activity quickly increased in young flowers. On days 5 and 6 postanthesis, the BSMT protein level decreased noticeably. These characteristic expression patterns were, in general, also found in independent control experiments using noninfested plants in two consecutive flowering periods (Supplemental Fig. S6).

Investigating flowers of the same age, it could be demonstrated that, at up to 10 d of larval feeding, the three parameters of BSMT expression also remained unaltered. As shown in Figure 8, 2-d-old flowers of noninfested plants exhibited similar transcript levels, protein levels, and enzyme activities on several subsequent days. After the introduction of larvae and ongoing green leaf herbivory, the daily reassessment of BSMT expression levels did not reveal any tendency toward decline or increase. Differences in noninfested plants could be noticed at some time points, but they were never consistent. The BSMT protein levels were very similar and the *bsmt* transcript levels and enzyme activities supported the same trend.

In conclusion, the molecular analysis of BSMT expression supported the results obtained from investigations of VOC emission. Green leaf herbivory did not provoke changes in floral VOC production, as demonstrated for methyl benzoate.

DISCUSSION

To investigate the impact of green leaf herbivory on floral scent production and emission, the tobacco *N. suaveolens* was infested with *M. sexta* larvae. This sphingoid moth is a well-characterized and widely used organism in animal and plant sciences. Native host

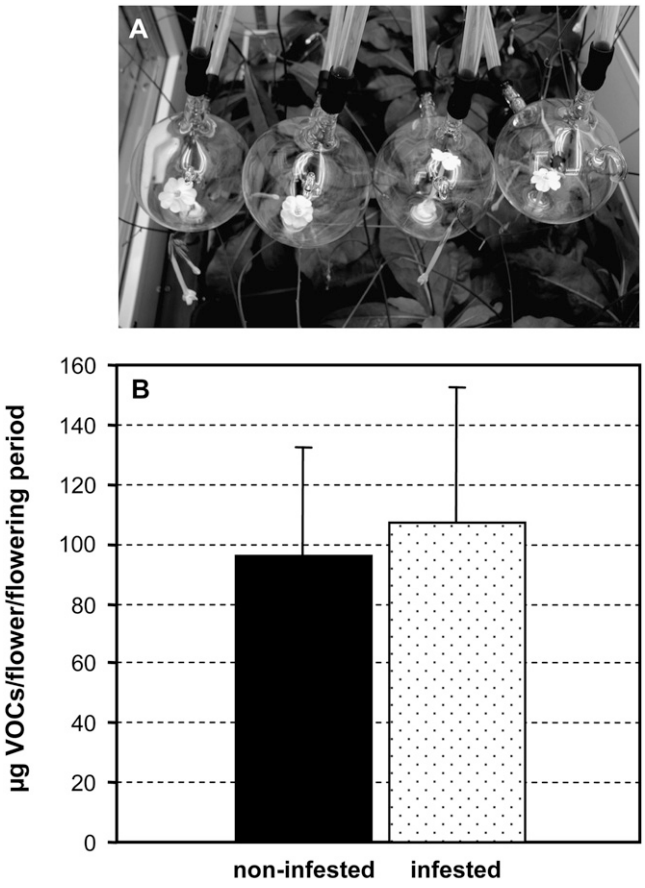

Figure 3. Collection and total amount of floral VOCs emitted from noninfested and *M. sexta*-infested *N. suaveolens* plants. A, Floral headspace device. B, Total VOC amount. Tests were performed using noninfested plants (black bar) and infested plants (spotted bar) in two consecutive flowering periods. For infestation, first instar *M. sexta* larvae were introduced to green leaves at 6 PM on the day of anthesis. $n = 6$; data = means ± SE.

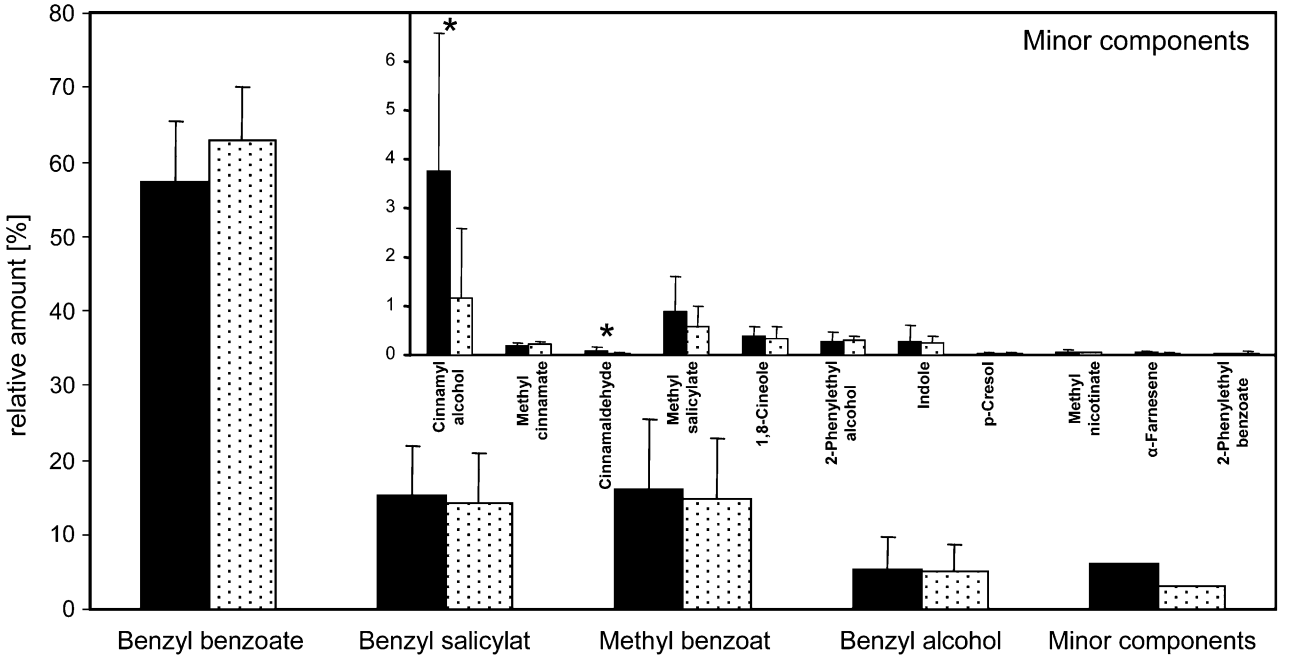

Figure 4. Relative amounts of main floral VOCs emitted from noninfested and *M. sexta*-infested *N. suaveolens* plants. Tests were performed using noninfested plants (black bars) and infested plants (spotted bars) in two consecutive flowering periods. For infestation, first instar *M. sexta* larvae were introduced to green leaves at 6 PM on the day of anthesis. The total amount of selected VOC was set at 100%. $n = 6$; data = means ± SE; asterisks mark significance ($\alpha = 0.05$).

