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VOLATILE COMPOSITION, EMISSION PATTERN, AND LOCALIZATION OF FLORAL SCENT EMISSION IN *MIRABILIS JALAPA* (NYCTAGINACEAE)[1]

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We elucidated scent components, daily emission patterns, and the localization of floral scent release of *Mirabilis jalapa*. Volatiles emitted by the whole plant as well as by detached flowers were investigated using dynamic headspace analysis and gas chromatography/mass spectrometry. Among several constituents including (*Z*)-3-hexenyl acetate, β-myrcene, (*Z*)-ocimene, and benzyl benzoate, the monoterpene (*E*)-β-ocimene was the major fragrance component. Fragrance release occurred in a time-dependent manner. The emission of volatiles, including (*E*)-β-ocimene, showed an evening-specific maximum (1700–2000 pm). The emission of (*Z*)-3-hexenyl acetate reached its maximum 3 h later. Histological (neutral red staining) and morphological studies (electron and light microscopy) of the flower surface and tissues of *M. jalapa* revealed differences in surface structures and tissue characteristics. The flower could be divided into four main sections, including the tube, the transition zone between tube and limb, a star-shaped center of the limb, and petaloid lobes of the limb. These petaloid lobes are the site of (*E*)-β-ocimene release. Stomata and trichomes found on the abaxial flower surface were not directly involved in fragrance release. Clear indications of osmophores involved in scent release could not be found. Thus, the results indicate that floral volatiles probably are released by diffuse emission in *M. jalapa*.

Key words: floral histology; (*Z*)-3-hexenyl acetate; *Mirabilis jalapa*; Nyctaginaceae; (*E*)-β-ocimene; petal morphology; rhythmic emission; scent emission.

Floral scent emission, in addition to color, shape, surface structure, and nectar guides, is one of crucial strategies plants employ to attract pollinators to assure reproduction. Fragrances typically are mixtures of volatile compounds belonging to the classes of isoprenoids, phenylpropanoids, and fatty acid derivatives, with characteristically low vapours pressure and low molecular weights (Knudsen et al., 1993; Dudareva et al., 2000; Pichersky and Gershenzon, 2002).

In vegetative tissues, production and release of volatiles are usually linked to certain tissues, organs, and compartments like glandular hairs, scales, oil cavities, and oil or resin ducts. This compartmentalization is well characterizied and has been intensively investigated in plant families with high essential oil content like Lamiaceae (Werker et al., 1985c; Werker, 1993; Voirin and Bayet, 1996; McConkey et al., 2000; Gershenzon et al., 2000; Turner et al., 2000; Gang et al., 2001). However, the mechanisms of volatile release from flowers have not been studied exhaustively and may differ from that of vegetative tissues.

Early investigations (Mazurkiewicz, 1913) described flower epidermis cells as the place of a diffuse volatile emission. Vogel (1962) reintroduced and established the term osmophore (odor = osmo; bearing = phore) for an enclosed area of floral tissue that is specialized in scent emission (Vogel, 1962). Since then, successful efforts have been devoted to the investigation of mechanisms of fragrance release via osmophores. Species that have been examined belong mostly to the families of *Araceae* and *Orchidaceae* in which strong or distructive floral scents are associated with pollinator attraction (Vogel, 1962; Pridgeon and Stern, 1983, 1985; Curry, 1987; Curry et al., 1988, 1991; Skubatz et al., 1995; Skubatz and Kunkel, 1999; Hadacek and Weber, 2002). Osmophores consist of a multilayered glandular epithelium (Vogel, 1962; Stern et al., 1987). Most remarkable are enormous deposits of starch or other storage compounds within the mesophyll. These deposits usually are missing in epidermis cells. This allows a distinction into production and emission layer, respectively. In contrast, flowers with diffuse emission, probably combine production and emission within the same epidermis cells (Vogel, 1962; Kolosova, 2001).

The diffuse emission of floral scent is probably a plesiomorphic character of flowers, whereas spatial patterns of emission, represented by osmophores, are most likely an apomorphic character (Vogel, 1962; Bergström et al., 1995). Also, temporal patterns of emission are rather apomorphic features and may reflect convergent evolutionary processes based on specialized relationships with certain pollinators (Baker, 1961; Whitten et al., 1986; Ollerton, 1996; Levin et al., 2003).

In addition to diffuse emission and osmophore based emission, both floral stomata and trichomes have to be considered as a medium to release scent. The function of stomata as a vehicle for flower scent release is mentioned from time to time. However, while vegetative monoterpene emission via

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stomata is well documented in Pinaceae (Kesselmeier and Staudt, 1999; Niinemets et al., 2002) and Fagaceae (Loreto et al., 1996), the involvement of floral stomata in scent emission is not agreed upon (Vogel, 1962; Kugler, 1970; Leins, 2000). The presence of glandular as well as nonglandular trichomes on flower surfaces has been frequently reported (Barthlott, 1980; Werker et al., 1985a, b; Dudai et al., 1988; Werker, 1993; Ascensão et al., 1999; Carpenter, 1999; Rodriguez, 2000; Kolosova et al., 2001). The direct proof that floral trichomes are the source of floral headspace fragrance is still missing, however the work of Werker and Werker et al. (Werker, 1993; Werker et al., 1985a, b) and Kolosova et al. (2001) provide an indication that floral trichomes might be involved in scent release.

An useful model for research on floral scent production and its release is *Mirabilis jalapa* (Nyctaginaceae). This plant is native to the tropical regions of America and shows perfect, but incomplete flowers. The perianth consists of a tube with a five-lobed limb, which is described as a corolla-like calyx (Woodson and Schery, 1961; Vanvinckenroye, 1993; Lu and Gelbert, 2003). Sepals obviously adopted the ability of attracting pollinators by olfactory and visual cues. Beyond that, the individual flower of *M. jalapa* displays a very unique mode of opening. Buds start to unfold in the late afternoon, stay open for one night and senescence develops after approximately 16–20 h the following morning. *M. jalapa* is pollinated by hawk moths (Cruden, 1970; Martinez del Rio and Burquez, 1986). The scent of *M. jalapa* consists mainly of the monoterpene (*E*)-β-ocimene with additional compounds such as α-farnesene, (*Z*)-3-hexenyl acetate and myrcene (Heath and Manukian, 1994; Levin et al., 2001), which means the floral volatile spectrum is relatively simple and studies should be tractable.

Here we report our findings on fragrance emission of *M. jalapa* flowers with special emphasis on the localization of floral volatile release. Volatiles emitted by the whole plant as well as detached flowers were analyzed with Gas Chromatography/Mass Spectrometry (GC/MS). Histological techniques such as neutral red (NR) staining and morphological studies of the flower surface and tissue using scanning electron microscopy (SEM), environmental scanning electron microscopy (ESEM) and light microscopy (LM) were employed to pinpoint the flower area or structure responsible for scent release.

