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RESEARCH PAPER

A simple floral fragrance and unusual osmophore structure in *Cyclopogon elatus* (Orchidaceae)

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ABSTRACT

We studied gland morphology, anatomy and the chemical composition of the floral fragrance in the sweat bee-pollinated orchid *Cyclopogon elatus*. This is apparently the first such analysis for any *Cyclopogon* species, and one of very few studies in which both odour and osmophore are characterised in a nectar-rewarding orchid. Structures responsible for floral scent production were localised with neutral red staining and histochemical assays for lipids and starch. Their morphology and anatomy were studied with scanning electron microscopy and light microscopy thin sections, respectively. Fragrance samples were collected using SPME fibres and analysed with GC-MS. Anatomical evidence suggests that two parallel oval-shaped patches of unicellular trichomes on the abaxial surface of the labellum are osmophores. These are rich in stored lipids, while the parenchyma surrounding the vascular bundles contains starch. Only freshly opened flowers produced odours, while buds and withered flowers lacked scent. The chemical composition of the odour was dominated (>99.8%) by a single compound, trans-4,8-dimethyl-nona-1,3,7-triene (DMNT). Gland anatomy and position on the outside of the perianth are unusual for scent glands in general. The presence of DMNT, a nearly ubiquitous compound in herbivore-induced vegetative emissions and one of the major floral volatiles of *Yucca*, is not surprising in view of hypotheses on the evolutionary origin of flower scents, suggesting that wound volatiles are utilised as kairomonal attractants by florivores whose activities result in pollination.

INTRODUCTION

Floral fragrances usually function as secondary floral attractants that announce to pollinators the presence and location of primary rewards (Faegri & van der Pijl 1979; Endress 1994; Proctor *et al.* 1996). A complex mixture of volatile compounds serve as attractants in plants pollinated by a varied fauna, including bats, beetles, butterflies, moths, bees and wasps (Knudsen & Tollsten 1993, 1995; Gibernau *et al.* 1999; Andersson *et al.* 2002; Petterson *et al.* 2004; Raguso 2004; Johnson *et al.* 2007). Flower parts engaged in scent production may be represented either by an undifferentiated epidermis, which diffusely emits fragrances (Mazurkiewicz 1913), or by osmophores, *i.e.* spatially localised and multilayered floral tissues (Stern *et al.* 1987; Vogel 1990).

The family Orchidaceae provides striking examples of several kinds of plant–pollinator interactions, including sexual and brood site deception, generalised and specialised food deception, as well as nectar and oil-based rewarding systems (van der Pijl & Dodson 1966; van der Cingel 1995, 2001). A large body of research on orchid fragrances has focused on species pollinated through sexual deception (Borg-Karlson 1990; Schiestl *et al.* 1999) and on euglossine bee pollination (Dressler 1982; Eltz *et al.* 1999). In contrast, there have been comparatively few studies on orchid scents in relation to an 'honest' rewarding system, in which one can expect a genuine floral scent, *i.e.* one that pollinators learn to associate with a flower reward (Patt *et al.* 1989; Tollsten & Bergström 1989; Kaiser 1993; Huber *et al.* 2005; Johnson *et al.* 2007).

Cyclopogon elatus (Sw.) Schltr. is a nectar-producing orchid that is pollinated by sweat bees (Halictidae) (Galetto *et al.* 1997; Benitez-Vieyra *et al.* 2006). The flowers of closely related species emit a strong sweet odour that is perceived as musk-like (Singer & Sazima 1999). As the plants grow concealed in the shady understorey of subtropical dry (Chaco) forest, their small brown flowers could easily pass unnoticed, and flower scents might play an important role in flower localisation by pollinators. The aims of this work were: (i) to localise the site of fragrance emission and production; (ii) to determine the morphological and anatomical nature of the scent-producing flower parts; (iii) to analyse the fragrance chemical composition; and (iv) to test the attractiveness of floral fragrances to bees. To our knowledge, this is the first chemical analysis of floral scent for any *Cyclopogon* species and one of very few studies in which odour and osmophore are characterised in a nectar-rewarding orchid.

MATERIALS AND METHODS

Study site

Studies were carried out in a wild population located near Cabana village, Córdoba province, Argentina (31°12′46″ S, 64°20′52″ W, 729 m) from 2005 to 2007.

Osmophore localisation and structure

To localise osmophore areas, buds, fresh and withered flowers were submerged in a solution of 1:10,000 neutral red:tap water for 12 h (Vogel 1990; Dobson *et al.* 2005) and then examined under a dissecting stereomicroscope. Histochemical assays for lipids were made with Sudan IV, after peeling off the abaxial epidermis of the labellum. To detect starch, the labella of flower buds, fresh and withered flowers were cleared with 3% NaOH for 3 h at 55 °C and then stained with Lugol (Bayley & Nast 1943).