plants are *Nicotiana attenuata* (Voelckel and Baldwin, 2004), *Datura wrightii* (Mira and Bernays, 2002), and the more recently discovered nonsolanaceous *Proboscidea parviflora* (Martyniaceae; Mira and Bernays, 2002), but as an oliphagous specialist, *M. sexta* accepts solanaceous plants in general (del Campo and Renwick, 1999; Voelckel and Baldwin, 2004). However, to assess and compare the susceptibility to *M. sexta*, the green leaf response of *N. suaveolens* was scrutinized. To adapt *M. sexta* larvae to *N. suaveolens* right from the beginning of the experiment (del Campo and Renwick, 2000) and to minimize stress due to diet change, eggs and hatchling larvae were kept off an artificial diet but reared on *N. suaveolens*. Infested leaves of *N. suaveolens* responded with the de novo synthesis of (*E*)-β-ocimene, α-bergamotene, and α- and β-farnesene as well as (*Z*)-3-hexenyl acetate and traces of (*Z*)-3-hexenyl butanoate and β-linalool, indicating that, in our experiments, the plant responded to larval herbivory. Interestingly, the composition of the mixture of these semiochemicals was similar, but not identical, to that of the VOC mixture induced by larval feeding of *Manduca quinquemaculata* on leaves of *N. attenuata* (Kessler and Baldwin, 2001). *N. attenuata* additionally emitted (*Z*)-3-hexenol and methyl salicylate, the latter in very small amounts. α-Farnesene was not present. This similarity of emitted compounds in both *Nicotiana* species is quite striking and supports the hypothesis that foliar feeding of *M. sexta* larvae or other herbivores triggers a specific set of semiochemicals in *Nicotiana* species (Kessler and Baldwin, 2001).

The diurnal rhythms of herbivory-induced green leaf VOCs has been frequently described (Loughrin et al., 1994). Because *M. sexta* larvae are known to show a sequence of continuous activities of food ingestion and exploration and longer nonfeeding periods are only noticeable during moult sleep, which occurs for the first instar larvae after approximately 6 d after eclosure (Reinecke et al., 1980; Bowdan, 1992), this rhythm is quite remarkable and must be endogenously regulated by the plant.

Green leaves of *N. suaveolens* responded with the well-known phenomenon of emission of semiochemicals as an immediate reaction to green leaf herbivory of *M. sexta*. The central question of this study was, however, whether the floral VOC production/emission would be comparably susceptible. The elucidation of nocturnally emitted floral volatiles from noninfested *N. suaveolens* plants resulted in the detection of 50 compounds. Most of the previously reported compounds (Loughrin et al., 1990, 1993; Raguso et al., 2003) were also identified in this study. Flowers of *N. suaveolens* predominantly emitted benzenoids, which seem to be a characteristic feature of floral scents of Australian *Nicotiana* species from the section *Suaveolentes* (Raguso et al., 2006). A high level of benzyl benzoate was noticeable. The monoterpenes 1,8-cineole, D-limonene, and (*E*)-β-ocimene belong to the so-called cineol cassette found to be characteristic of the *Nicotiana* section *Alatae* (Raguso et al., 2003, 2006), but also detected in *N. suaveolens* (Raguso et al., 2003). Recently, a 1,8-cineole-synthase was isolated and characterized

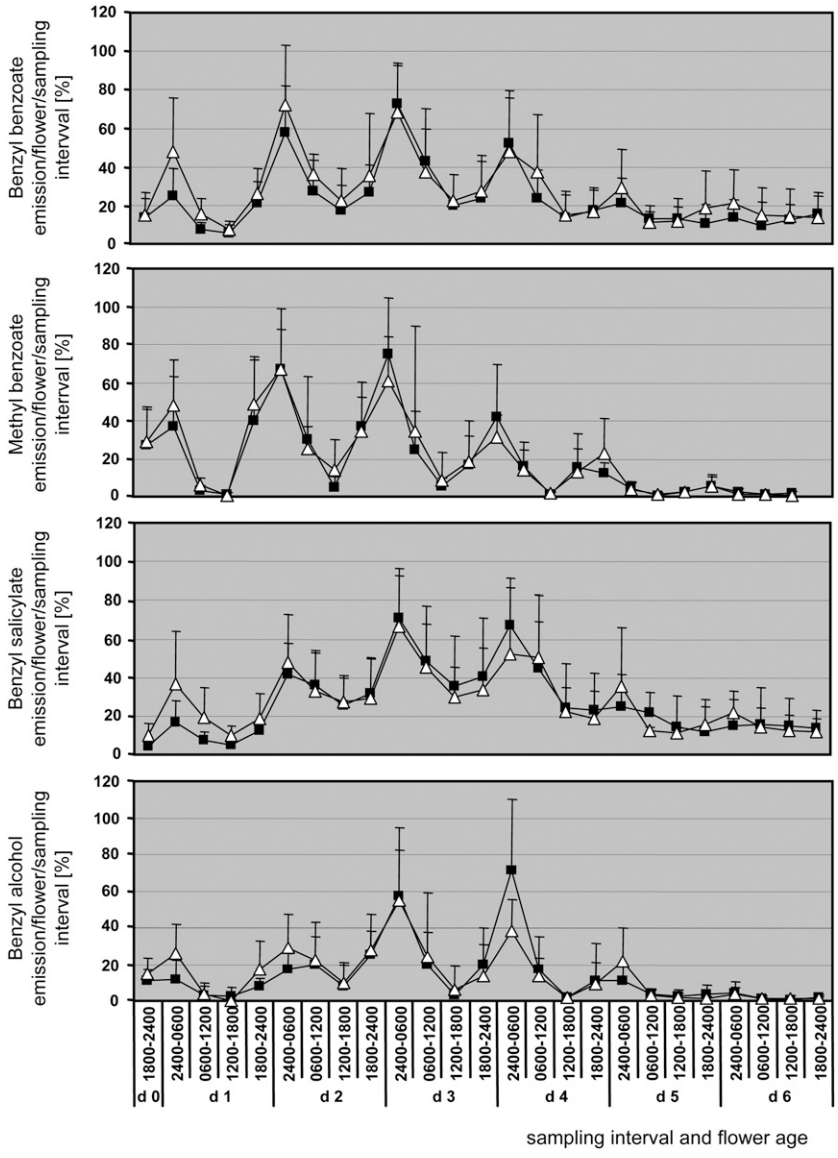

Figure 5. Emission pattern of the major floral VOCs emitted from noninfested and *M. sexta*-infested *N. suaveolens* plants. Tests were performed using noninfested plants (■) and infested plants (△) in two consecutive flowering periods. For infestation, first instar *M. sexta* larvae were introduced to green leaves at 6 PM on the day of anthesis. d 0 = day of anthesis; $n = 6$; data = means ± SE.

from flowers of *N. suaveolens* (Roeder et al., 2007). In vitro, this monoterpene synthase is responsible for the formation of 1,8-cineole, β-myrcene, sabinene, D-limonene, α-terpineol, α-pinene, and (*E*)-β-ocimene. It is likely that the concentration of the missing monoterpenes of the cassette were just under the limit of detection/identification in this study. Elucidation of the nocturnally emitted floral volatiles of *M. sexta* larvae-infested *N. suaveolens* plants revealed similar results. The floral VOC production and emission were not influenced by green leaf herbivory. This was not necessarily expected because two scenarios of interference could be hypothesized.