MATERIALS AND METHODS

Plant material and growth conditions—*Mirabilis jalapa* L. seedlings of a white- blooming variety were kept under greenhouse conditions (temperature day/night: 22°C/17°C ± 5°C, photoperiod: 14 h; illumination: ca. 300 μE · m^{-2} · s^{-1}). Just before flowering, plants were transferred into narrow 3-L pots and grown under controlled conditions in a plant chamber (temperature day/night: 22°C; photoperiod: 14 h; illumination: 100 μE · m^{-2} · s^{-1}; Percival Scientific, Perry, Iowa, USA).

Collection and analysis of headspace volatiles emitted from whole plants—Volatiles were collected using a headspace collection system established by Heath and Manukian (1994) and further described by Röse et al. (1996) which was mounted into a plant chamber. Two plants were investigated in parallel. Each of them was placed into a glass chamber. The bottom of the chamber was closed by a guillotine-like base around the stem leaving the pot outside the chamber. Pure humidified air (60%) entered the system through an air diffuser at the top end of the chamber, providing a uniform flow (20 L/min). Eight collector traps, each containing 100 mg Super-Q (Alltech Associates, Deerfield, Illinois, USA) as an adsorbent, were inserted through tight fittings at the base of the chamber, allowing a maximum of eight collection periods over 24 h in order to study time-dependent variation of scent emission of *M. jalapa*. Approximately 30% of the air was sucked through the traps (7 L/min); the remaining excess air escaped through the base of the chamber. Trapped volatile compounds were eluted with 2 × 100 μL methylene chloride. Nonyl acetate and n-octane were added as internal standards.

Samples were analyzed using a Hewlett Packard GC 6890 equipped with a HP 5973 Mass Selective Detector and a DB-5MS column (30 m × 0.25 mm × 0.25 μm; J & W Scientific, Folsom, California, USA). Splitless injections of 1 μL were performed at an injector temperature of 220°C. The column temperature was initially set at 40°C for 3 min, followed by a gradient of 5°C/min up to 220°C. Helium was used as carrier gas at a constant flow rate of 2 mL /min and a linear velocity of 51 cm/s.

Collection and analysis of headspace volatiles from flowers and flower parts—Flowers of *M. jalapa* were harvested immediately after full opening and dissected into tube, transition zone, and limb as indicated in Fig. 7A. For more detailed investigation, the limb was divided as shown in Fig. 7C into the star-shaped center and petaloid lobes. The lobes were further divided into the edges along the star and the corresponding remaining part, and the lobe rim, also with the corresponding remaining part. The different flower parts were separately sealed into a 20-mL headspace vial. Three flowers were used for one sample. For comparison, three flowers that had been cut into the same pieces as the sample were sealed as a whole in a second vial. Because of ongoing emission from the flower parts and therefore accumulation of volatiles in the vial during GC/MS measurements, only one flower part at the time was evaluated and directly compared with the corresponding cut whole flower.

Analysis was conducted using a GC/MS-QP5000 (70 eV; Shimadzu, Kyoto, Japan) equipped with a headspace injection system. Vials were equilibrated for 10 min at 35°C, and 500 μL of the headspace air were directly transferred to the injection port and splitless applied to a DB-5MS column (60 m × 0.25 mm × 0.25 μm; J & W Scientific) at 200°C, with a sampling time of 1 min. Carrier gas was helium, with a column flow of 1.3 mL/min and a linear velocity of 29 cm/s. The temperature program started at 35°C with a gradient of 10°C/min up to 240°C followed by a gradient of 20°C/min up to 300°C with a hold for 1 min at 300°C.

Experiments were replicated 6 times unless otherwise noted. The emission was estimated per milligram fresh mass per square centimeter. The emission of the identically cut whole flower was set to 100%.

Extraction and analysis of trichome volatiles—Part of the transition zone between flower tube and limb was cut away and placed onto a cooled stage in the chamber of a XL30 ESEM-FEG (Phillips, Eindhoven, Netherlands), and trichomes were harvested using a sharp needle tip (0.2 nm). The needle was attached to a holder of a nanomanipulator (Nanotechnik Kleindiek, Reutlingen, Germany) consisting of three nanomotors that allowed a three- dimensional movement of the needle with a minimum distance of 1 nm. Trichomes were hooked at the anchoring cell and torn off. The tip of the needle carrying the trichome was immediately immersed into methanol and snapped into the solvent. A total of 19 trichomes were pooled and extracted with 80 μL of methanol. Samples of the extract (4 μL) were splitless injected at 200°C into a port of a Shimadzu GC/MS-QP5000 onto a DB-5MS column (60 m × 0.25 mm × 0.25 μm; J & W Scientific), with a sampling time of 3 min. The carrier gas was helium, at a flow of 1.2 mL/min and linear velocity of 28 cm/s. The program was initiated by an isothermal step at 35°C (1 min) followed by a gradient of 10°C/min up to 280°C and 20°C/min up to 300°C with a hold for 8 min at 300°C.

Compound identification—Mass spectra were obtained using the scan modus (total ion chromatogram, mass range 40–300). Confirmation of compound identity was based on comparisons with mass spectra in the Wiley or National Institute of Standards and Technology libraries or on direct comparison with mass spectra and retention times of standards, if available.

Neutral red staining—Detached flowers were stained with neutral red (NR), which is a weak cationic dye that penetrates membranes by nonionic

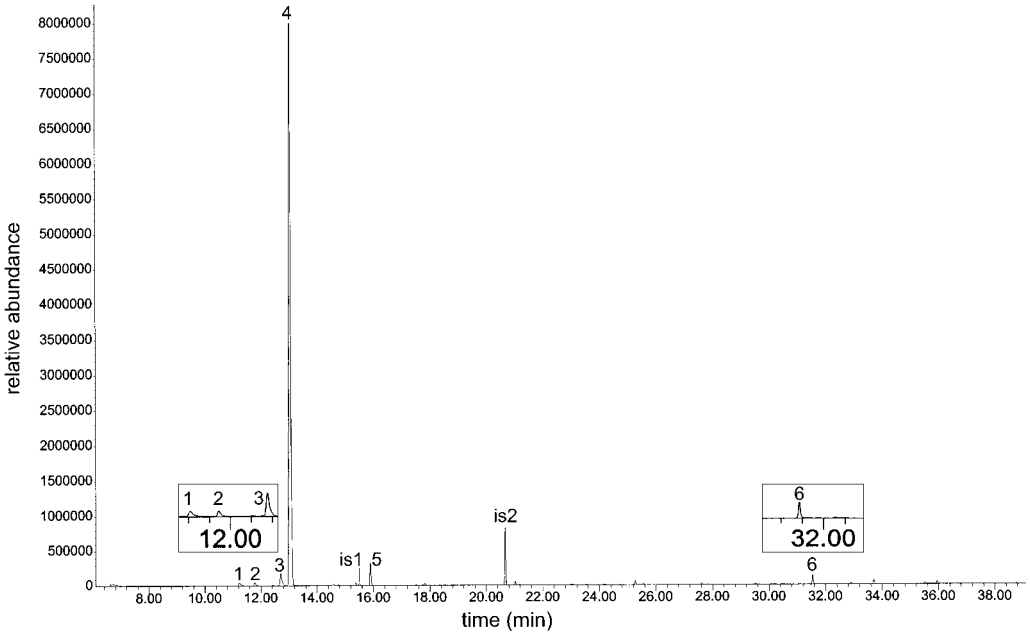

Fig. 1. Volatile profile of *Mirabilis jalapa*. Headspace volatiles emitted between 1700–2000 from a whole plant with several flowers, trapped on Super-Q. (1) β-myrcene, (2) (*Z*)-3-hexenyl acetate, (3) (*Z*)-ocimene, (4) (*E*)-β-ocimene, (5) (*E*)-epoxy-ocimene, (6) benzyl benzoate; Internal standards were n-octane (is1) and nonyl acetate (is2).

diffusion and accumulates intracellulary. Vogel (1962) emphasized this quick and selective staining of intact tissue with NR as a very characteristical feature of osmophores. Flowers were immersed in an aqueous NR solution (0.1%, tap water). The optimal staining time was determined by a time course of 5, 10, 15, and 20 min of staining. Afterwards, flowers were rinsed with tap water and photographed (Olympus C-3030 Zoom, Tokyo, Japan).

