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Michael Euler · Ian T. Baldwin

The chemistry of defense and apparency in the corollas of *Nicotiana attenuata*

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Abstract The morphological and chemical characteristics of flowers which attract pollinators present a dilemma for plants; advertising may increase the "apparency" of plants to their predators and some pollinators are also predators. We explore how a self-compatible disturbance species, *Nicotiana attenuata*, copes with this potential dilemma by examining the changes in emission of chemicals from flowers in response to pollination and herbivory. We propose that chemical changes induced by herbivory and pollination reflect the function of the chemicals in the plant. The emission of a single compound, benzyl acetone (BA, 4-phenyl-2-butanone), by flowers increases dramatically (50×) in the evening, peaking just after dark – a pattern of emission characteristic of moth-pollinated flowers. Pools of BA were found only in the outer lip of the corolla where pollinators come in contact with the flower, and diurnal changes in the size of the corolla pool closely paralleled the amount emitted by intact flowers throughout the day, as determined by headspace sampling. Pollination dramatically decreases both the pools of BA in the corolla and its emission from flowers. Similarly, nicotine, a broadly biocidal defense metabolite and an induced defense in vegetative and reproductive tissues, is also found in the headspace of flowers and is principally localized in the basal parts of the corolla below the attachment of the filaments and the nectar reward. Moreover, the dynamics of the corolla pools of BA and nicotine throughout the day are consistent with their roles in advertisement and defense, respectively. The corolla pools of nicotine are stable throughout the day except during the period of peak BA production and emission when nicotine pools decrease significantly. The coordinated increase in BA emission and decline in nicotine pools are not inexorably linked, because herbivory or mechanical damage to corolla tissue rapidly increases corolla nicotine pools without affecting the increase in BA pools. Similarly, leaf damage results in a slower, systemic increase in corolla nicotine pools during reproductive growth but again does not affect BA pools. Excised flowers emitted BA in a manner similar to that of intact flowers, and excision of a majority of flowers from a plant did not alter the BA emission patterns of the remaining flowers. We conclude that although *N. attenuata*'s defensive and advertisement chemistries respond synchronously to some environmental stimuli, the flowers' chemical responses to pollinators and herbivores are distinct and the differences reflect their ecological roles. We propose that the cost-benefit framework of the optimal defense and apparency theories can be fruitfully applied to the allocation of defense metabolites and floral volatiles that function in pollinator attraction, and that this framework can be readily tested by manipulating the patterns of the emissions of plants in the field.

Key words Floral volatiles · Benzyl acetone · Apparency · Plant defense · Nicotine

Michael Euler · Ian T. Baldwin (✉)
Department of Biological Sciences, SUNY,
University at Buffalo, Buffalo, NY 14260, USA
fax: (716) 645-2975

Introduction

The "apparency" of plants to herbivores – as defined by their growth form, density, longevity, and persistence – may determine the types and patterns of defense used by plants to protect themselves from predation (Feeny 1976; Rhoades and Cates 1976; Chew and Courtney 1991). More recently, plants have been found to change their olfactory apparency to carnivores by systemically emitting volatile compounds when attacked by herbivorous insects; these compounds function as "alarm calls" and increase the hunting efficiency of insect parasitoids (Dicke 1994; Tumlinson et al. 1993; Drukker et al. 1995). Moreover, plants change the apparency of their reproductive structures to pollinators and dispersal agents as these structures mature. While considerable evidence demonstrates that changes in apparency and defense during fruit ripening are coordinated (Heftmann and Schwimmer 1972; Pearce et al. 1988), little information is available on how plants coordinate the potentially conflicting

requirements of defense and pollinator attraction during flowering.

Floral odors are complex blends of volatile metabolites. Most blends consist of compounds with both attractant and repellent qualities to particular insect species. Extensive anecdotal and experimental evidence indicates that volatile compounds emitted from flowers play an important role in attracting pollinators (Dobson 1994). However, these floral volatiles may function in more than a purely synomonal role as pollinator attractants. Many of these compounds function allomonally to repel potential attackers. This role may have been the ancestral function of floral volatiles (Pelmyr and Thien 1986; Pelmyr et al. 1991). Last, floral volatiles are known to function kairomonally by serving as orientation cues: helping herbivorous animals to locate feeding, mating or oviposition sites (Metcalf and Metcalf 1991). Volatile feeding deterrents produced by flowers may have been co-opted by animals and first used as orientation cues to locate feeding, mating and oviposition sites, and only later for communication with pollination mutualists (Pelmyr et al. 1991). While the composition of floral blends may help maintain species-specific pollination mutualisms (Williams 1982), the specificity with which insects choose to visit flowers may be the result of the quantitative relationship between the attractant and repellent components in the blend. For example, pollination and predation are thought to maintain distinct odor-morphs within a single interbreeding population in the dimorphically-scented *Polemonium viscosum* (Galen and Newport 1988). The sweet-smelling odor-morph of this flower attracts bumblebees, which are efficient pollinators, as well as nectar-thieving ants, while the skunky odor-morph attracts flies, which are inefficient pollinators, and repels ants. Apparently the presence of ants in an area selects for the skunky odor-morph, while the sweet odor-morph dominates in the absence of ants. Although the chemical basis of these particular odor-morphs remains undescribed, clearly a great deal of similarity exists among the chemical structures that function as repellents and those that function as attractants (Rodriguez and Levin 1976). Moreover the compounds which attract one insect may repel another (Berenbaum and Seigler 1992), which makes it difficult to generalize the functional roles of allelochemicals from their structures.

The plasticity of synthesis, emission, and release of allelochemicals in response to environmental signals informs the functional roles that particular allelochemicals play in the life of a plant. For example, a synomonal role for floral volatiles is suggested by the high correlation between their emission and the activity of the plant's pollinators (Jakobsen and Olsen 1994) and by the abrupt decrease in emission after pollination (Tollsten and Bergstrom 1989). The correspondence between emission of particular compounds and pollinator activity is particularly close in hawkmoth-pollinated flowers. These exhibit striking diurnal emission patterns; the largest emissions occur in the evening when these moths are active (Loughrin et al. 1990; Heath et al. 1992; Knudsen and Tollsten 1993). This pattern in scent production, in addition to informing pollinators of the reward status of the flower, may also help minimize the biosynthetic and ecological costs of advertising for pollinators. Similarly, an allomonal role in plant defense is strongly suggested for metabolites that are produced in response to herbivory (Baldwin 1994; Tallamy and Raupp 1991), particularly when the allelochemicals are known to be broadly toxic or biocidal.