Based on the first scenario, the floral metabolism could be influenced by green leaf herbivory due to the loss of photosynthetic source tissue and subsequent insufficient supply of sinks with photosynthates and/or impaired translocation of photosynthates. The latter has been shown for silverleaf whitefly infestations, whose feeding on leaves caused reduced photosynthate export from source tissues (Lin et al., 2000). Also, the flower should be an example sink tissue. Experiments using $^{14}CO_2$ or ^{14}C-Suc showed that buds prior to anthesis were indeed strong sinks. However, evidence was provided that the sink intensity declined dramatically upon anthesis (Ho and Nichols, 1977; Robinson et al., 1980; Brun and Betts, 1984). These findings indicate that there is no constant allocation of photosynthates to flowers. Only the developing flower tissue prior to anthesis seems to represent a sink. After anthesis, floral metabolism rests upon storage compounds, a condition that grants metabolic autonomy after flower maturation. Two observations support this hypothesis. (1) Osmophores, floral tissue specialized in VOC synthesis and emission, accumulate enormous deposits of starch, which disappear upon VOC emis-

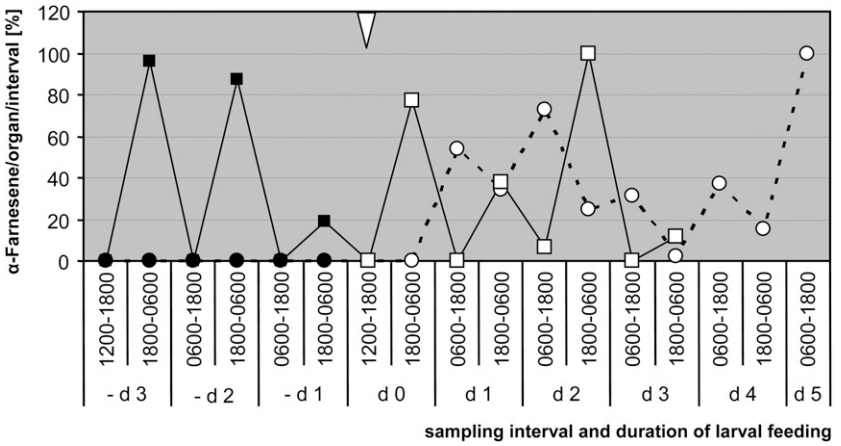

Figure 6. Emission patterns of floral and herbivore-induced foliar α-farnesene in noninfested and *M. sexta*-infested *N. suaveolens* plants. Floral (■) and foliar (●) volatiles of noninfested plants were sampled for 3 d (−d 3 to −d 1). First instar larvae of *M. sexta* were introduced on day 0 at noon (▽) and floral (□) and foliar (○) volatiles were collected for the following days as indicated. Floral volatile collection started on the day of anthesis (−d 3 and d 0). Time-controlled sampling allowed two collection intervals per day. Data represent one set of $n = 2$. One hundred percent relates to 14 ng of floral and 189 ng of herbivore-induced α-farnesene.

sion (Vogel, 1962; Stern et al., 1987); and (2) prior to anthesis, a large number of amyloplasts have been found in the mesophyll cells of petals. These starch deposits also disappeared in mature flowers (Bergougnoux et al., 2007; U. Effmert, unpublished data). With regard to our investigations, this would explain why floral VOC production and emission remained unaltered in infested plants. At the time, when larvae began to feed on leaves, flowers were at the stage of anthesis (day 0, used for analysis) or older and had sufficiently accumulated photosynthates in their petals. Also, the 2-d-old flowers, which were monitored up to 10 d of larval feeding, still managed to accumulate sufficient amounts of carbon sources. Although plants suffered considerable loss of vegetative source tissue, no signs of derogated VOC production and emission were visible. The duration of floral ontogeny in *N. suaveolens* of approximately 5 to 6 d supports this assumption. Upscaling of photosynthate allocation toward the developing flowers upon green leaf herbivory could also

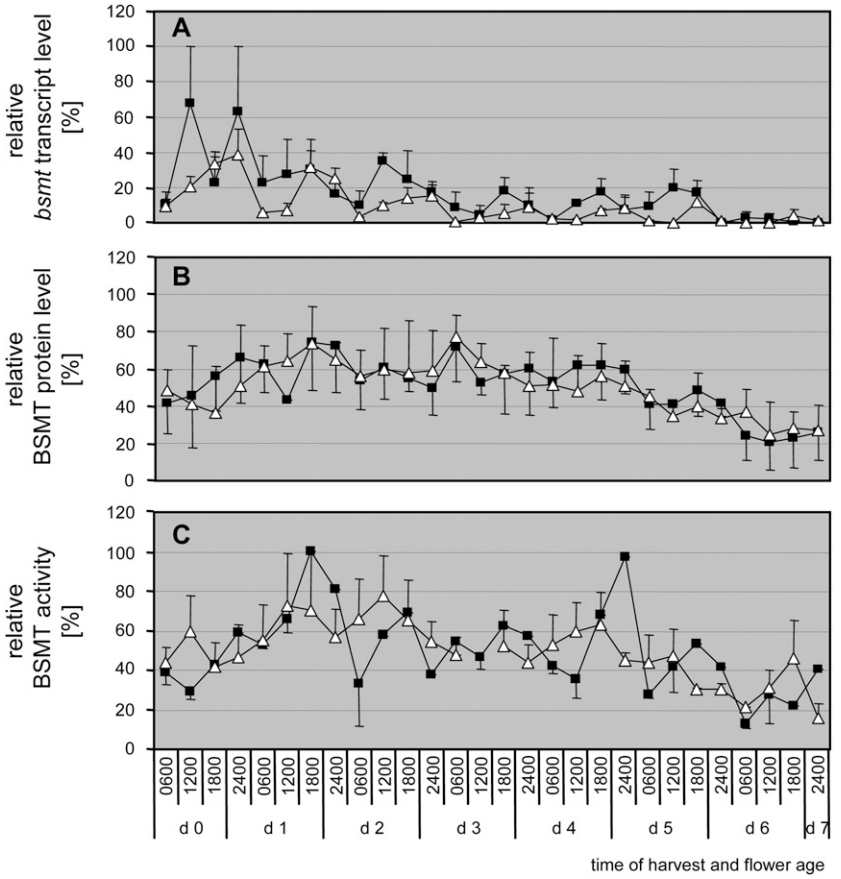

Figure 7. BSMT expression in flowers of noninfested and *M. sexta*-infested *N. suaveolens* plants. Tests were performed using noninfested plants (■) and infested plants (△) in two consecutive flowering periods. For infestation, first instar *M. sexta* larvae were introduced to green leaves at 6 PM on the day of anthesis. d 0 = day of anthesis. Steady-state levels of *bsmt* mRNA were analyzed using northern-blot techniques (A). Analyses of the amount of BSMT protein were performed using western dot-blot techniques with BSMT-specific antibodies (B). In vitro enzyme activity assays were conducted using freshly prepared crude extracts, radioactive-labeled *S*-adenosyl-L-Met as a methyl donor, and benzoic acid as substrate (C). $n = 2$; data = means ± difference.