Electron microscopy—*Scanning electron microscopy (SEM)*—Different areas of the perianth of fresh flowers were cut as indicated in Fig. 4 and immediately fixed in 4% glutaraldehyde in 0.1 mol/L Tris-HCl (pH 8.0), and dehydrated in ethanol (100%). After critical-point drying and sputter coating with gold, samples were studied using the scanning electron microscope DSM960A (Carl Zeiss, Oberkochen, Germany).

Environmental scanning electron microscopy (ESEM)—This method provided the opportunity to investigate living material. Different parts of the perianth (Fig. 4) were fixed with wax onto a cooled stage and examined using a XL30 ESEM (Phillips, Eindhoven, Netherlands).

Light microscopy—For light microscopy (LM), different parts of the perianth were fixed in 4% glutaraldehyde in Tris-HCl (pH 8.0) and rinsed with 0.1 mol/L sodium phosphate buffer. Post-fixation was done with 1% osmium tetroxide. After repeated rinsing with distilled water, the samples were dehydrated with acetone and embedded in epoxy resin ''Araldid'' (Fluka, Basel, Switzerland). Sections of 500 nm were prepared using an Ultramicrotom (LKB, Uppsala, Sweden) and stained with toluidine blue. Samples were examined using an Zeiss Axioplan II optical microscope (Carl Zeiss, Oberkochen, Germany) and documented with a Coolpix 995 (Nikon, Tokyo, Japan).

Investigation of floral stomata—In order to check a possible role of floral stomata in scent release, part of the transition zone between flower tube and limb of fresh flowers was excised and immediately fixed in 4% glutaraldehyde in Tris-HCl (pH 8.0). To investigate both surfaces of the epidermis over the course of bloom, two samples were taken every hour, starting directly after flower opening until 6 h after opening; a last sample was excised 12 h after flower opening. The sections were prepared for SEM as described above. The condition of all stomata found in the prepared flower part was assessed and the mean condition was determined. Stomatal conditions were classified into three categories: (0) closed, (0.5) half-open, and (1) open. Scent emission was estimated subjectively by olfactory perception (by two persons). Levels of emission were classified into five categories: (1) for the maximum and (0.75), (0.5), (0.25) and (0) for progressively lower levels.

RESULTS

Analysis of scent components and daily emission patterns—The volatile emission of a whole *M. jalapa* plant with open flowers, monitored over a time period of 48 h, showed that the scent was strongly dominated by the monoterpene (*E*)-β-ocimene (Fig. 1, number 4). Clearly detectable amounts of β-myrcene (number 1), (*Z*)-3-hexenyl acetate (number 2), (*Z*)-ocimene (number 3), (*E*)-epoxy-ocimene (number 5), and benzyl benzoate (number 6) were also identified. Minor scent components (not shown in the chromatogram) included allo-ocimene, (*Z*)-3-hexenol, and methyl salicylate.

When volatiles were collected from whole plants in 3 h intervals, a time-dependent variation of scent emission became evident. A time course of (*E*)-β-ocimene and (*Z*)-hexenyl acetate emission is presented in Fig. 2. The onset of scent emission was observed in the early afternoon between 1400 and 1700, which correlated well with the timing of flower opening around 1600. The major compound (*E*)-β-ocimene reached its maximum emission between 1700 and 2000 and could be detected until 0200, but only at trace levels thereafter. The same emission patterns were observed for benzyl benzoate, epoxy-ocimene, (*Z*)-ocimene, and β-myrcene (data not shown). Most interestingly, the emission of (*Z*)-3-hexenyl acetate followed a similar pattern, but with a shift of one sampling period. Its emission peaked later between 2000 and 2300. It was almost undetectable between 0200 and 1400. The evening-specific emission of fragrance components from *M. jalapa* plants suggested that the volatiles were originating from the flowers. Additional analysis of detached flowers confirmed that (*E*)-β-

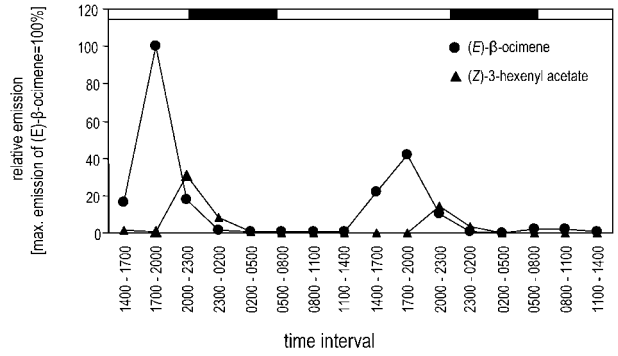

Fig. 2. Time course of (*E*)-β-ocimene and (*Z*)-3-hexenyl acetate emission from *Mirabilis jalapa* plants. Headspace volatiles emitted in 3-h intervals over a 48-h sampling period from a whole plant with several flowers, trapped on Super-Q. Open bars: day/light. Solid bars: night/dark. Results from one representative plant are shown, $N = 2$.

ocimene was released by flowers, while (*Z*)-3-hexenyl acetate could not be detected (data not shown). Sampling on two consecutive days revealed the same emission patterns, but the overall amount of volatile released declined on day 2 compared to day 1. This might be due to fewer open flowers but most likely to a general response of the plant being placed in the volatile collection chamber.

Histochemical studies with neutral red—NR staining of detached flowers was used for visualization of putative osmophore areas and gave a first indication of the location of scent emanation of a *M. jalapa* flower. The staining solution penetrated first an area of the very upper part of the adaxial flower tube (= transition zone, Fig. 3A; see also Fig. 7A). Subsequently, reddish coloring developed along the edges of a star-shaped tissue area that forms the center of the limb (Fig. 3B and C). With increasing time of staining, the reddening extended over the petaloid segments between the star-shaped center, while the tissue of the star remained unstained (Fig. 3D). The abaxial side of the flower showed the same coloring pattern, but it was less intensive. These results indicate that the permeability of tissues to NR differed among areas of the *M. jalapa* flower and enhanced permeability is correlated with volatile emission.