Several *Nicotiana* sp. have been found to be exceptionally plastic in both their defensive chemistry (alkaloids, phenolics, sesquiterpenoid phytoalexins, proteinase inhibitors, PR proteins; references in Baldwin 1994) and in their emission of floral volatiles (Loughrin et al. 1990, 1991). In this paper, we report that the flowers of *N. attenuata* emit dramatically greater quantities of volatiles at night and that a single compound, benzyl acetone (4-phenyl-2-butanone; BA), accounts for a vast majority of that change. BA is produced and emitted from the outer lobes of the corolla, and the emissions decrease dramatically after pollination. BA has been reported as a minor component of the floral headspace of only one other plant species, the orchid *Dendrobium superbum* (Flath and Ohinata 1982), and is structurally similar to amyl salicylate and amyl benzoate – compounds reported to attract tobacco hornworm adults, *Manduca sexta* (Morgan and Lyon 1928). Therefore, we use BA emissions as a proxy for allocation to floral "advertisement". Nicotine is also found in the corollas and to a lesser extent in the headspace of these flowers, and since it is a potent nerve toxin with broad insecticidal properties (Schmeltz 1971), which the plant uses as an inducible defense (Baldwin 1991; Baldwin and Ohnmeiss 1993; Baldwin and Karb 1995), we use this compound as a proxy for a plant's allocation to floral defense. We explore the potentially conflicting requirements of advertisement for pollinators and defense against herbivores by examining the changes in BA and nicotine pools in the corollas of plants subjected to simulated and real herbivory and pollination.

Materials and methods

Plant natural history

N. attenuata is an herbaceous annual native to North America which can be found in dry washes and recently disturbed or burned areas throughout the Great Basin desert (Goodspeed 1954; Baldwin and Morse 1994). As it matures it produces a central racemose or narrowly paniculate indeterminate inflorescence with self-compatible perfect flowers that have the characteristics of a typical moth-pollinated flower (Knudsen and Tollsten 1993). The flowers take 3 days to fully expand and open their corollas, which then remain open for 2–3 nights, occasionally closing during the day. The fruit, a capsule, matures and dehisces 10–300 small (120–160 μg) seeds approximately 16 days after pollination. Hummingbirds, hawkmoths, and bees visit these flowers, and hawkmoth larvae cause extensive leaf damage (Wells 1959; Baldwin and Ohnmeiss 1993; I. Baldwin unpublished work).

Plant growth

N. attenuata seeds from the 3rd generation of glasshouse-grown self-pollinated plants started from seed collected in 1988 on the DI

Ranch in southwestern Utah (section T40S R19W) were soaked in an aqueous extract of wood smoke with 9.8 $mmol^{-1}$ KNO_3 to stimulate germination (Baldwin et al. 1994b), germinated in flats of Cornell mix A (Boodley and Sheldrake 1977) and transplanted to individual pots (0.375 or 4.0 l) after three weeks of growth. Plants were maintained in a glasshouse with supplemental lighting from 400-W high-pressure sodium lamps or 1500-W quartz halogen lamps for 16 h/day (5 a.m.–9 p.m.). The supplemental lighting of provided a minimum photosynthetically active radiation (PAR) of 220 $\mu mol\ m^{-2} s^{-1}$.

Analysis of headspace volatiles

Two types of traps were used: Tenax traps for the identification of headspace volatiles and activated charcoal traps for the quantification of diurnal fluctuations of volatiles identified in the Tenax traps. Tenax and thermal desorbtion analysis of headspace volatiles provide excellent quantitative information but is too time-consuming for the large sample sizes required for the analysis of diurnal fluctuations. The trapping of volatiles on activated charcoal and their elution with solvent is readily amenable to the automation of sample analysis, but had the drawback that we were not able to extract nicotine quantitatively from these traps. Hence, even though nicotine was detected in the headspace of flowers trapped on Tenax traps, we had to rely on measures of nicotine pools in the corolla to measure the dynamics of this metabolite.

Tenax traps

Volatiles were collected for identification by passing headspace air through Tenax adsorbant [101.6±0.5 mg (mean ± SE) 60–80 mesh; Hewlett-Packard, Kennett Square, Pa.] secured in stainless steel short path thermal desorbtion tubes (Scientific Instrument Services, Ringoes, N.J.) with silanized glass wool. Tenax traps were conditioned at 250°C for 10 min under continuous He flow of 20 ml/min. Trapped headspace volatiles were thermally desorbed (250°C for 5 min) directly into a 200°C splitless injection port and cryo-focused on a capillary column (50 m×0.2 mm HP-5 or 25 m×0.2 mm HP-1) maintained at 5°C with liquid CO_2. After thermal desorbtion, the column was heated to 50°C at 15°C/min and from 50°–225°C at 5°C/min. Compounds eluting from the column were detected with a mass selective detector (Hewlett Packard 5971) and identified by comparison to mass spectra found in the Wiley 138 K mass spectra database and with the retention times and mass spectra of authentic compounds. Volatiles identified in this manner were collected from: (1) whole plants enclosed in 4.4-l bell jars; (2) individual flowers attached to plants enclosed in 30-ml open-ended test tubes; and (3) freshly excised flowers with their pedicels submerged in water in a 0.1-l Wheaton purge and trap system (Scientific Instrument Services). For whole plant (method 1) and individual flower (method 2) collections, headspace air was drawn through attached Tenax traps with a vacuum pump at 928±36 ml/min. For excised flower collections (method 3), activated charcoal-filtered air was forced through the Wheaton trap containing the flowers and through an attached Tenax trap at 20 ml/min. A filter paper disk with 10 µg methyl benzoate as an internal standard was also placed in the Wheaton trap. Methyl benzoate is diurnally emitted by *N. sylvestris* flowers (Loughrin et al. 1990) but was not detected in headspace collections from *N. attenuata* plants or flowers. Tenax traps were sealed and stored at 5°C until thermal desorbtion.

Activated charcoal traps

Volatiles were trapped for quantification on 12.7±0.2 mg of 100-mesh activated charcoal secured in borosilicate glass pasteur pipets with fine pore polyester packing foam. We found that this trap optimized both the efficiency of processing and the accuracy of our quantification. Prior to collecting volatiles 421±4 ng of 1-phenyl-2-butanone (11.97±0.11 mg of a solution containing 46.48 µg 1-phenyl-2-butanone/ml CH_2Cl_2) was added to each charcoal trap under vacuum as an internal standard. 1-phenyl-2-butanone is structurally similar to BA (4-phenyl-2-butanone), the dominant component of *N. attenuata*'s nocturnal floral volatile emission, and was not detected in headspace collections from *N. attenuata*. After collection of volatiles, charcoal traps were stored at 5°C until extraction; traps were never reused. BA standard curves were prepared by adding 11.97±0.11 mg of internal standard solutions containing 4.56 to 452.27 µg/ml BA to fresh charcoal traps. These traps were then processed and analyzed with the headspace samples. Acetophenone and decanal standard curves were prepared in the same manner. The amounts of BA, acetophenone, and decanal were quantified because they were detected in Tenax collections of floral headspace. Volatiles were extracted from the activated charcoal by immersing the traps in 1 ml of CH_2Cl_2 and sonicating for 15 min. The eluent was transferred to a 2 ml crimp=top vial and the samples stored at 5°C until analysis.