For light microscopy (LM), samples were fixed for 24 h in gluteraldehyde-buffer solution, stored in 70% ethanol and embedded in resins according to the manufacturer's instructions (Technovit Histo-Technique 7100, Heraeus Holding GmbH, Hanau, Germany). Microtomed 5-μm thick sections were stained with toluidine blue (Sakai 1973). For scanning electron microscopy (SEM), labella were critical point dried and sputter-coated with gold–palladium using conventional protocols.

Volatile collection and analysis

For odour samples, intact, live inflorescences containing either flower buds, freshly opened or withered flowers (four replicates, *ca*. 160 flowers) were enclosed in nylon resin oven bags (Reynolds Inc., Richmond, VA, USA) and equilibrated for increasing periods of time (from 20 to 40 min) until no new peaks appeared in the chromatograms (see Goodrich *et al.* 2006). Subsequently, fragrances were exposed for 30 min to a 'stableflex' solid phase microextraction fibre (SPME: Supelco Co., PA, USA) (Zhang & Pawliszyn 1993) coated with divinylbenzene and polydimethylsiloxane (65-μm film thickness). SPME is an appropriate technique to capture minor components that is increasingly used for floral fragrance analysis and avoids the loss of components and contamination through use of intermediary solvents and vials (Flamini *et al.* 2003; Goodrich *et al.* 2006). Control samples were collected in parallel from leaves and empty bags. Volatiles were desorbed within the splitless injection port of a gas chromatograph connected to a quadrupole mass spectrometer (Perkin Elmer Q-Mass 910; Perkin Elmer Inc., Waltham, MA, USA). The exposed fibre was cleaned for 5 min between samples at 240 °C in the GC injection port (Raguso *et al.* 2003). Volatile compounds were separated in the GC using an EC-WAX column (Econocap brand GC column with poly(ethylene glycol) as a polar stationary phase) (length 30 m, ID = 0.25 mm, Alltech Associates, Inc., Deerfield, IL, USA, now a division of W.R. Grace Inc., Columbia, MD, USA) and helium as carrier gas (flow rate 1 $ml \cdot min^{-1}$). The oven temperature programme used was 40 °C for 3 min, increasing 10 °C min^{-1} to 240 °C, where it was held for 5 min. Volatiles were identified by comparing the mass spectra with those in computer libraries (NIST, Wiley, Adams) and published spectra (Degenhardt & Gershenzon 2000).

Pollinators

Pollinator activity in the *C. elatus* population was recorded during three consecutive years, totalling 34 man-hours (6 h in 2005, 8 h in 2006 and 20 h in 2007). Observations on pollinator behaviour were made during spring 2007. To test if pollinators were able to find *C. elatus* flowers by means of scent alone, in the absence of visual cues appropriate to the flowers, we observed pollinator visits to enclosed spikes in scentless chimney-like paper tubes, which allowed the diffusion of odours through the open apical end. Observations were made in five patches consisting of six to 11 flowering plants from 11:00 to 15:00 h, totalling 20 man-hours. In each patch, we recorded the visits to one enclosed plant, to one empty tube as a negative control, and to the rest of the plants within the patch as positive controls. Shapiro–Wilks tests showed non-normal distribution of raw data, so we carried out non-parametric tests. We tested (and rejected) the hypothesis that the number of pollinator visits differed significantly among patches (Kruskall–Wallis test: $H = 5.05$, $p = 0.15$, n.s.). Thus, we pooled the data and used Wilcoxon tests to compare between-treatment differences (enclosed plants and positive controls, since no visits were recorded in negative controls).

RESULTS

Osmophore localisation and structure

Neutral red staining was positive in flower buds and freshly opened flowers, showing high metabolic activity in two