Morphological studies of the adaxial and abaxial flower epidermis—Exploration of the flower surface showed wide variation in epidermal features (Fig. 4A–H). The adaxial epidermis (Fig. 4A–D) of the upper petaloid lobe between the arms of the star-shaped center was characterized by a bullate surface (Fig. 4A). On the adaxial epidermis toward the transition zone, puzzle-like cells were observed, which lent an increasingly planar appearance to the epidermis (Fig. 4B). Cells of the transition zone itself were elongated and tightly packed (Fig. 4C). Long bundled cells formed a very rough epidermis at the adaxial side of the floral tube (Fig. 4D).

The abaxial epidermal surface of the *M. jalapa* flower displayed a somewhat different morphology (Fig. 4E–F). Cells near the rim showed characteristics comparable to the cell type described for the adaxial epidermis of the upper petaloid lobe, but were less bullate (Fig. 4E). Moving toward the transition zone, cells developed an elongated and puzzle-like shape (Fig. 4F), which was strongly suggestive of the leaf epidermis (not shown). The abaxial transition zone showed long cells forming a dense and rugose epidermis (Fig. 4G), and the abaxial tube revealed long cells organized in parallel files (Fig. 4H).

A typical feature of both epidermal layers was the appearance of stomata. In the adaxial epidermis, stomata were observed in the upper part of the transition zone and their number increased towards the tube (Fig. 4C–D). In the abaxial epidermis, they occurred in the lower petaloid lobe (Fig. 4F), as well as in the transition zone (Fig. 4G) and abaxial tube (Fig. 4H). In contrast to leaf stomata, flower stomata were not embedded into the epidermis and appeared to be elevated above it (see Fig. 4H and insert Fig. 4C and G).

Trichomes were frequently observed in the abaxial epidermis and less often on the adaxial epidermis (Figs. 4B and 5A). They clustered in the abaxial transition zone, along the star-shaped area and veins. These trichomes were fragile and collapsed easily under the SEM. They appeared to be multicellular and uniseriate, as indicated in Fig. 5A (right). The apical cell was enlarged and formed a single-cellular head. After electron beam treatment under the ESEM, a shrinkage of the

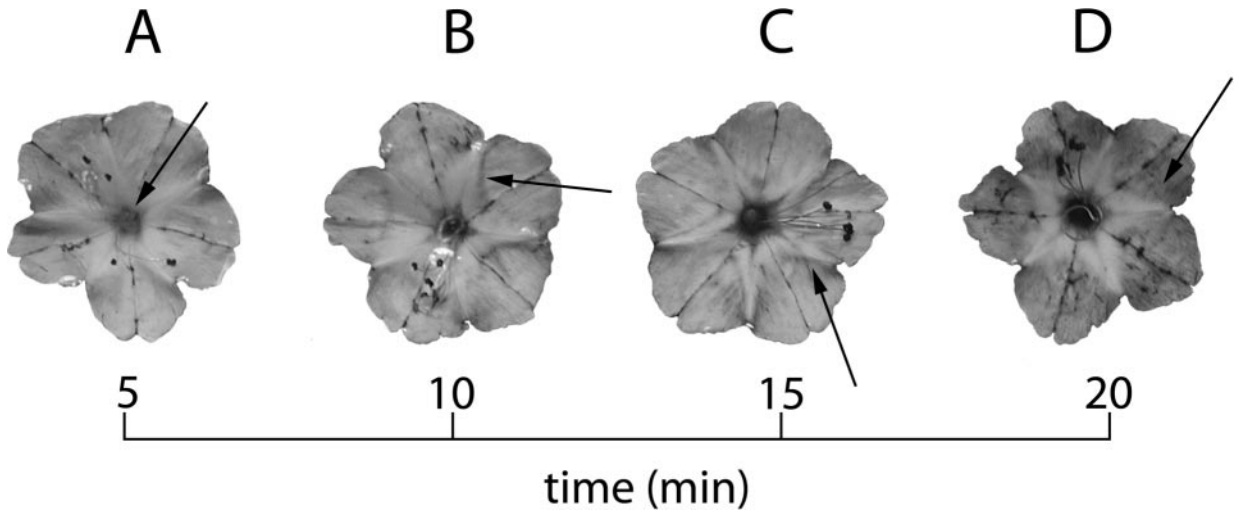

Fig. 3. Neutral red staining of detached *Mirabilis jalapa* flowers. Flowers were immersed in an aqueous neutral red solution (0.1%), followed by rinsing with tap water. Arrowheads point to stained areas.