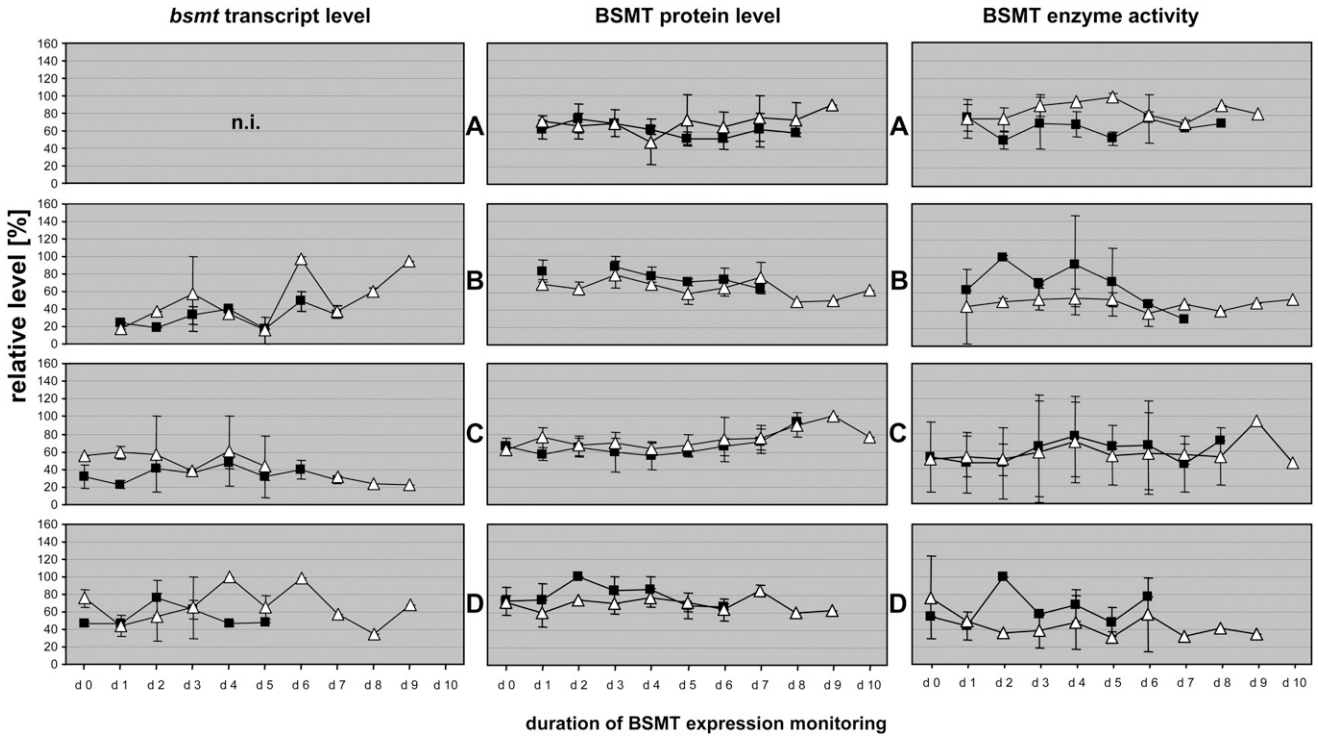

Figure 8. BSMT expression in 2-d-old flowers of noninfested and *M. sexta*-infested *N. suaveolens* plants during progression of larval damage. Flowers were harvested for several days at midnight (A), 6 AM (B), noon (C), 6 PM (D). After control series using noninfested plants (■), first instar *M. sexta* larvae were introduced to green leaves at 6 PM on day 0 and test series were conducted for 10 d of herbivory (△). Steady-state levels of *bsmt* mRNA were analyzed using northern-blot techniques. Analyses of the amount of BSMT protein were performed using western dot-blot techniques with BSMT-specific antibodies. In vitro enzyme activity assays were conducted using freshly prepared crude extracts, radioactive-labeled *S*-adenosyl-L-Met as a methyl donor, and benzoic acid as substrate. $n = 2$; data = means ± difference. n.i., Not investigated.

maintain a sufficient photosynthate supply; however, this is highly speculative at this stage of investigation, especially because it was recently demonstrated that, upon green leaf herbivory, photosynthates were increasingly allocated to the roots (Schwachtje et al., 2006). This gave the plant the advantage of delayed senescence and prolonged flowering with even larger late flowers compared to the noninfested control.

A second scenario would include a floral response as a consequence of the plant's adaptation to a changing biotic environment and would imply intraplant and/or interplant signaling between leaves and flowers similar to the signaling between vegetative organs (Gershenzon, 2007). Interplant (intraspecific) signaling requires volatile signals as described for the information transfer between herbivore-infested and noninfested plants (Dicke and Bruin, 2001; Kost and Heil, 2006) or pathogen-infected and uninfected plants (Shulaev et al., 1997). Signals such as the green leaf volatiles (*Z*)-3-hexenyl acetate and (*E*)-2-hexenal, the monoterpenoid (*E*)-β-ocimene, the fatty acid derivatives (*Z*)-jasmone and methyl jasmonate, or the benzenoid methyl salicylate are assumed to prime or even trigger the defense system in leaves of noninfested receiver plants (Shulaev et al., 1997; Farmer, 2001; Engelberth et al., 2004; Baldwin et al., 2006). The same phenomenon could be shown within a plant. Intraplant signaling (autosignaling) can trigger an induction of defense reactions in yet unaffected plant organs. This mechanism depends on volatile signals (Arimura et al., 2000; Farmer, 2001; Heil and Bueno, 2007) and/or is mediated via a wound-signaling pathway that includes systemin as a long-distance signal and jasmonic acid/methyl jasmonate necessary for recognition and generation of long-distance wound signals (Röse et al., 1996; Thaler, 1999; Seo et al., 2001; Li et al., 2002; Ryan and Moura, 2002; Röse and Tumlinson, 2005). The possibility of interplant and intraplant signaling via volatiles can be excluded due to our experimental design because analyzed flowers were enclosed in glass containers and were therefore not amenable to leaf-born volatile signals. However, the experimental setup would have provided evidence for systemic signaling that could provoke a floral response due to leaf-herbivore interactions. Because floral VOC emission traits remained unaltered in infested plants, it can also be concluded that this hypothesis is not supported. The metabolism of matured flowers seems to be independent and not susceptible to systemic signaling. The opposite oscillations

of foliar and floral α-farnesene emission can be taken as further indication of floral metabolic autonomy postanthesis. Whereas the floral α-farnesene was emitted at night, the leaf-born α-farnesene was released at daytime. This supports an independent flower inherent regulation of floral VOC production and emission.