Of each CH_2Cl_2 sample 1 µl was injected into a 250°C injection port with an automatic sampler (Hewlett Packard 7673) with a 0.5-min splitless injection; constituents were separated on a 25 m×0.2 mm HP-1 column maintained at 50°C for 5 min and subsequently heated at 15°C/min to 200°C, with a continuous He flow of 0.6 ml/min, and detected with a selective ion monitoring (SIMS) acquisition tuned to the following target ions: 148 m/z for 1-phenyl-2-butanone and BA (4-phenyl-2-butanone), 120 m/z for acetophenone and 82 m/z for decanal, at the following retention times: 10.4, 10.6, 8.1 and 10.3 min, respectively. A minimum of three sets of standard curve samples were analyzed with each experiment for the determination of relative response factors (RRFs) and to verify retention times. The coefficient of variability (CV) for the RRFs was low, varying from 1 to 3%.

Quantification of BA and nicotine pools in corolla tissue

Corollas were weighed immediately after excission to the nearest 0.2 mg and extracted in 1.18±0.06 g of CH_2Cl_2 containing 50.8 ng/ml methyl benzoate as an internal standard for 24–36 h at 5°C. Samples were sonicated for 5 min and the eluent was transferred to a 2-ml crimp-top vial and stored at 5°C until analysis. This extraction method optimized both extractable BA and nicotine. Extracts of corollas were analyzed using the same GC program described for the quantification of headspace volatiles; however, the SIMS target ions were 105 m/z for methyl benzoate and BA and 84 m/z for nicotine at RTs of 8.6, 10.6 and 11.8 min respectively. Internal standard solutions containing 26.4–528.5 ng/ml BA and 265.7–5315.0 ng/ml nicotine were used to prepare standard curves. A minimum of three standard curves were run with each experiment, and the CVs for the RRFs were 4.7% for BA and 8.1% for nicotine.

Experimental protocols

Distribution of BA and nicotine within the corolla

Five flowers were harvested from a single plant at 9:30 p.m. (the time of peak BA emission), their corollas and attached filaments were separated from the calyx, weighed and extracted after being dissected into the following three sections: the flared lip of the corolla, and the corolla tube both above and below the point of attachment of the filaments. The flared lip of the corolla has five distinct lobes; these lobes were further dissected into inner and outer sections. Concentrations of BA and nicotine were expressed as a percentage of wet mass.

Diurnal changes in the emission of volatiles and the corolla pools of BA and nicotine

Diurnal changes in the emission of volatiles from seven newly-opened flowers on seven plants were individually quantified. Each flower was enclosed in an open-ended test tube and air was drawn

over the flower and through an activated charcoal trap attached to the end of the test tube at 119±6 ml/min. From each flower five consecutive 2-h collections were made between 5 p.m. and 3 a.m., followed by a single 5-h collection till 8 a.m. Air was collected from a single open-ended test tube without a flower during each time interval to determine ambient concentrations of volatiles in the glasshouse. Emissions were expressed as ng/h/corolla above the background.

The relationship between BA emission and BA pools in corollas was examined by quantifying diurnal changes in corolla BA pools over the same time interval during which emissions were quantified. Twelve plants with more than seven newly opened flowers were used in this study. A single corolla was sampled from each plant at 1.5-h intervals between 5 p.m. and 2 a.m. Extractable pools were expressed as ng/corolla.

Response to pollination

The effect of pollination on BA and nicotine pools in corollas and on BA emissions from corollas was examined using paired flowers that were either pollinated or not. Mature but un-opened flowers – those that would be open and receptive by the next dark period – were paired on four large plants by their position on branches. In order to prevent self-pollination, these flowers were emasculated at 2 p.m. by cutting the corolla one half its length and removing the anthers. Between 5:30 and 6:30 p.m., after the flowers had opened, one flower from each pair of emasculated flowers was pollinated. Pollination was accomplished by removing pollen from the dehiscent anthers of unmanipulated flowers and applying it to the stigmatic surface of the emasculated flowers with a glass pipette tip filled with glass-wool. Unpollinated flowers received the same manipulation with a new glass-wool filled pipette tip which did not contain pollen. Corollas were harvested at 9:30 p.m. for BA and nicotine pool determinations, and headspace samples were collected between 7 p.m. and 7 a.m. on activated charcoal traps from individual flowers attached to plants by enclosing them in open-ended test-tubes as previously described. Nine pairs of flowers were analyzed for emissions and corolla pool sizes.

Response to floral damage

The effect of corolla herbivory and mechanical simulations thereof on corolla BA and nicotine pools were examined in two experiments. Since corolla pools of BA closely tracked BA emissions, these experiments quantified corolla pools. The first experiment examined the influence of herbivory and mechanical damage to immature elongating corollas on the BA and nicotine pools of the corollas at the time of maturity. Flowers were labeled on six plants at 1 p.m. These were expected to open for the first time the next evening and randomly assigned to the following three treatment groups on each plant: undamaged, razor-damaged and caterpillar-damaged. Each plant had a minimum of five flowers in each treatment group. Razor-damage consisted of an incision one half the length of the corolla tube. One 2nd or 3rd instar *Manduca sexta* larvae was placed on each flower in the caterpillar-damage treatment group. Caterpillars that did not initiate feeding within 30 min were replaced, ensuring that the flower did receive feeding damage. Flowers were damaged between 1:30 p.m. and 3:30 p.m. and harvested 30 h later at 9:30 p.m. Corollas were separated from the calyx and pistil before extraction. Flowers which aborted or were obviously senescent when sampled were not included in the analysis. Abortion rates were 7% (3 of 46), 23% (11 of 48) and 36% (19 of 53) for undamaged, razor-damaged and caterpillar-damaged treatments, respectively.

Short-term responses to mechanical damage were examined in a second experiment in which flowers were damaged with a razor prior to opening and harvested the same evening during their time of peak emission. At 3 p.m. four flowers on ten plants were labeled and two flowers on each plant were damaged by means of an incision one half the length of the corolla tube; the other two were left undamaged. Corollas were harvested for chemical analysis at 9:30 p.m.