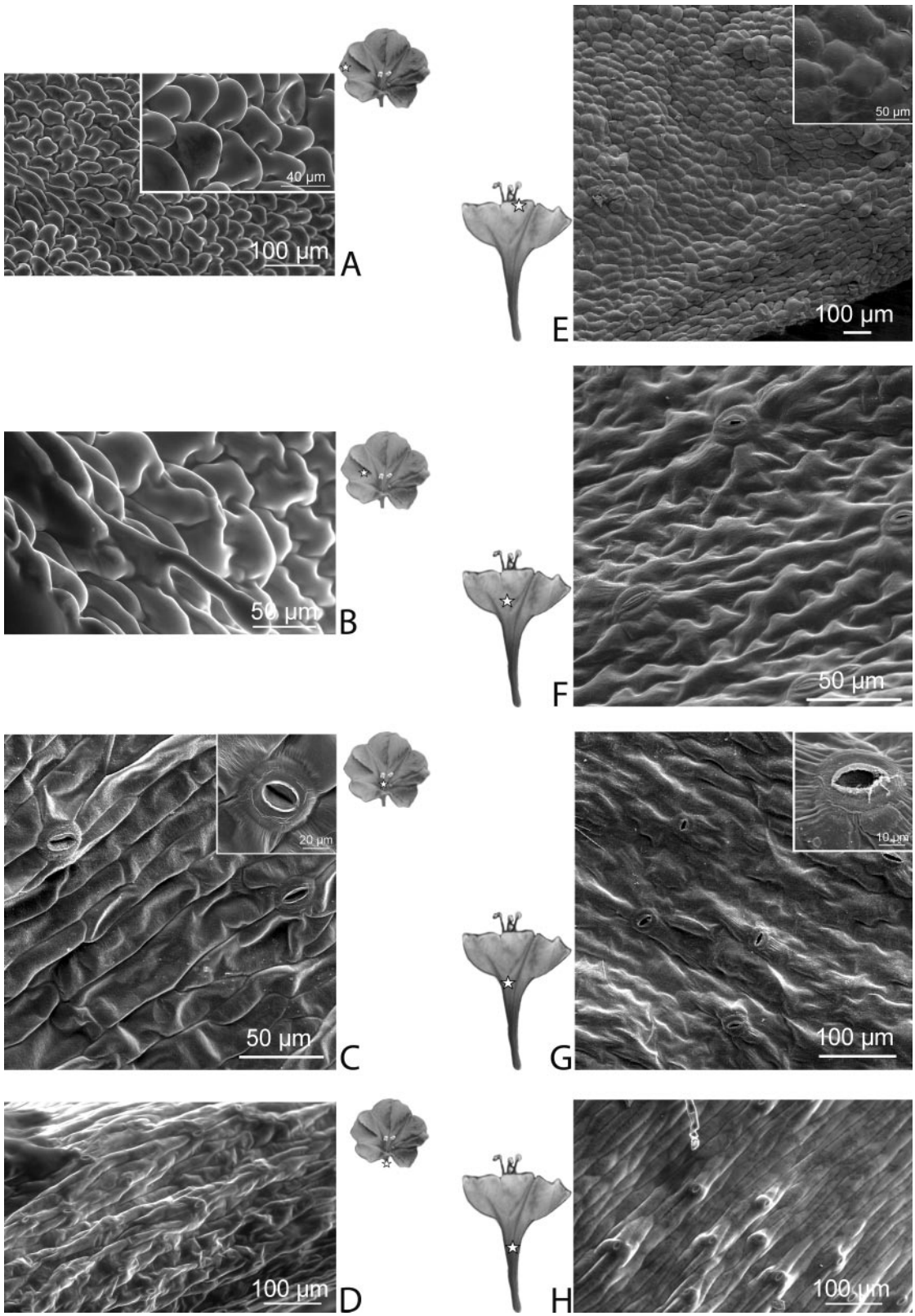

Fig. 4. Morphological characteristics of the *Mirabilis* flower surface. The area selected is indicated in the depicted flower (☆). (A–D): Adaxial epidermis. (E–F): Abaxial epidermis. (A + E), Upper petaloid lobe. (B + F), Lower petaloid lobe. (C + G), Transition zone between tube and limb. (D + H), Tube. A, B, D, H: environmental scanning electron microscope. C, E, F, G: scanning electron microscope.

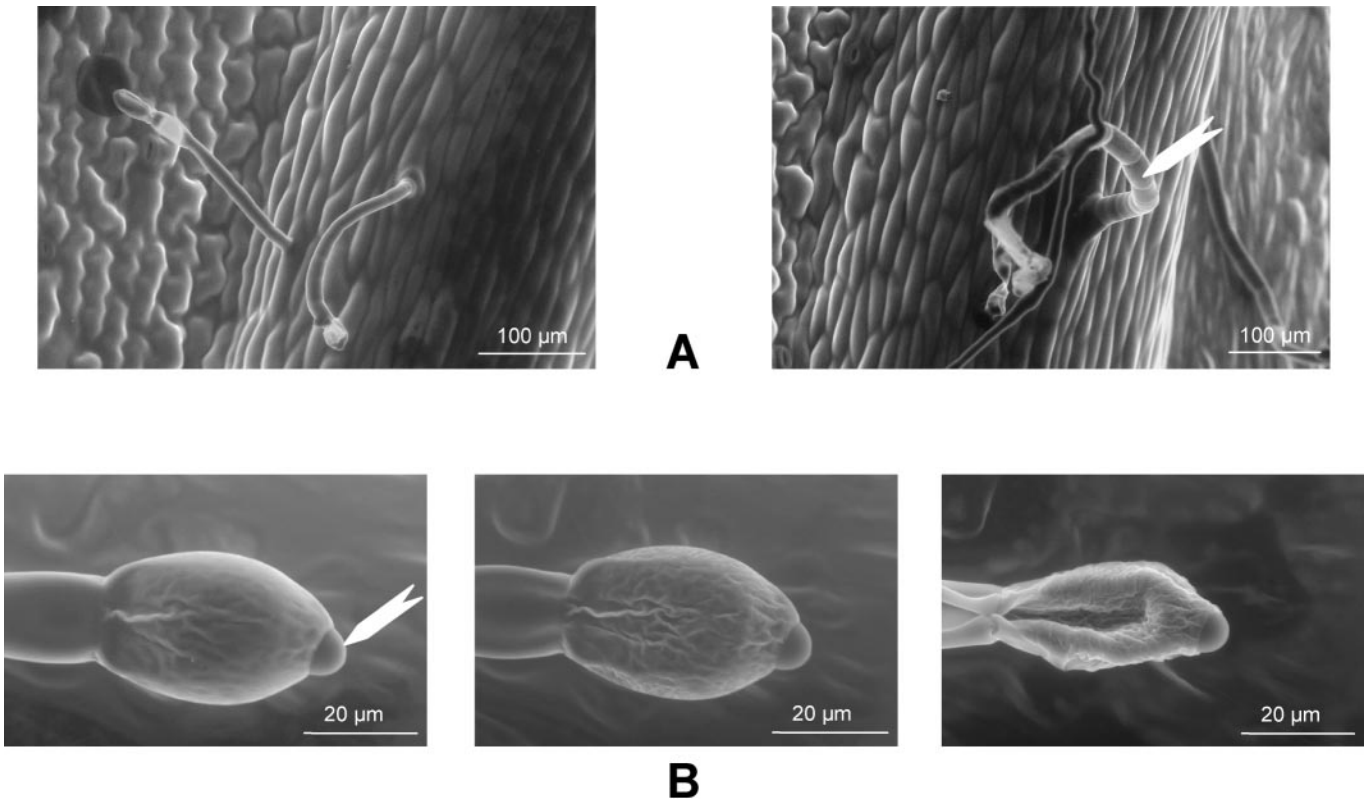

Fig. 5. Morphological characteristics of floral trichomes of *Mirabilis jalapa*. Micrographs were taken using an environmental scanning electron microscope. (A), Two examples of uniseriate trichomes on the abaxial flower epidermis. The arrowhead marks a single cell. (B), Shrinkage of the apical cell caused by electron beam treatment within 30 s (left to right). The arrowhead indicates the apical plug of the trichome.

head cell was observed (Fig. 5B). The tip was formed by an apical pore, which seemed to be a gateway for organic material and implied a glandular character of the trichomes (see arrowhead Fig. 5B, left).

Anatomical studies of the flower tissue using light microscopy—Cross-sections within the (1) upper part of the petaloid lobe and the (2) transition zone of the flower provided further information, which tissue or cell type might be responsible for