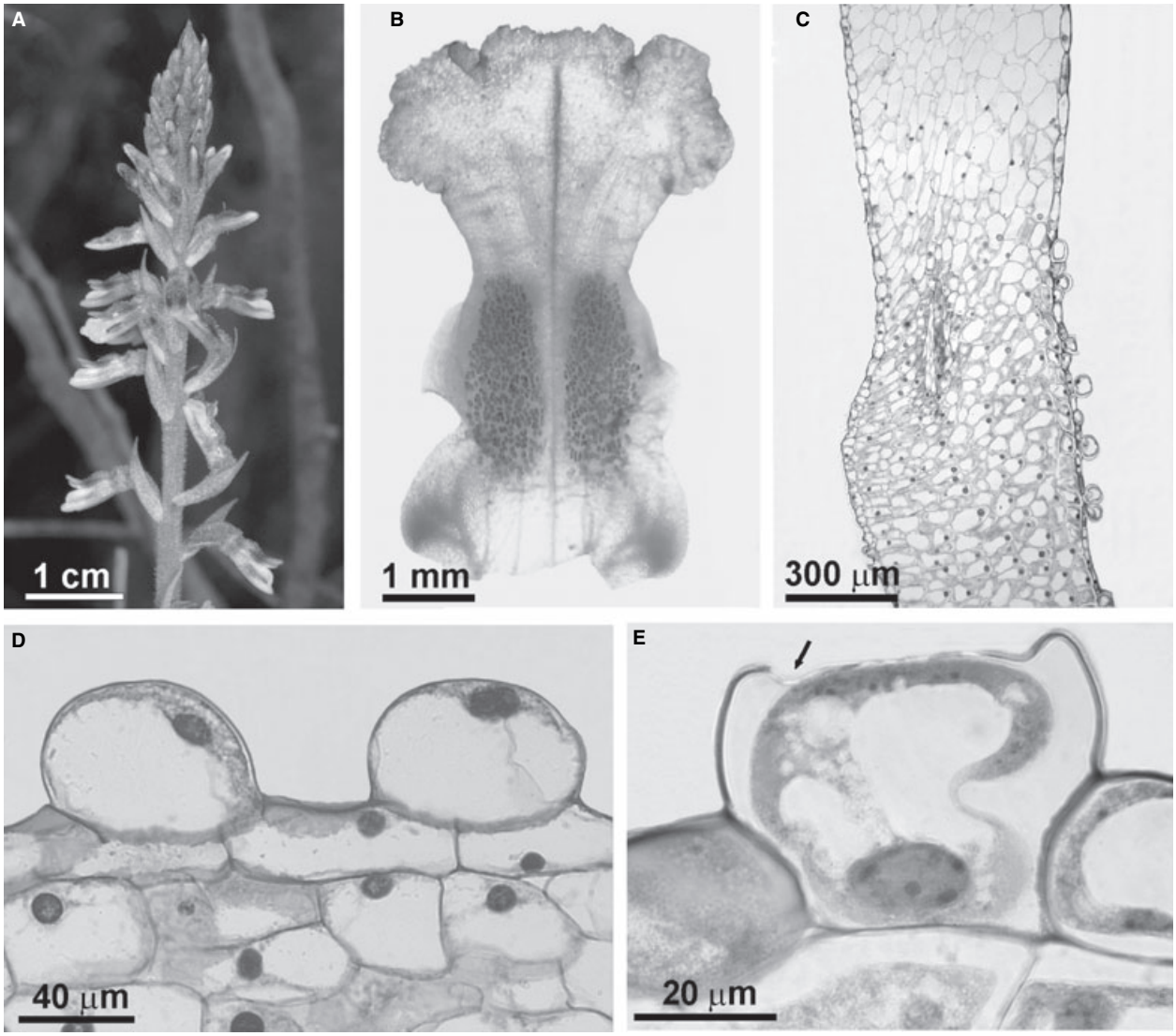

Fig. 1. *Cyclopogon elatus*. A: Spike with buds and opened flowers; B: Clear field of a labellum. Notice the two osmophore patches covered with trichomes. C–E: LM sections of the osmophore. C: Longitudinal section showing the glandular (bottom) and non-glandular (top) areas. D: Globular trichomes of a bud. E: Detail of a trichome from an opened flower. Notice the indentation at the top, where the cell wall is lacking (arrow).

parallel oval-shaped areas located on the abaxial surface of the labellum. Clear field, LM and SEM photographs revealed that these areas were covered with globular trichomes (Figs 1B,C and 2A, respectively). When these areas in freshly opened flowers were rubbed with a needle, an intense fragrance was perceived. When seen from above using the SEM photographs, trichome shape varied from spherical near the base of the labellum to nearly teardrop-shaped towards its apex, with the globular portion oriented towards the base of the labellum (Fig. 2A).

Sections of buds and open flowers observed under LM revealed that trichomes were unicellular, with dense cytoplasm, a prominent nucleus (mean diameter 19 ± 4 μm in buds and 17 ± 2 μm in freshly opened flowers, n = 30 each) with conspicuous chromocentres and a large vacuole. In contrast to neighbouring epidermal cells proper, trichomes had a smooth cuticle and a thin and low-optical density cell wall. From buds to the open flower stage, the trichome cytoplasm became detached from the outer cell wall and accumulated many coarse lipid-rich vesicles (Fig. 1D and E), as revealed by Sudan IV staining. SEM showed an apical, nearly circular, sharply delimited wall indentation (Fig. 2B and C). In LM sections, this feature corresponded to a patch-like area lacking cell wall where the cuticle collapsed and shrank in the open flower stage (Fig. 1E). The anticlinal walls, when in contact with the epidermal cells proper, showed conspicuous primary pit fields.