The effect of leaf damage and flower excision on corolla BA and nicotine pools

The results of the second experiment demonstrated that pools of BA and nicotine in the corolla are highly regulated and exhibit coordinated changes over ontogeny. This experiment was designed to examine (1) the effect of damage to vegetative tissues (leaves) on corolla chemistry and (2) the relationship between flower number and corolla chemistry. Nicotine is thought to be synthesized in the roots of *Nicotiana* plants (Baldwin 1989 references therein) and transported throughout the shoot via the xylem stream. Since leaf damage is known to dramatically increase whole-plant nicotine production and increase the nicotine concentrations of leaves (Baldwin et al. 1994a) and of the calyx of flowers (Baldwin and Karb 1995), we sought to determine whether leaf damage increased corolla nicotine concentrations as well. If nicotine accumulates in corolla tissues passively as a function of the amount produced in the shoot, we predict higher corolla concentrations in the flowers of plants which have had a majority of their flowers excised. To test these predictions, we randomly assigned forty plants in the early stages of stalk elongation to one of four treatment groups: (1) both leaf damage and flower excision, (2) flower excision only, (3) leaf damage only, (4) undamaged control. The leaf damage treatment consisted of one row of evenly distributed fabric pattern-wheel (Dritz, Spartanburg, S.C., USA) damage applied to alternate sides of the midribs of alternate leaves. Every leaf was damaged every other day throughout the period of stalk elongation and flowering. One row of pattern-wheel damage results in 4.51 1-mm^2 holes/cm of leaf (Ohnmeiss and Baldwin 1994). By the end of the experiment plants had received an average of 16.8±0.8 days of leaf damage. The first 29 mature flowers were excised from plants in the flower excision groups on their first night open by cutting through the pedicel at the base of the calyx with a razor blade. The flower excision treatment lasted an average of 8.1±0.3 days. All plants were inspected daily, newly matured flowers were labeled and the number of newly open flowers was recorded every evening. Flowers were harvested at the same developmental stage and at specific intervals of flower production in order to track the developmental stage of each plant. Hence from each plant in all four treatment groups, nine flowers (the first three flowers, the 14th–16th and the 27th–29th flowers produced on each plant) were harvested for chemical analysis by cutting through the pedicel with a razor blade at 9:30 p.m. on their first night open. The corollas were separated from the calyces and extracted. The mean BA and nicotine pools in the corollas of each set of three flowers from each plant was used for analysis. Three plants, one from each treatment group except the undamaged control, developed root infections and were excluded from the analysis.

Statistical analysis

Diurnal changes in corolla chemistry and flower emissions within plants were compared with repeated measures ANOVAs. Contrasts from these ANOVAs were used to compare changes between sampling intervals. Paired *t*-tests were performed on the paired flowers of the pollination experiment. Comparisons of treatments in the two floral damage experiments were made with two-way ANOVAs with plant and treatment as factors. The repeated damage experiment was analyzed with two-way ANOVAs with leaf damage and flower excision as factors. Percentage and proportion data were arcsine-transformed before statistical analysis. All data analysis was performed with the MGLH module of the SYSTAT statistical package (Evanston, Ill., USA).

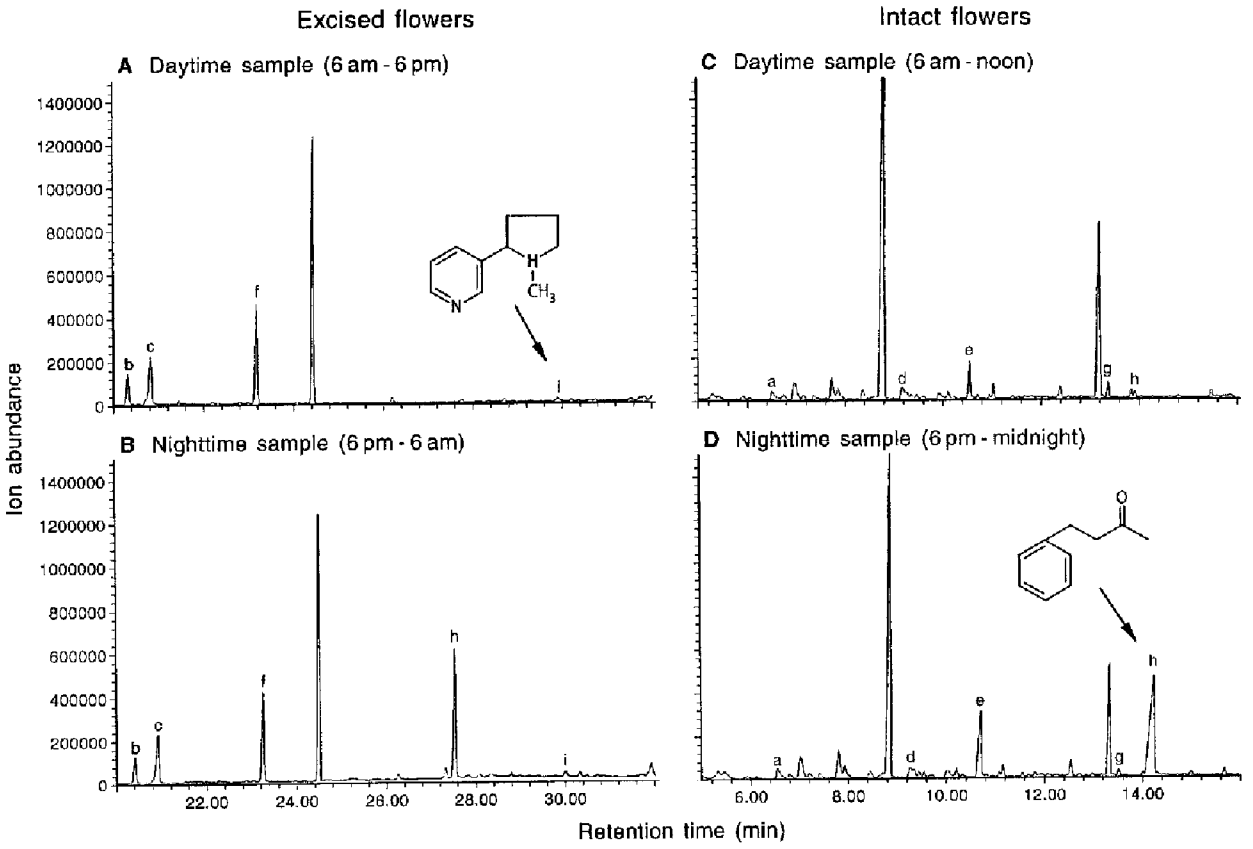

Fig. 1 Gas chromatograph (GC) separations of volatiles from **A** daytime and **B** nighttime headspace samples from ten excised flowers, and **C** daytime and **D** nighttime collections from a single intact flower. *Labeled peaks*: *a* benzaldehyde, *b* 2-ethyl-1-hexenal, *c* benzene methanol, *d* acetophenone, *e* nonanal, *f* methyl benzoate (internal standard), *g* decanal, *h* benzyl acetone, *i* nicotine. *Unlabeled peaks* are either clearly background or were not readily identified by their mass spectra. Differences in retention times are due to differences in chromatographic conditions. Note the dramatic increase in benzyl acetone (*h*) in the nighttime collections

Results

Identification of diurnally variable headspace volatiles

Many compounds exhibited slight changes in their emissions but none showed greater variation than BA (Fig. 1). While decanal emission increased 2 to 3-fold during the day and the emission of other compounds (acetophenone and nonanal) increased 1 to 3-fold at night, BA emissions increased nearly 50-fold at night. Since excised flowers (Fig. 1A, B) and flowers attached to plants (Fig. 1C, D) had the same pattern of emission, and no BA was found in the headspace of either vegetatively-growing plants or flowering-stage plants with all their mature flowers excised (M. Euler and I. Baldwin unpublished data), we conclude that flowers are the source of BA emissions. Nicotine was identified as a minor component in headspace collections from whole plants (M. Euler and I. Baldwin unpublished work) and excised flowers (Fig. 1A, B), but it did not exhibit diurnal changes in headspace concentration.