To summarize, the results of this work indicate that, despite considerable damage of green leaf tissue by *M. sexta*, *N. suaveolens* plants retained the quality of the VOC composition as well as the quantity and emission patterns of their floral VOCs. There was no immediate floral response or delayed interference with the floral VOC production/emission due to green leaf herbivory. Within the time frame investigated, the plant's capacity to invest in VOC production and emission remained unchanged. The pattern of photosynthate allocation between green leaf source tissue and sink tissues, like roots, and the developing flower as well as the ability to assimilate storage in petal cells, might play a key role in this phenomenon. VOC emission would probably only be affected if adequate accumulation and storage of photosynthates in petal tissues failed prior to anthesis.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seedlings of *Nicotiana suaveolens* Lehmann (Solanaceae) were raised on vermiculite (Deutsche Vermiculite Dämmstoff GmbH) in a growth room (temperature, 20°C ± 2°C; photoperiod, 16/8 h; illumination, 150 μmol m^{-2} s^{-1}). At the onset of flowering, plants were transferred to the analysis plant chamber with a headspace collection device (temperature, 24°C ± 1°C; photoperiod, 14/10 h; illumination, 100 μmol m^{-2} s^{-1}). Plants were watered with Hoagland solution (Hoagland and Aronon, 1938). The analysis of VOCs usually started 11 weeks (±3 weeks) after sowing, as soon as sufficient flowering was observed.

Rearing of Larvae

Eggs of *Manduca sexta* (Lepidoptera, Sphingidae), kindly provided by Markus Huss (University of Osnabrück, Germany), were placed on leaves of young *N. suaveolens* plants and incubated at 27°C and a photoperiod of 16/8 h. Larvae hatched after 3 to 4 d. These young first instar larvae were used for experiments.

Experimental Setups

Influence of Larval Leaf Damage on Floral VOC Emission and BSMT Expression

Flower Age-Dependent Analysis. Analysis was performed using 10- to 14-week-old plants that had started flowering and were adapted to the conditions of the analysis plant chamber. Sixteen individual plants belonged to one batch. Control experiments were conducted analyzing two consecutive flowering periods (periods 1 and 2) of noninfested plants. Test series were performed analyzing two consecutive flowering periods of noninfested plants (period 1) and *M. sexta* larvae-infested plants (period 2).

Floral VOCs were collected and analyzed according to the flowering time of individual flowers, starting with the first collection interval from 6 PM to midnight on the day of anthesis. For the next 6 d, flowers were labeled on their day of anthesis. On day 6, two to eight flowers of every age (day of anthesis to 6 DPA) were harvested at 6 AM, noon, 6 PM, and midnight. Flowers were weighed and divided. The two halves were separately pooled and used for protein and RNA extraction to assess BSMT activity and amounts as well as *bsmt* mRNA levels. The tissue for protein extraction was processed and immediately used for activity assays. Aliquots of the crude protein extracts were then stored at −70°C for subsequent use in dot-blot western experiments. The tissue for RNA extraction was immediately submerged into liquid nitrogen and stored at −70°C until processing. After the last collection interval of flowering period 1, new buds were inserted into glass containers and flowering period 2 was investigated. In case of use of infested plants during flowering period 2, six to 10 first instar *M. sexta* larvae were placed onto young rosulate leaves of each plant at 6 PM on the day of flower anthesis. Floral VOCs were collected starting with the first interval (6 PM to midnight) on the day of anthesis. Again, for the next 6 d, flowers were labeled on their day of anthesis, harvested, and processed as described above.

Analysis of BSMT Expression in Flowers of the Same Age during the Progression of Larval Damage. The accumulation of *bsmt* transcripts (northern-blot analysis), BSMT amounts (western dot-blot analysis), and BSMT activities was investigated using flowers of the same age (2 DPA). Eight- to 12-week-old *N. suaveolens* plants that had started flowering were adapted to the conditions of the analysis plant chamber. Two-day-old flowers (two to seven) were harvested for several days at midnight, 6 AM, noon, and 6 PM, weighed, and divided. The halves were separately pooled and processed for protein and RNA extraction as described above. Thereafter, five to 13 first instar larvae of *M. sexta* were distributed at 6 PM on young rosulate leaves of each plant of the batch and the test series was subsequently conducted. Again, 2-d-old flowers were harvested at midnight, 6 AM, noon, and 6 PM for up to 10 d while larval feeding was ongoing. Flowers were weighed, divided, and processed as described above.

The VOC collections were replicated six times ($n = 6$). The corresponding control measurements using noninfested plants in both consecutive flowering periods were carried out in triplicate ($n = 3$). The investigations of *bsmt* transcript accumulation, BSMT amount, and BSMT activity were performed in duplicate.

Analysis of VOCs Emitted from Leaves during Larval Herbivory

Floral and green VOCs were collected in parallel. Two flower buds and two young rosulate leaves were inserted into round-shaped and tube-shaped glass containers, respectively, and VOCs were collected starting with the interval 6 PM to 6 AM on the day of flower anthesis (control experiment). After 3 d and after the last control collection (6 PM to 6 AM), new buds were inserted into round glass containers and, at noon, three first instar *M. sexta* larvae were directly placed inside the tube-shaped glass containers on the same leaves used in the control series. Additionally, up to 17 larvae were distributed on the rosulate leaves of each plant of the batch. The test series started with the first collection interval from noon to 6 PM and ran for 5 d. Experiments were performed in duplicate ($n = 2$).

Collection of Floral and Green Leaf Headspace VOCs

Sampling and accumulation of VOCs of *N. suaveolens* flowers were performed with an open loop system as described by Heath and Manukian (1994). On the day of anthesis, four flower buds were placed into four round glass containers (Ø 8 cm), each of which was softly clamped with a loose-fitting plug of purified cotton wool. Charcoal-purified air entered the containers through an inlet lug at the opposite of the flower inlet. A compressor (Schneider Werkstatt- und Maschinenfabrik) provided a constant inlet air flow of 5.6 L min^{-1}, which was divided between the four glass containers. Volatile-enriched air streams merged into a collection trap that contained 100 mg SuperQ (Alltech Associates). A vacuum pump (KNF Neuberger) sucked the volatile-enriched air with 2.8 L min^{-1} through the trap. The remaining access air vanished through the flower inlet. Isoversinic (Kleinfeldt Labortechnik) fittings connected Teflon tubing (Roth) with the glass containers and the trap. Time-controlled sampling allowed four collection periods of 6 h/d up to 6 d. After adding nonyl acetate as an internal standard (1.5 μg), the trapped volatiles were consecutively eluted with 200 and 100 μL dichloromethane and analyzed by gas chromatography (GC)-mass spectrometry (MS).

To collect headspace VOCs emitted from the leaves of *N. suaveolens*, the setup was slightly modified. The glass containers were adapted to the oblanceolate shape of the leaves (Ø 6 cm, length 14 cm), with the leaf inlet on one side, the air inlet on the opposite, and the air outlet on the top of the

glass container. VOCs of two containers were combined to yield one green leaf VOC sample. Because flowers were analyzed in parallel, independent floral and green air flows were established. Each flow was supplied with charcoal-purified air delivered by aquarium pumps (1.1 L min^{-1}). Volatile-enriched air was sucked over the trap using membrane pumps (Riechle), which provided a constant flow of 0.7 L min^{-1}. Time-controlled sampling allowed two collection periods of 12 h/d for 5 d.