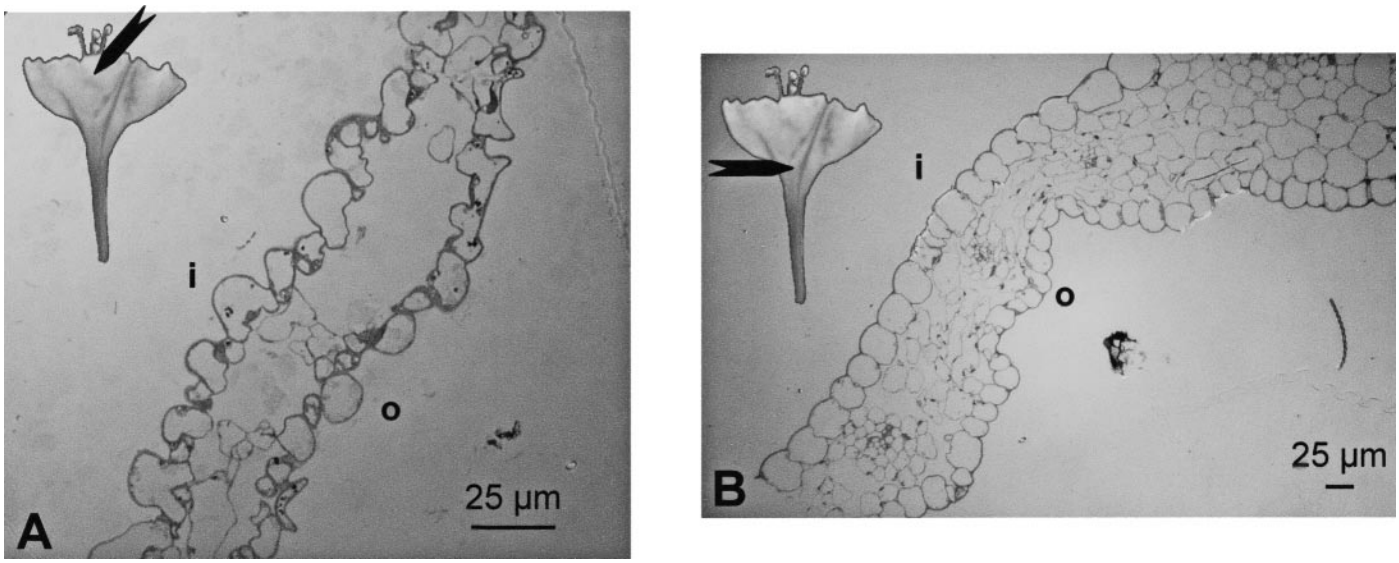

Fig. 6. Anatomical characteristics of the *Mirabilis jalapa* flower. Cross-sections of the indicated flower parts. (A), Upper petaloid lobe. (B), Transition zone between tube and limb. i = adaxial epidermis, o = abaxial epidermis.

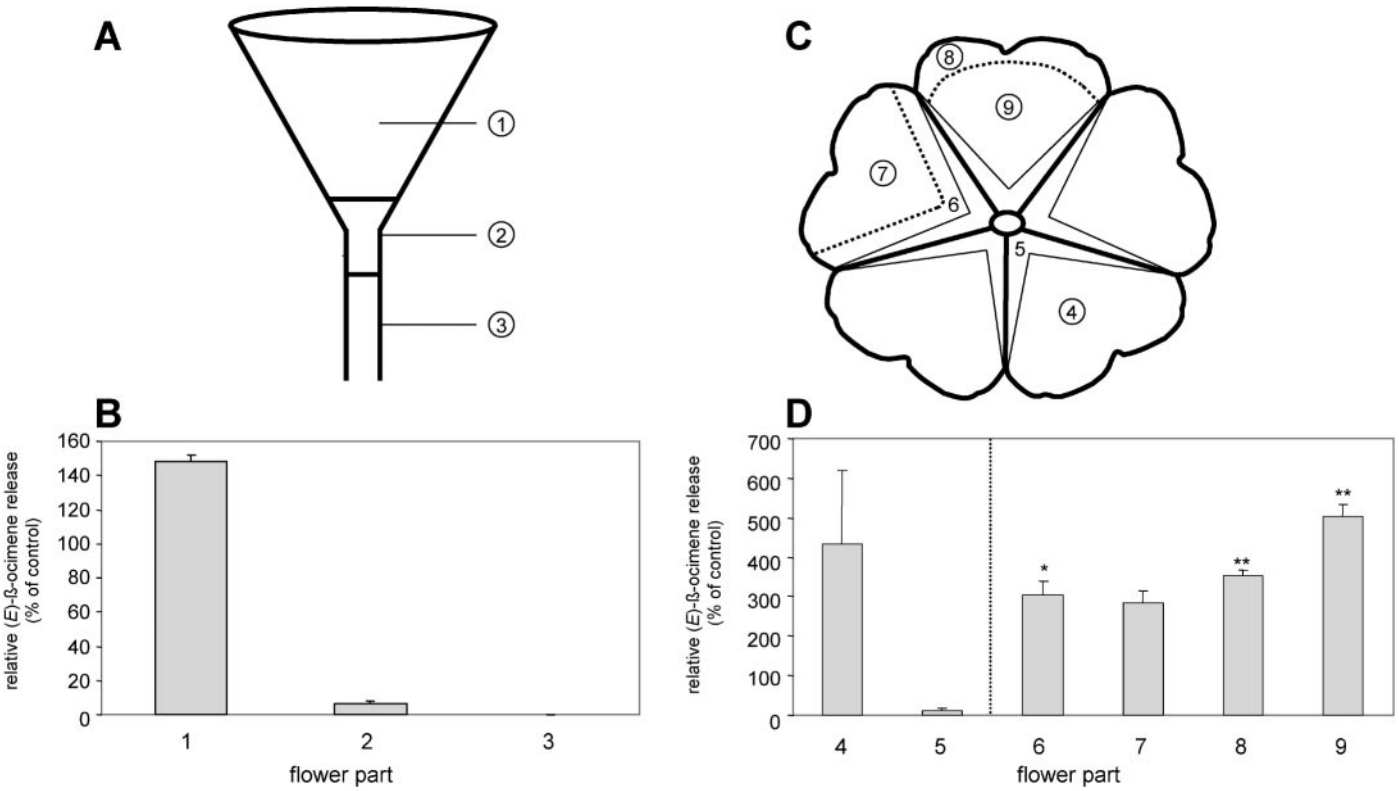

Fig. 7. (*E*)-β-ocimene release from different flower parts of *Mirabilis jalapa*. (A + B) Whole flower sectioned in three parts: 1 Limb, 2 Transition zone, 3 Tube. (C + D) Limb sectioned into six parts: 4 Petaloid lobe, 5 Star-shaped center, 6 Edge along the star, 7 Petaloid lobe without edge of the star, 8 Petaloid lobe rim, 9 Petaloid lobe without rim. (*E*)-β-ocimene release from each flower part was independently and directly compared with the emission of a identically cut whole flower (control = 100%). The emission was estimated per milligram fresh mass per square centimeter. $N = 6$; $*N = 3$; $**N = 8$.

scent emission of *M. jalapa* (Fig. 6). The sections within the upper part of the petaloid lobe (Fig. 6A) confirmed the delicate bullate epidermis of both the adaxial and abaxial side of the flower, as observed with the electron microscopy (Fig. 4A and E). Furthermore, only a few mesophyll cells were observed. They were separated by an extensive intercellular system. In the transition zone, the cells of the adaxial and abaxial epidermis formed an even compact cell layer (Fig. 6B) as seen previously under the electron microscope (Fig. 4C and G), whereas the mesophyll revealed several cell layers with less intercellular space.