Distribution of BA and nicotine in the corolla

The vast majority of the BA in the plant is found in the flared lip of the corolla ($F_{2,12} = 9.26$; $P < 0.01$; Fig. 2A), with the highest concentration located on the outer lobes of the corolla lip ($t_6 = 8.78$; $P < 0.001$; Fig. 2B). Extractable nicotine pools were greatest in the base of the corolla below the point of attachment of the filaments ($F_{2,12} = 7.34$; $P < 0.01$; Fig. 2A). The inner surface of this section of the corolla tube has the greatest density of trichomes (M. Euler personal observations), suggesting that the nicotine may be sequestered in these trichomes, as is the case with the trichomes found on the leaves and calyx of this species (Baldwin and Karb 1995).

Diurnal changes in BA emission and pools of nicotine and BA in corollas

The diurnal changes in the emission of BA from flowers closely paralleled the diurnal changes in extractable pools of BA in the corollas (Fig. 3A). The correlation between extractable pools of BA and the averaged hourly emissions had a slope of 0.997 and $r^2 = 0.76$, which demonstrates that extractable pools of BA estimate the averaged hourly emission with a high degree of accuracy (Fig. 3A). Repeated measures ANOVAs of BA pools in corollas on the same plant and the emission of BA from

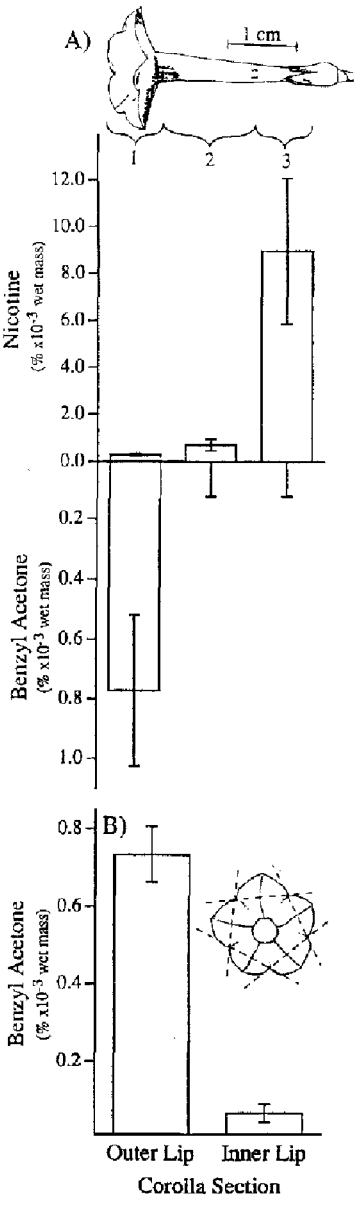

Fig. 2 **A** Nicotine (*upper*) and benzyl acetone (BA) (*lower*) concentrations of corolla sections illustrated in the diagram inserted in **A**: *section 1* is the flared lip of the corolla, *2* is the corolla tube from the point of insertion of the filaments to the lip, *3* is the corolla tube below the point of insertion of the filaments. **B** Concentrations of BA in the lip of the corolla (*section 1*). The diagram inserted in **B** illustrates the sectioning of the corolla lip into inner and outer sections. Values are means (±SEM) of the sections of five flowers for **A** and four flowers for **B** collected at 9:30 p.m. on the flowers' first night open

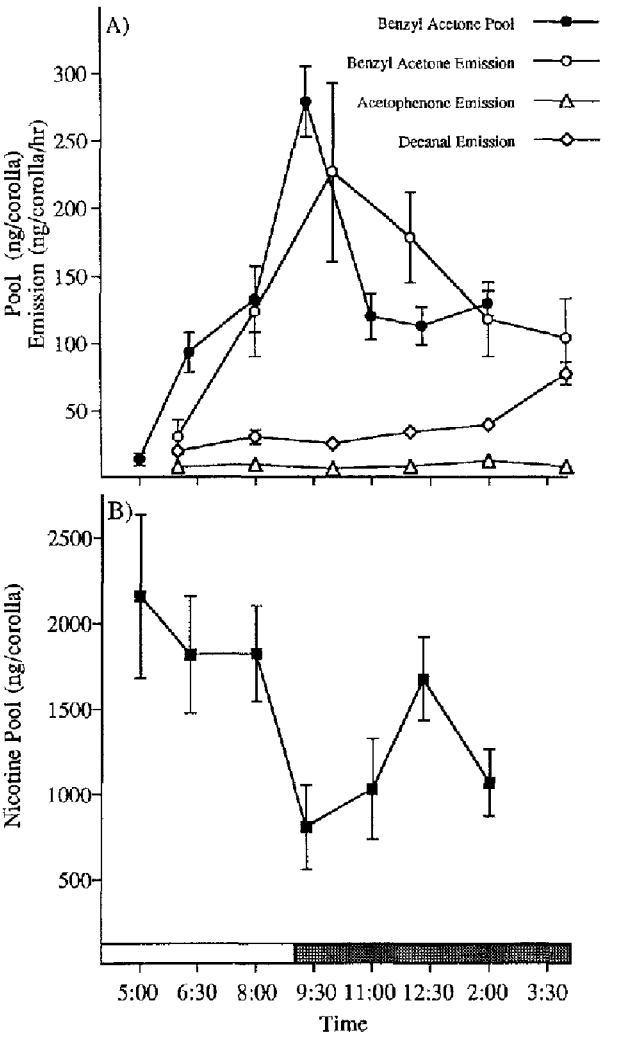

Fig. 3 Diurnal patterns in **A** volatiles (benzyl acetone, acetophenone and decanal) emitted from individual flowers and pools of **A** BA and **B** nicotine in corollas. The *filled symbols* are mean (±SEM) corolla pools of nicotine or BA (ng/corolla) extracted from 12 corollas on 12 plants at each of the designated times. The open symbols are mean (±SEM) headspace emissions (ng/corolla/h) of seven individual flowers on each of seven plants; the values between 5 p.m. and 3 a.m. are averages of 2-h collections, the values at 4 a.m. are averages of 5-h collections. The *shaded bar* on the *x-axis* indicates when lights were turned off