Analysis and Identification of VOCs

Samples were analyzed using a GC-MS-QP5000 from Shimadzu (70 eV; Kyoto) equipped with a DB5-MS column (60 m × 0.25 mm × 0.25 μm; J&W Scientific). Splitless liquid injection of 1 μL of sample was performed at 200°C with a sampling time of 2 min using a CTC autosampler (CTC Analytics). The initial column temperature was set at 35°C, followed by a ramp of 10°C min^{-1} up to 280°C, with a final hold for 15 min at 280°C. Helium was used as the carrier gas. The column flow was set at 1.1 mL min^{-1}, with a linear velocity of 28 cm^{-1}.

Mass spectra were obtained using the scan modus (total ion count, 40–280 *m/z*). The confirmation of compound identity was done by comparison of mass spectra and retention times with those of available authentic standards or essential oils, by comparison of retention indices (Kovats indices), and by comparison of obtained spectra with spectra in the library of the National Institute of Standards and Technology (NIST147).

RNA Extraction and Northern-Blot Analysis

RNA was extracted as described by Chang et al. (1993), which included the extraction of frozen and ground floral material with CTAB buffer (2% [w/v] hexadecyltrimethyl-ammonium bromide, 2% [w/v] PVP-40, 25 mM EDTA, 2 M NaCl, 100 mM Tris-HCl, pH 8.0; 2.7% [v/v] β-mercaptoethanol) at 65°C, chloroform/isoamylalcohol (24:1 [v/v]) extraction of nucleic acids, and lithium chloride precipitation (final concentration 2 M LiCl) of RNA. Five micrograms of the samples were electrophoretically separated and transferred onto a nylon membrane (Roche Diagnostics). The *bsmt* and control probe (18S rDNA) were Dig-dUTP labeled by PCR (Roche Diagnostics) using the *bsmt*-specific primers int1fw (TCCTTACTATTTCCGAACTC) and int3rev (CTCAAGTTACGCTCAATAGA), which delivered a 421-bp fragment, as well as the 18S rDNA primers Pa_rDNA1 (GGTCGCAAGGCTGAAACTT) and Pa_rDNA2 (TTATTGCCTCAAACTTCC) derived from the highly conserved 18S rDNA sequence of *Picea abies*, which delivered a fragment of 300 bp using *N. suaveolens* DNA as a template.

Hybridization was performed at 50°C in DIG-Easy-Hyb (Roche Diagnostics) overnight. After washing in 2× SSC (0.3 M NaCl, 30 mM sodium citrate, 0.1% [w/v] SDS, pH 7.0) at room temperature for 3 × 5 min and in 0.1× SSC (15 mM NaCl, 1.5 mM sodium citrate, 0.1% [w/v] SDS, pH 7.0) at 50°C (55°C for 18S probe) for 2 × 15 min, the membranes were incubated with anti-DIG antibodies linked to alkaline phosphatase (Roche Diagnostics) and subsequently with CDP-Star (Roche; 0.25 μM) in the dark. They were then exposed to the luminescent image analyzer LAS-1000 (Fujifilm), and the luminescence was read for 30 to 40 min (5–10 min for 18S probe). Quantification was performed using the appending Fujifilm Image Gauge software.

Enzyme Extraction and Activity Assays

Crude floral protein extracts were prepared as described by Wang et al. (1997). After harvesting, the flower tissues were immediately submerged in freshly prepared ice-cold extraction buffer (50 mM BisTris-HCl, pH 6.9; 14 mM β-mercaptoethanol, 1% [w/w] PVP-40, and 5 mM $Na_2S_2O_5$; 5 μL mg^{-1} fresh weight) and protease inhibitor was added (completemini, EDTA-free; Roche Diagnostics). The tissue was homogenized in a chilled mortar with one-fifth volume of quartz sand and centrifuged for 15 min at 12,000*g*. The supernatant was immediately used for enzyme assays or, after addition of 10% (v/v) glycerol, stored at −20°C for further processing. Protein quantification was done according to Bradford (1976).

The enzyme assay was performed according to Wang et al. (1997). The reaction contained 10 μL crude enzyme extract, 10 μL assay buffer (250 mM HEPES, 25 mM KCl, pH 6.9), 1 μL benzoic acid (50 mM in ethanol), 1 μL *S*[methyl-^{14}C]adenosyl-L-Met (20 μCi mL^{-1}; 52 mCi $mmol^{-1}$; Hartmann Analytics) and water to a final volume of 50 μL. Samples were incubated for 40 min at 25°C. The reaction was terminated by adding 3 μL HCl (6 M), and the product was extracted with 100 μL ethyl acetate. Thirty microliters of ethyl acetate were mixed with 2 mL scintillation liquid (Perkin-Elmer) and radioactivity was read using a liquid scintillation counter (Tri-Carb 2100TR; Packard).

Preparation of Antibodies

The *bsmt* gene was amplified by reverse transcription-PCR and cloned into the vector pET101/D-TOPO (Invitrogen), which carries a C-terminal hexa-His (Pott et al., 2004). Plasmids were transformed into the *Escherichia coli* overexpression strain HM174(D3). The overexpressed protein was purified to homogeneity by Ni-NTA affinity chromatography (Qiagen) as a native protein. The polyclonal antibody was produced by Davids Biotechnology. It was highly specific and delivered a single signal, which is a requirement for western dot-blot analysis.

Western-Blot Analysis

Western-blot experiments were performed using the dot-blot technique. Crude native protein extracts were spotted onto a nylon membrane (Roche Diagnostics; 3 μg/dot) using a dot-blot device. The membrane was treated for at least 2 h with blocking solution containing 1% (v/v) blocking reagent (Roche Diagnostics) in maleic buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5). After a 2-h incubation with the BSMT-specific antibody diluted 1:10,000 in Tris-buffered solution containing 0.05% (v/v) Triton X-100 (TBS-T) and repeated washing with TBS-T, the membrane was incubated with the secondary anti-rabbit alkaline phosphatase-conjugated antibody (Sigma-Aldrich; diluted 1:20,000 in TBS-T) and again washed with TBS-T and TBS. The membrane was then equilibrated in detection buffer (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$) and incubated with CDP-Star (Roche; 0.25 μM in detection buffer) in the dark. Luminescence was read and quantified using the luminescent image analyzer LAS-1000 and the appending Fujifilm Image Gauge software (Fujifilm).

VOC Quantification and Statistics

VOCs were quantified per flower and per sampling interval. For total amounts, VOC amounts of the entire flowering period of an individual flower were summarized. Assessment of significance of floral VOC emission between noninfested and infested plants was performed using the Wilcoxon-Mann-Whitney test ($\alpha = 0.05$).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AJ628349.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Flower weight of noninfested and *M. sexta*-infested *N. suaveolens* plants.

Supplemental Figure S2. Weight of 2-d-old flowers of noninfested and *M. sexta*-infested *N. suaveolens* plants during progression of larval damage.