Ocimene emission from different flower segments—As a result of the histological and morphological findings, the perianth could be divided into different areas, in which the petaloid lobe because of its morphology (Fig. 4A, E) and special NR staining pattern (Fig. 3B and C), as well as the transition zone because of its pronounced NR staining (Fig. 3A–D) and multilayered mesophyll (Fig. 6B) seemed to be sites of potential scent emission. In order to link these flower parts with the actual sites of emission, flowers were dissected (Fig. 7A and C) and separately examined for (*E*)-β-ocimene release (Fig. 7B and D). Investigations of the limb (1), transition zone (2) and lower tube (3) demonstrated that the limb was the main site of ocimene emission, whereas the transition zone contributed only a minor part to the ocimene release (Fig. 7A and B). When the limb was divided as indicated in Fig. 7C into the lobes (4) and the star-shaped center (5), the lobes emitted the majority of ocimene (Fig. 7D, left panel). After subdividing the lobe into the edge along the star-shaped center (6) and the corresponding remains (7), and into the petaloid lobe rim (8) and the remaining part of the lobe (9), a clear assignment of emission to any of these regions was not possible (Fig. 7D, right panel). However, results confirmed that the petaloid lobe was the main source of (*E*)-β-ocimene emission, while the star-shaped center contributed insignificantly to the emission, which correlated with the results of NR staining and morphological studies. The transition zone, despite showing pronounced NR staining, emitted little ocimene.

Are floral stomata or floral trichomes involved in scent release?—Monitoring the degree of stomatal opening in the adaxial and abaxial epidermises did not show a clear correlation between the open stomata state and the time of a high scent emission (Fig. 8). Therefore, it seems very unlikely that stomata are directly involved in volatile emission. However, in the beginning of scent release, stomata of the adaxial and abaxial epidermises were mostly open indicating an enhanced gas exchange during volatile production as described e.g., for osmophores due to elevated metabolism. (Vogel, 1962).

The investigation of floral trichomes revealed that the major component of the trichome content was (*E*)-β-farnesene, while four other minor compounds could yet not be identified. However, none of the floral fragrance components were detected in the trichomes. These results indicated that floral stomata and trichomes did not contribute to the fragrance emission of *M. jalapa*.

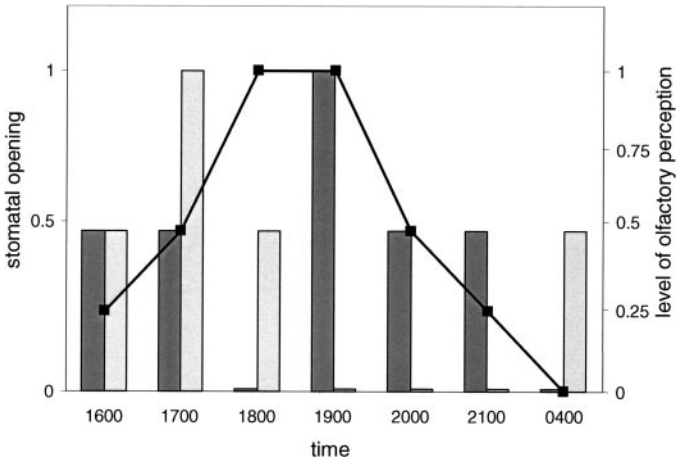

Fig. 8. Stomatal opening in correlation with the time course of scent emission. Three categories of stomatal condition were defined: (0) closed, (0.5) half-open, (1) opened. Levels of olfactory perception was classified as (1) for the maximum and (0.75), (0.5), (0.25) and (0) for progressively lower levels. ■ stomata of the inner epidermis. ■ stomata of the outer epidermis. –■– scent perception. $N = 1$.

DISCUSSION

As previously described by Heath and Manukian (1994) and Levin et al. (2001), findings from our fragrance analysis using both the whole plant and separate flowers showed (*E*)-β-ocimene to be the prominent scent component of flowering *M. jalapa*. We also identified myrcene and (*Z*)-3-hexenyl acetate in accordance with both authors. However, the presence of α-farnesene, which was emitted in almost equal amounts compared to (*E*)-β-ocimene (Levin et al., 2001), could not be confirmed. In addition, like Levin et al. (2001), we could not confirm the presence of indol and benzaldehyde found by Heath and Manukian (1994). Furthermore, Levin et al. (2001) detected 14 other scent compounds in which we also found (*Z*)-ocimene, (*Z*)-3-hexenol, benzyl benzoate and traces of methyl salicylate. We also detected epoxy-ocimene, which neither Heath and Manukian (1994) nor Levin et al. (2001) mentioned.

These variations in scent composition could be due to intraspecific differences, but also to different growth conditions. Although our results were obtained using the sampling method established by Heath and Manukian (1994), the latter conducted the experiment under greenhouse conditions with a variability of light intensity and temperature, whereas our experiments were performed in growth chambers supplying defined constant conditions. Also Levin et al. (2001) performed their studies in the greenhouse, where the influence of environmental conditions including stress could be responsible for differences in the volatile blend (Gouinguené and Turlings, 2002). This might especially apply to the absence of α-farnesene as a fragrance constituent of plants that were investigated under stable plant chamber conditions. α-farnesene has been found to be released in response to environmental stress such as herbivore damage (Loughrin et al., 1994; Röse et al., 1996; Rodriguez-Saona et al., 2001). Moreover, it increased mortality and reduced reproduction of aphids (Harrewijn et al., 1996). Differences in fragrance analysis in various studies could also be caused by different periods of sampling. Levin et al. (2001) collected for 12 h, which provides the advantage of longer accumulation of minor volatiles, whereas Heath and Manukian (1994) and our investigations were based on eight collections with 3-h intervals.

The temporal emission patterns we observed for (*E*)-β-ocimene and almost all minor components corresponded with those reported by Heath and Manukian (1994). The peak of emission matched nicely with the flower opening and activity of crepuscular pollinators, which are hawk moths like *Erinnyis ello* and *Hyles lineata* (Martinez del Rio and Burquez, 1986). One unusual result was that (*Z*)-3-hexenyl acetate had its own rhythm and reached its maximum of release later than (*E*)-β-ocimene. (*Z*)-3-hexenyl acetate is known to be a volatile emitted after herbivory as well as mechanical wounding (Loughrin et al., 1994; Röse et al.,1996; Thomas, 2000; van Poecke et al., 2001; D'Auria et al., 2002). In *M. jalapa*, this volatile was found to be released by the vegetative tissue (Levin et al., 2001). De Moraes et al. (2001) could show that (*Z*)-3-hexenyl acetate is emitted by *Nicotiana tabacum*, where it discourages conspecific female moths (*Heliothis virescens*) from placing their eggs on damaged leaves (De Moraes et al., 2001; Ryan, 2001; Pichersky and Gershenzon, 2002). Following this scenario, the emission of (*Z*)-3-hexenyl acetate from green leaves of *M. jalapa*, although undamaged, could represent a strategy to prevent unwelcome visits of night-flying female moths after the optimal time for pollination has passed. This applies most presumably to the period after maximal (*E*)-β-ocimene emission. The release of (*Z*)-3-hexenyl acetate as a result of injured leaf tissue following the transfer of plants into the collecting chamber can be excluded, because the emission showed a persistent rhythm. Release of (*Z*)-3-hexenyl acetate as a wounding signal occurs immediately after damage and a rhythmic emission would not be expected in this case (Loughrin et al., 1994; Thomas, 2000; D'Auria et al., 2002).