intact flowers demonstrate that the diurnal changes were significant (univariate ANOVAs; $Fs_{6,66 \text{ and } 4,24}$ = 23.11 and 7.77 respectively; Ps < 0.001). Difference contrasts demonstrated that peak BA pools occurred at the 9:30 p.m. sampling time, 0.5 h after the lights were turned out (Table 1). Interestingly, corolla nicotine pools (Fig. 3B) dropped significantly to their lowest values during peak BA emissions (univariate ANOVA; $F_{6,66}$ = 3.604; P < 0.005; multivariate Wilks' lambda $F_{6,6}$ = 14.578; P < 0.005; for contrasts between sampling intervals see Table 1). A weak positive correlation was found between BA pool and corolla mass when all harvest times were pooled ($F_{1,82}$ = 7.45; P < 0.01; r^2 = 0.083). However, when each sampling time was analyzed independently, the correlation was significant only for the 8 p.m. and 11 p.m. samples ($F_{1,10}$s >5.99; Ps < 0.05; r^2s > 0.375). Corolla mass did not differ significantly between sampling times ($F_{6,77}$ = 1.81; P > 0.1), and no significant relationship was found between corolla mass and nicotine pool ($F_{1,82}$ = 0.0004; P > 0.1).

Table 1 *F* statistics and *P* values for differences between consecutive sampling times as determined by contrasts from a repeated measures ANOVA of extractable benzyl acetone and nicotine pools in corollas of freshly opened flowers sampled at seven times throughout the 12-h period as depicted in Fig. 3A and B. The statistics are for the change in extractable pools between the times indicated. (NS, $P>0.1$)

	Time interval					
	5:00-6:30	6:30-8:00	8:00-9:30	9:30-11:00	11:00-12:30	12:30-2:00
Benzyl acetone						
$F_{1,11}$	24.839	1.413	30.904	60.93	0.591	2.098
P	0.0004	NS	0.0002	0.00008	NS	NS
Nicotine						
$F_{1,11}$	0.373	0.0002	8.917	20.507	1.665	4.241
P	NS	NS	0.0124	0.0009	NS	0.0639

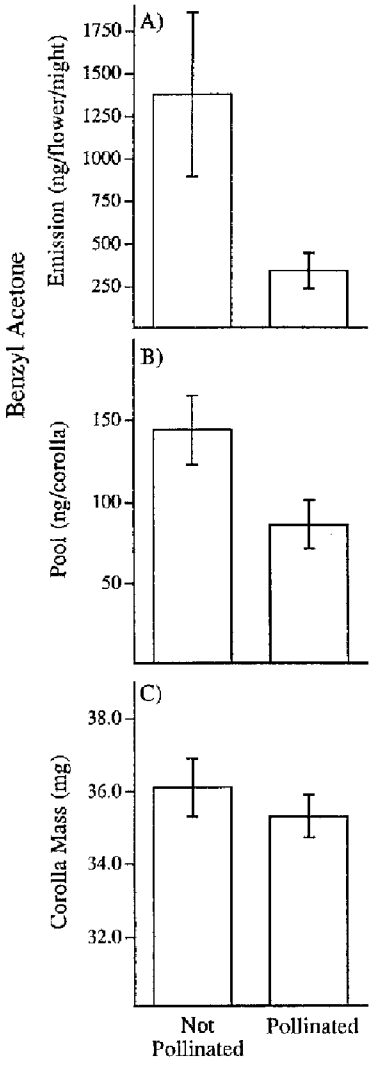

Fig. 4A–C Effect of pollination on BA emissions and pools. **A** Mean (±SEM) BA in 12-h headspace collections of 9 pairs of individual flowers (ng/corolla/night). All flowers were emasculated before anthesis and one flower of each pair was hand pollinated; the other flower was manipulated but not pollinated. **B** Pools (mean ±SEM) in the corollas of ten pairs of individual flowers 3 h after pollination treatment (ng/corolla). **C** Mean (±SEM) corolla wet mass of flowers harvested in **B** (mg/corolla)

Response to pollination

Pollination decreased total BA emissions by 75% as determined by the 12 h headspace collections (Fig. 4A; mean difference = 1036.0±34.3 ng BA flower^{-1} night^{-1}; paired $t_8 = 2.65$; $P < 0.05$). An hour and a half after pollination, corolla pools of BA were 33% greater in unpollinated flowers than in pollinated flowers (Fig. 4B; mean difference = 59.1±7.1 ng BA corolla^{-1}; paired $t_9 = 3.74$; $P < 0.005$). Corolla mass was positively correlated with BA pool ($F_{1,18} = 10.90$; $P < 0.005$; $r^2 = 0.322$) but was not affected by pollination (Fig. 4C; paired $t_9 = 1.80$; $P > 0.10$). Corolla nicotine pools did not differ between pollinated and unpollinated flowers (paired $t_9 = 0.24$; $P > 0.1$).

Response to floral damage

Both mechanical and herbivore damage to immature flowers 30 h prior to peak BA emission resulted in a 2-fold increase in nicotine pools in the mature corollas ($F_{2,86} = 8.19$; $P < 0.001$; Fig. 5B) and decreased BA pools ($F_{2,86} = 6.52$; $P < 0.005$; Fig. 5A) and corolla masses ($F_{2,86} = 13.99$; $P < 0.0001$; Fig. 5C). The plant from which flowers were sampled had a significant influence on corolla nicotine pools ($F_{5,86} = 2.66$; $P < 0.05$) but not on BA pools or corolla mass ($F_{5,86}$s < 1.76; Ps > 0.1). There were no interactions between plant and the damage treatment ($F_{10,86}$s < 0.97; Ps > 0.1). BA pools were again positively correlated with corolla mass ($r^2 = 0.16$; $F_{1,102} = 20.03$; $P < 0.0001$), which suggested that the decrease in BA pools was a result of the smaller mass of the damaged corollas. To test this suggestion an ANCOVA on BA pools with corolla mass as a covariate was performed. The ANCOVA demonstrated that the decrease in corolla mass could account for the decrease in BA pools in damaged flowers ($F_{2,85} = 1.48$; $P > 0.10$ for damage treatment and $F_{1,85} = 10.39$; $P < 0.005$ for corolla mass). No difference was found between mechanically damaged flowers and those damaged by caterpillars in the context of increase in nicotine pools or decrease in BA pools (contrast $F_{1,86}$s < 1.49; Ps > 0.1). The differ-