Supplemental Figure S3. Total amount of floral VOCs emitted from noninfested *N. suaveolens* plants.

Supplemental Figure S4. Relative amounts of main floral VOCs emitted from noninfested *N. suaveolens* plants.

Supplemental Figure S5. Emission pattern of the major floral VOCs emitted from noninfested *N. suaveolens* plants.

Supplemental Figure S6. BSMT expression in flowers of noninfested *N. suaveolens* plants.

Supplemental Table S1. Herbivory-induced headspace volatiles emitted from green leaves of *N. suaveolens*.

Supplemental Table S2. Headspace volatiles emitted from flowers of noninfested and *M. sexta*-infested *N. suaveolens* plants.

Supplemental Table S3. Relative amounts of selected main VOCs within the floral blend emitted from noninfested and *M. sexta*-infested *N. suaveolens* plants in two consecutive flowering periods.

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LITERATURE CITED

Arimura GI, Ozawa R, Shimoda T, Nishioka T, Boland W, Takabayashi J (2000) Herbivory-induced volatiles elicit defence genes in lima bean leaves. Nature **406:** 512–515

Baldwin IT, Halitschke R, Paschold A, von Dahl CC, Preston CA (2006) Volatile signaling in plant-plant interactions: ''talking trees'' in the genomics era. Science **311:** 812–815

Baldwin IT, Kessler A, Halitschke R (2002) Volatile signaling in plant-plant-herbivory interactions: what is real? Curr Opin Plant Biol **5:** 351–354

Bergougnoux V, Caissard JC, Jullien F, Magnard JL, Scalliet G, Cock JM, Hugueney P, Baudino S (2007) Both the adaxial and abaxial epidermal layers of the rose petal emit volatile scent compounds. Planta **226:** 853–866

Bowdan E (1992) Behavior as a window on physiology: a simple apparatus for recording caterpillar feeding. Phys Educ **7:** 9–14

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Brun WA, Betts KJ (1984) Source/sink relations of abscising and non-abscising soybean flowers. Plant Physiol **75:** 187–191

Canto A, Parra-Tabla V, Garcia-Franco JG (2004) Variations in leaf production and floral display of *Anthurium schlechtendalii* (Araceae) in response to herbivory and environment. Funct Ecol **18:** 692–699

Carroll AB, Pallardy SG, Galen C (2001) Drought stress, plant water status, and floral trait expression in fireweed, *Epilobium angustifolium* (Onagraceae). Am J Bot **88:** 438–446

Chang S, Puryear J, Cairney J (1993) A simple and efficient method for isolating RNA from pine trees. Plant Mol Biol Rep **11:** 113–116

Cole DH, Ashman TL (2005) Sexes show differential tolerance to spittlebug damage and consequences of damage for multi-species interaction. Am J Bot **92:** 1708–1713

Cresswell JE, Hagen C, Woolnough JM (2001) Attributes of individual flowers of *Brassica napus* L. are affected by defoliation but not by intraspecific competition. Ann Bot (Lond) **88:** 111–117

De Moraes CM, Lewis WJ, Paré PW, Alborn HT, Tumlinson JH (1998) Herbivore-infested plants selectively attract parasitoids. Nature **393:** 570–573

del Campo M, Renwick JAA (1999) Dependence on host constituents controlling food acceptance by *Manduca sexta* larvae. Entomol Exp Appl **93:** 209–215

del Campo M, Renwick JAA (2000) Induction of host specificity in larvae of *Manduca sexta*: chemical dependence controlling host recognition and developmental rate. Chemoecology **10:** 115–121

Dicke M, Bruin J (2001) Chemical information transfer between plants: back to the future. Biochem Syst Ecol **29:** 981–994

Dobson HEM (1994) Floral volatiles in insect biology. *In* E Bernays, ed, Insect-Plant Interactions, Vol 5. CRC Press, Boca Raton, FL, pp 47–81

Dobson HEM (2006) Relationship between floral fragrance composition and type of pollinator. *In* N Dudareva, E Pichersky, eds, Biology of Floral Scent. CRC Press, Boca Raton, FL, pp 147–198

Dudareva N, Pichersky E (2006) Floral scent metabolic pathways. Their regulation and evolution. *In* N Dudareva, E Pichersky, eds, Biology of Floral Scent. CRC Press, Boca Raton, FL, pp 55–78

Dufaÿ M, Anstett MC (2003) Conflicts between plants and pollinators that reproduce within inflorescences: evolutionary variations on a theme. Oikos **100:** 3–14

Engelberth J, Alborn HT, Schmelz EA, Tumlinson JH (2004) Airborne signals prime plants against insect herbivore attack. Proc Natl Acad Sci USA **101:** 1781–1785

Euler M, Baldwin IT (1996) The chemistry of defense and apparency in the corollas of *Nicotiana attenuata*. Oecologia **107:** 102–112

Farmer EE (2001) Surface-to-air signals. Nature **411:** 854–856

García MB, Ehrlén J (2002) Reproductive effort and herbivory timing in a perennial herb: fitness components at the individual and population levels. Am J Bot **89:** 1295–1302

Gershenzon J (2007) Plant volatiles carry both public and private messages. Proc Natl Acad Sci USA **104:** 5257–5258

Heath RR, Manukian A (1994) An automated system for use in collecting volatile chemicals released from plants. J Chem Ecol **20:** 593–608

Heil M, Bueno JCS (2007) Within-plant signalling by volatiles leads to induction and priming of an indirect plant defense in nature. Proc Natl Acad Sci USA **104:** 5467–5472

Ho LC, Nichols R (1977) Translocation of ^{14}C-sucrose in relation to changes in carbohydrate content in rose corollas cut at different stages of development. Ann Bot (Lond) **41:** 227–242

Hoagland DR, Aronon DL (1938) The water-culture method of growing plants without soil. California Agriculture Experimental Station Circular **374:** 1–39

Ivey CT, Carr DE (2005) Effects of herbivory and inbreeding on the pollinators and mating system of *Mimulus guttatus* (Phrymaceae). Am J Bot **92:** 1641–1649

Japan Tobacco Inc. (1994) The Genus *Nicotiana* Illustrated. Japan Tobacco Inc., Tokyo

Kessler A, Baldwin IT (2001) Defensive function of herbivore-induced plant volatile emissions in nature. Science **291:** 2141–2144

Kost C, Heil M (2006) Herbivore-induced plant volatiles induce an indirect defence in neighbouring plants. J Ecol **94:** 619–628

Leege LM, Wolfe LM (2002) Do floral herbivores respond to variation in flower characteristics in *Gelsemium sempervirens* (Loganiaceae), a distylous vine? Am J Bot **89:** 1270–1274

Li L, Li C, Lee GI, Howe GA (2002) Distinct roles for jasmonate synthesis and action in the systemic wound response of tomato. Proc Natl Acad Sci USA **99:** 6416–6421

Lin TB, Wolf S, Schwartz A, Saranga Y (2000) Silverleaf whitefly stress impairs sugar export from cotton source leaves. Physiol Plant **109:** 291–297