What mechanism is involved in the release of the sweet floral fragrance of *M. jalapa*? In order to check the presence of osmophores, detached flowers were stained with neutral red (NR). The selective uptake and retention of this stain by intact tissue are considered to be an indication for osmophores (Vogel, 1962; Pridgeon and Stern, 1983; Stern et al., 1986), and it is assumed to be caused by increased permeability of their epidermis cell wall and long-lasting storage ability of vacuoles. Vacuoles act like a NR ion trap because of their slightly acidic pH-value. Applied nonionic NR molecules can diffuse through the tonoplast, but then cannot penetrate vice versa. Therefore, putative osmophoric flower areas or parts would take on red color. The staining experiments pointed to the petaloid lobes between the star-shaped area (Fig. 7B, see 4) and the so called transition zone (Fig. 7A, see 2) as the most likely sites of emission. The coloring of the petaloid segment always initiated along the edges of the tips of the star-shaped center (Fig. 7B, see 6).

The central role of the petaloid lobe in emission was also supported by morphological studies using SEM and ESEM. This segment was the only flower area that displayed a distended epidermis surface (Fig. 4A), which was strongly suggestive of the conical cells found in the epidermis of rose and *Petunia* petals (F. Ehrig, unpublished data) and in snapdragon (Kolosova et al., 2001). This structure has also been described for lobe margins of *Bougainvillea stipitata* flowers (Nyctaginaceae; López and Galetto, 2002). This typical bullate epidermis would also fulfill a feature found in osmophores, because surface enlargement is supposed to be a precondition for optimal volatile emanation (Vogel, 1962). However, cross-sections of the upper petal segment (Fig. 6A) examined by LM

confirmed a fragile structure of both epidermises of the upper petaloid lobe supporting facilitated emission, but the nearly missing mesophyll cell layers between the epidermises indicate that the scent has a diffuse emission rather than one by osmophores. The planar, elongated and sinoid epidermis cells of the lower limb part (Fig. 4B and F) observed in the SEM/ESEM studies, as well as the presence of stomata and trichomes primarily along the abaxial star-shaped center were found to be similar to those of the *Mirabilis* leaf surface (unpublished data). This could be explained simply as evidence of the evolutionary history of *Nyctaginaceae* flowers, since their perianth is interpreted as a calyx with corolloid appearance (Vanvinckenroye et al., 1993; López and Galetto, 2002).

Dividing the flowers into parts based on the findings of the staining experiments and microscopy to evaluate each for scent release confirmed the crucial role of the petaloid segment (Fig. 7B, see 4) in ocimene emission. Compared to the whole flower, this area was responsible for most of the emission. Although the star-shaped area (Fig. 7B, see 5) contributed a minor amount to the overall (*E*)-β-ocimene emission, this finding may be due to an artefact in our method, when small portions of the petaloid segment along the edge of the star (Figs. 7B, 6) sometimes remained attached to the star-shaped center after flower dissection and probably contributed to ocimene release in this area. Therefore, the emission of the star-shaped center was probably lower than measured, which is further supported by the finding that scent emission of the star-shaped center plus narrow edge tissue did not significantly differ from that released from the edge alone (U. Effmert, unpublished data). Dividing the flower into three parts according to Fig. 7A, it could be verified that the ocimene emission is limited to the limb (Fig. 7A see 1). The floral tube (Fig. 7A see 3) was not important for ocimene release and, surprisingly, also scent release from the transition zone (Fig. 7A see 2) could not be confirmed, even though this area took up NR stain and showed a multilayered mesophyll and intercellular channels, which would be typical for an osmophore structure (Vogel, 1962; Hadacek and Weber, 2002). A possible connection between NR staining and the presence of nectaries in this area, which can also be colored by NR (Comba et al., 1999) can be excluded here, since Vanvinckenroye et al. (1993) reported the nectariferous tissue of *M. jalapa* to be located at the base of the stamens, which have fused filaments that form a staminal tube where the nectar is secreted through nectarostomata at the inner side of the tube. A positive staining of the transition zone due to chemical reactions as described by Stern et al. (1987) might be possible, but remain speculative at this point of the investigations.

Because sepals act like petals in *M. jalapa* and indeed still show some morphological characteristics of green tissues, a connection between floral scent release and floral stomata was not dismissed right away. In addition, the appearance of stomata on the epidermis of flowers is not that unusual. A concentration of stomata is often observed on the abaxial epidermises of osmophores, where they manage the extensive gas exchange due to intensive metabolism caused by volatile production (Vogel, 1962; Skubatz et al., 1995). However, stomata were observed both in the adaxial and abaxial epidermis of the *M. jalapa* flower, suggesting that the presence of stomata should be discussed beyond the evolutionary aspect mentioned earlier. Could they be involved in sent release as generally mentioned by Kugler (1970) and Leins (2000)? If so, preferentially the adaxial stomata should at least be open during the time of emission in order to guide pollinators to the inside of the flower. There was, however, no clear correlation between fragrance emission and stomatal opening. Instead, both inner and outer stomata were open at the beginning of scent release, which might be correlated with an enhanced gas exchange, and were closed later, despite the ongoing scent release. The influence of the conditions in the growth chamber on stomatal closure or opening should be excluded, since changes in light intensity, temperature, and humidity can be neglected.

Mirabilis jalapa showed a large number of single, uniseriate, and multicellular hairs that might be involved in volatile emission, in spite of the fact that they did not seem to be flower-specific and most of them were located on the outer surface. When trichomes were isolated and contents were analyzed by GC/MS analysis, however, none of the fragrance components of *M. jalapa* could be detected. This indicated that these trichomes, although displaying glandular character, did not contribute to the scent of *M. jalapa* flowers, but they obviously were used for β-farnesene storage and/or synthesis. The role of this sesquiterpene as a semiochemical is well described. Elevated levels are emitted by cotton and maize plants after herbivore attack (Turlings et al., 1990; Loughrin et al., 1994; Röse et al., 1996; Rodriguez-Saona et al., 2001). Most remarkable were findings that assigned the use of β-farnesene as an alarm pheromone for aphids. By taking advantage of the aphid alarm signal, plants are able to repel herbivores as reported for the wild potato *Solanum berthaultii* (Gibson and Pickett, 1983; Ave et al., 1987; Crock et al., 1997; Mondor et al., 2000). Since β-farnesene could not be detected as a headspace fragrance constituent of *M. jalapa*, we speculate that it could play a role as repellent, which is released upon damage of the trichomes, e.g., by herbivores. After all, keeping in mind that the flower of *M. jalapa* is derived from sepals and that green leaves bear a very similar trichome type, it should be considered that the defense purpose of β-farnesene might be originally assigned to the leaf.

In summary, *M. jalapa* shows both temporal emission patterns of floral scent and spatial patterns of scent release. Only the petaloid lobes of the perianth limb were involved in scent emanation. The question of diffuse emission vs. localized emission from a specific part of the lobe with osmophore characteristics has not been definitely answered. However, results obtained so far suggest a diffuse emission of scent from the petaloid lobes. It must be concluded that only the entirety of analytical, morphological/anatomical, and histochemical studies, together with the localization of the enzyme activity and the enzyme itself responsible for (*E*)-β-ocimene formation will give a complete picture of scent production and emanation of the *M. jalapa* flower.

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