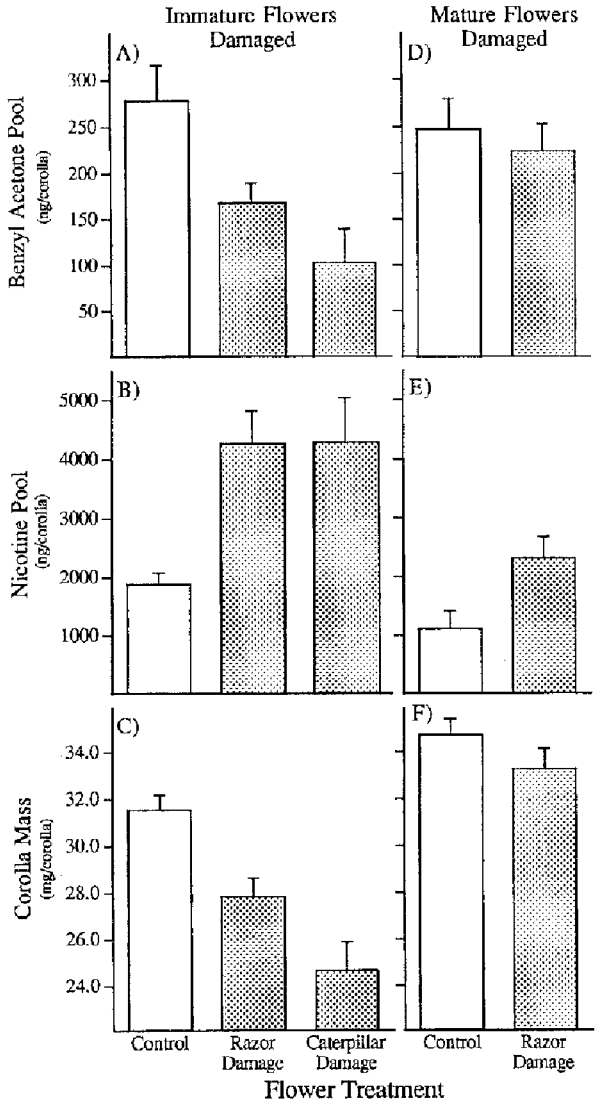

Fig. 5 Response to floral damage: **A** benzyl acetone and **B** nicotine pools (ng/corolla) and **C** corolla mass (mg/corolla) 30 h after damage to immature flowers. **D** Benzyl acetone and **E** nicotine pools (ng/corolla) and **F** corolla mass (mg/corolla) 6 h after damage to mature flowers. Values are mean (±SEM) of at least five flowers from each of five plants for **A**, **B** and **C** and two flowers from each of ten plants for **D**, **E** and **F**. All flowers were sampled at 9:30 p.m. on their first night open

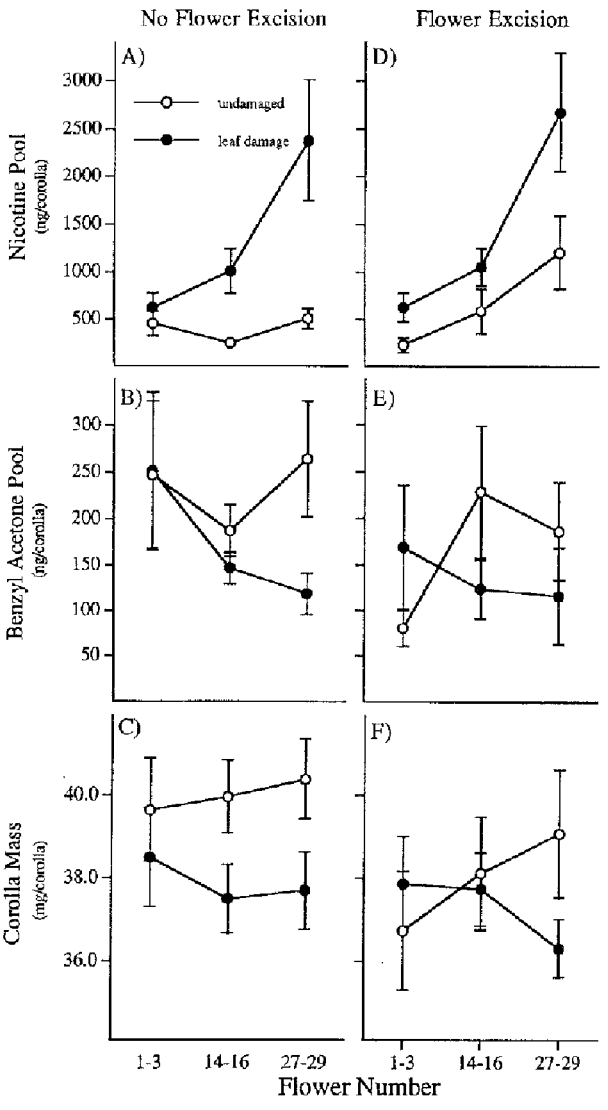

Fig. 6 Effect of **A**, **D** leaf damage and flower excision on corolla nicotine and **B**, **E** benzyl acetone pools (ng/corolla), and **C**, **F** corolla mass (mg/corolla) of flowers 1–3, 14–16 and 27–29 produced on plant that received daily leaf damage (filled circles) and plants that did not (open circles). The *x* axis designates the sequential number of flowers opening on the plants. Values are means (±SEM) of nine plants in each treatment. Data presented in the *left panels* (**A**, **B** and **C**: no flower excision) were from plants that had only the sampled flowers excised for chemical analysis, while the *right panels* (**D**, **E** and **F**: flower excision) depict data from plants that had all their mature flowers excised

ence between the mass of mechanically damaged corollas and the mass of those eaten by caterpillars was significant (contrast $F_{1,86} = 4.59$; $P < 0.05$; Fig 5C).

In the second floral damage experiment, where mature flowers were mechanically damaged 6 h prior to the period of peak BA emission, damaged flowers had nicotine pools 2× greater than undamaged flowers (Fig. 5E; $F_{1,15} = 5.92$; $P < 0.05$). Damage did not influence either BA pools or corolla mass ($F_{1,15}$s < 2.22; Ps > 0.1; Fig. 5D, F). In this experiment plant did not influence corolla nicotine or BA pools or the mass of the corollas (all $F_{9,15}$s < 1.01; Ps > 0.1), and no interactions between plant and the damage treatment (all $F_{9,15}$s < 1.42; Ps > 0.1) were found.