Loughrin JH, Hamilton-Kemp TR, Andersen RA, Hildebrandt DF (1990) Headspace compounds from flowers of *Nicotiana tabacum* and related species. J Agric Food Chem **38:** 455–460

Loughrin JH, Hamilton-Kemp TR, Burton HR, Andersen RA (1993) Effect of diurnal sampling on the headspace composition of detached *Nicotiana suaveolens* flowers. Phytochemistry **32:** 1417–1419

Loughrin JH, Manukian A, Heath RR, Turlings TCJ, Tumlinson JH (1994) Diurnal cycle of emission of induced volatile terpenoids by herbivore-injured cotton plants. Proc Natl Acad Sci USA **91:** 11836–11840

Mattiacci L, Ambühl Rocca B, Scascighini N, D'Alessandro M, Hern A, Dorn S (2001) Systemically induced plant volatiles emitted at the time of ''danger''. J Chem Ecol **27:** 2233–2252

Mira A, Bernays EA (2002) Trade-offs in host use by *Manduca sexta*: plant characters vs natural enemies. Oikos **97:** 387–397

Ornelas JF, Gonzalez C, Jimenez L, Lara C, Martinez AJ (2004) Reproductive ecology of distylous *Palicourea padifolia* (Rubiaceae) in tropical montane cloud forest. II. Attracting and rewarding mutualistic and antagonistic visitors. Am J Bot **91:** 1061–1069

Paré PW, Tumlinson JH (1999) Plant volatiles as a defense against insect herbivores. Plant Physiol **121:** 325–331

Pellmyr O, Tang W, Groth I, Bergström G, Thien LB (1991) Cycad cone and angiosperm floral volatiles: inferences for the evolution of insect pollination. Biochem Syst Ecol **19:** 623–627

Pichersky E, Gershenzon J (2002) The formation and function of plant volatiles: perfumes for pollinator attraction and defense. Plant Biol **5:** 237–243

Pott MB, Hippauf F, Saschenbrecker S, Chen F, Ross J, Kiefer I, Slusarenko A, Noel JP, Pichersky E, Effmert U, et al (2004) Biochemical and structural characterization of benzenoid carboxyl methyltransferases involved in floral scent production in *Stephanotis floribunda* and *Nicotiana suaveolens*. Plant Physiol **135:** 1946–1955

Raguso RA, Levin RA, Foose SE, Holmberg MW, McDade LA (2003) Fragrance chemistry, nocturnal rhythms and pollination ''syndromes'' in *Nicotiana*. Phytochemistry **63:** 265–284

Raguso RA, Schlumpberger BO, Kaczorowski RL, Holtsford TP (2006) Phylogenetic fragrance patterns in *Nicotiana* sections *Alatae* and *Suaveolentes*. Phytochemistry **67:** 1931–1942

Reinecke JP, Buckner JS, Grugel SR (1980) Life cycle of laboratory-reared

tobacco hornworms, *Manduca sexta*, a study of development and behaviour, using time-lapse cinematography. Biol Bull **158:** 129–140

Ribnicky DM, Shulaev V, Raskin I (1998) Intermediates of salicylic acid biosynthesis in tobacco. Plant Physiol **118:** 565–572

Robinson M, Harav I, Halevy AH (1980) Distribution of assimilates from various source leaves during the development of *Gladiolus grandiflorus*. Ann Bot (Lond) **45:** 113–122

Roeder S, Hartmann AM, Effmert U, Piechulla B (2007) Regulation of simultaneous synthesis of floral scent terpenoids by the 1,8-cineol synthase of *Nicotiana suaveolens*. Plant Mol Biol **65:** 107–124

Röse USR, Lewis WJ, Tumlinson JH (1998) Specificity of systemically released cotton volatiles as attractants for specialist and generalist parasitic wasps. J Chem Ecol **24:** 303–319

Röse USR, Manukian A, Heath RR, Tumlinson JH (1996) Volatile semiochemicals released from undamaged cotton leaves. Plant Physiol **111:** 487–495

Röse USR, Tumlinson JH (2004) Volatiles released from cotton plants in response to *Helicoverpa zea* feeding damage on cotton flower buds. Planta **218:** 824–832

Röse USR, Tumlinson JH (2005) Systemic induction of volatile release in cotton: how specific is the signal to herbivory? Planta **222:** 327–335

Ryan CA, Moura DS (2002) Systemic wound signaling in plants: a new perception. Proc Natl Acad Sci USA **99:** 6519–6520

Sampson BJ, Cane JH (1999) Impact of enhanced ultraviolet-B-radiation on flower, pollen, and nectar production. Am J Bot **86:** 108–114

Sanchez-Lafuente AM (2007) Corolla herbivory, pollination success and fruit predation in complex flowers: an experimental study with *Linaria lilacina* (Scrophulariaceae). Ann Bot (Lond) **99:** 355–364

Schwachtje J, Minchin PEH, Jahnke S, van Dongen JT, Schittko U, Baldwin IT (2006) SNF1-related kinases allow plants to tolerate herbivory by allocation carbon to the roots. Proc Natl Acad Sci USA **103:** 12935–12940

Seo HS, Song JT, Cheong JJ, Lee YH, Lee YW, Hwang I, Lee JS, Choi YD (2001) Jasmonic acid carboxyl methyltransferase: a key enzyme for jasmonate-regulated plant responses. Proc Natl Acad Sci USA **98:** 4788–4793

Shulaev V, Silverman P, Raskin I (1997) Airborne signalling by methyl salicylate in plant pathogen resistance. Nature **385:** 718–721

Steets JA, Ashman TL (2004) Herbivory alters the expression of a mixed-mating system. Am J Bot **91:** 1061–1069

Stern WL, Curry KJ, Pridgeon AM (1987) Osmophores of *Stanhopea* (Orchidaceae). Am J Bot **74:** 1323–1331

Thaler JS (1999) Jasmonate-inducible plant defences cause increased parasitism of herbivores. Nature **399:** 686–688

Theis N (2006) Fragrance of Canada thistle (*Cirsum arvense*) attracts both floral herbivores and pollinators. J Chem Ecol **32:** 917–927

van Poecke RMP, Dicke M (2004) Indirect defence of plants against herbivores: using *Arabidopsis thaliana* as a model plant. Plant Biol **6:** 387–401

Voelckel C, Baldwin IT (2004) Generalist and specialist lepidopteran larvae elicit different transcriptional responses in *Nicotiana attenuata*, which correlate with larval FAC profiles. Ecol Lett **7:** 770–775

Vogel S (1962) Duftdrüsen im Dienste der Bestäubung: Über Bau und Funktion der Osmophoren. Abhandl Math-Naturwiss Kl Akad Wiss Mainz **10:** 1–165

Wang J, Dudareva N, Bhakta S, Raguso RA, Pichersky E (1997) Floral scent production in *Clarkia breweri* (Onagraceae). II. Localization and developmental modulation of the enzyme *S*-adenosyl-L-methionine: (iso)eugenol *O*-methyltransferase and phenylpropanoid emission. Plant Physiol **114:** 213–221