The effect of leaf damage and flower excision on corolla BA and nicotine pools

The results of this experiment further illustrate the control that *N. attenuata* has over its corolla chemistry. The flower excision treatment did not significantly affect BA or nicotine pools or corolla mass at any harvest (all $F_{1,33}$s < 3.31; Ps > 0.05; Fig. 6D, E, F), and no significant interactions between damage and excision were found (all $F_{1,33}$s < 1.05; Ps > 0.1). In contrast, leaf damage increased the nicotine pool in corollas at each harvest ($F_{1,33}$s $= 4.59$–12.49; Ps $= 0.04$–0.001; Fig. 6A, D) to

such an extent that the 27th–29th flowers produced by plants with leaf damage had 4 times more nicotine in their corollas than did comparable flowers on undamaged plants. Leaf damage significantly decreased BA pools ($F_{1,33} = 4.57$; $P = 0.04$; Fig. 6B, E) and corolla mass ($F_{1,33} = 6.36$; $P = 0.017$; Fig. 6C, F) only in the final harvest. Again, the effect of leaf damage on corolla mass is likely responsible for the decrease in BA pools because corolla mass and BA pools were significantly correlated at all three harvests ($F_{1,109}$s = 4.97–26.82; Ps = 0.03–0.00001; r^2 = 0.044–0.23). This proposition is supported by the fact that when BA corolla pools are expressed as a percentage of corolla mass, BA concentrations in the corollas of flowers from the final harvest did not differ between damaged and undamaged plants ($F_{1,33} = 3.75$; $P = 0.061$). A repeated measures ANOVA on BA concentration at the three harvests supported this conclusion (univariate $F_{1,33}$s = 0.85; $P > 0.1$; multivariate Wilks' lambda $F_{2,32} = 0.0043$; $P > 0.1$).

Discussion

In insect-pollinated plants, the corolla is the tissue which must reconcile the conflicting requirements of advertisement and avoidance of herbivory during flowering. The corolla protects the stigma, anthers, and nectar rewards from inimicable abiotic environments. It also functions as a disposable tissue which must attract pollinators without attracting herbivores and prevent opportunistic herbivory by pollinators or nectar thieves. The spatial location and dynamics of BA and nicotine pools in the corolla reflect how *N. attenuata* solves this potential dilemma.

The location of BA in the outer lobes of the flared lip of the corolla and its precisely regulated pattern of emission is consistent with its role in attracting pollinators, such as hawkmoths, which are active shortly after sundown. A similar diurnal pattern of emission is also found for other volatile phenylpropanoid-derived metabolites released from the flowers of *N. sylvestris* and *N. suaveolons* (Loughrin et al. 1990, 1991). Moreover, the rapid decrease in BA pools after pollination, which results in a 4-fold decrease in emission in recently pollinated flowers, is also consistent with the role of BA in pollinator attraction. The coordinated release of BA with pollinator activity and its rapid decrease with pollinator performance suggest that the costs of unregulated emission are high. These costs could reflect the biosynthetic expenses associated with the synthesis and release of the volatiles, but in the case of *N. sylvestris* and *N. suaveolons*, this possibility seems unlikely: the corolla senesces after self-pollination with pools of glycosidically-bound precursors for the phenylpropanoid-derived volatiles that are many times larger than the quantities emitted (Loughrin et al. 1992). Therefore, for these species, the plant has already invested the resources for the production of these metabolites, and this resource investment is not likely to be reduced by decreasing emission. The ecological costs of increased "apparency" may have selected for the rapid up-and-down regulation of the BA emission. These ecological costs could be a consequence of greater discovery rates by non-pollinating herbivores or the opportunistic herbivory by pollinators. The latter possibility may be particularly germane for *N. attenuata* because a common insect herbivore on this species is the larvae of *Manduca* spp. (Baldwin and Ohnmeiss 1993), the adults of which visit and presumably pollinate flowers.

The distribution of nicotine in the corolla, with the highest concentrations found in the lower regions in the corolla tube that surround the base of pistil and lower concentrations where the pollinators interact with the corolla, is consistent with the predictions of optimal defense theory (McKey 1974; Krischik and Denno 1983; Zangerl and Bazzaz 1992). This theory argues that defense metabolites are distributed within plants so that their fitness benefits are optimized in light of their fitness costs. As a consequence they are allocated preferentially to tissues with high fitness value to the plant and a high probability of attack. Ovules are clearly tissues with a high fitness value for this annual plant and, because of their high nutritional value, likely to be attacked. However, because high concentrations of nicotine in the corolla could produce sufficient nicotine in the headspace to repel, or even poison, potential pollinators, deploying this defense poses a significant ecological cost. The distribution of nicotine in the corolla (Fig. 2) may optimize the benefits of the defensive use of nicotine while minimizing nicotine's potential ecological costs in terms of lost pollinations. Moreover, the dramatic drop in nicotine pools during peak BA emission (Fig. 3) is consistent with the ecological costs of using nicotine as a defense in a tissue whose function is to advertise for pollinators.

If the insects that are attracted to BA emissions turn out to be herbivores and the corolla is wounded, nicotine pools in the corolla increase rapidly without any biomass-corrected alteration in BA emission. The fact that herbivory by *Manduca sexta* larvae or wounding does not attenuate BA emission suggests that the ecological costs associated with increased apparency to herbivores may be less important than the loss of pollinators which are attracted to BA emissions. Similarly, the protective benefit from wound-induced increases in the corolla's nicotine pool must outweigh the potential costs of interfering with the pollinator-attraction function of the corolla. In summary, when *N. attenuata* flowers are damaged by an herbivore, the plant increases its concentrations of a defense metabolite but does not change its pollinator-attraction chemistry. Once a plant is wounded by an herbivore, it has already been "discovered," and a reduction in volatile emissions at this time is not likely to confer a net fitness benefit.

The increase in corolla nicotine pools in response to wounding of leaves or flowers could be a passive consequence of the systemic increase in whole-plant nicotine pools; however, two lines of evidence suggest that this is unlikely:

1. The increase in corolla nicotine pools in response to corolla damage is localized to the damaged flower. Damaged and undamaged flowers were from the same plants and no difference would have been discerned had the response been systemic.
2. The increase in corolla nicotine pools occurred within one evening, too rapid a period for a whole-plant response [it usually takes 2 days to discern a change in nicotine pools (Baldwin et al. 1994a)].

The slower response in corolla nicotine pools to leaf damage occurred within the time frame of a systemic response. However, if it had been a passive response, one would expect plants in the floral excision treatment, which had all but three of their flowers excised, to have significantly higher corolla nicotine pools than those of plants that did not have any flowers excised. This, however, was not the case (Fig. 6). Therefore, the changes in corolla nicotine pools are likely to be highly regulated, much the same way that corolla BA pools apparently are.

In conclusion, we propose that the cost-benefit framework which forms the basis of the optimal defense and apparency theories of plant chemical defense should be applied to the volatile emissions that function in pollinator attraction. While the benefits of these emissions are clearly associated with the benefits of outcrossing, their costs are likely to be ecological, associated with increases in apparency, rather than biochemical, because the quantities of chemicals emitted are so small (see Dicke and Sabelis 1989). While testing the cost-benefit framework for chemical defense has proven difficult due to the difficulties associated with measuring the cost of chemical defense (Zangerl and Bazzaz 1992), this framework may be much easier to test for floral emissions that are regulated rapidly up and down, because the manipulation of these diurnal patterns is tractable, even under field conditions.

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