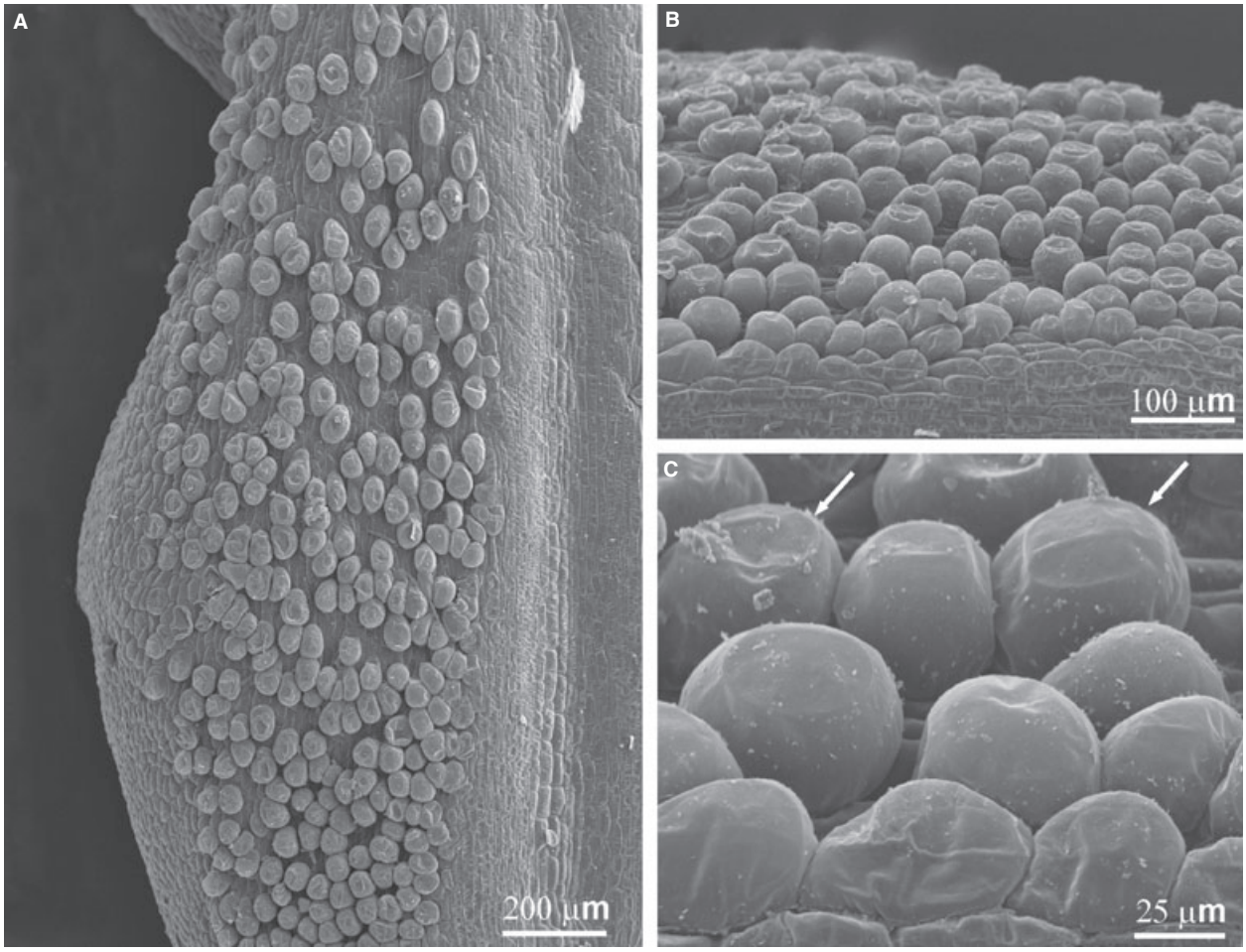

Fig. 2. SEM photographs of *Cyclopogon elatus* osmophore in an opened flower. A: General view of one osmophore located in the abaxial side of the labellum. B: Detailed view of osmophore surface showing trichomes. C: Close-up of trichomes with indentations (arrows).

The parenchyma underlying the trichome fields was compact, *i.e.* with small intercellular spaces, and consisted of cytoplasm-rich cells also containing vesicles, one or two large vacuoles and a large nucleus (mean diameter 14.5 ± 1.9 μm in open flowers, n = 30) with many chromocentres. Cells surrounding the vascular bundles had abundant amyloplasts. Lugol staining showed progressive consumption from flower buds to withered flowers of starch grains in the labella. In contrast, the non-glandular parenchyma at the tip of the labellum consisted of cytoplasm-poor cells containing one large vacuole and reduced nuclei (Fig. 1C). The cytoplasm of trichomes, epidermal cells proper and parenchyma cells was noticeably reduced in withered flowers, while the cuticle and the cell wall of the trichomes remained unchanged from open to withered flower stage.

Flower odour

Flowers of *C. elatus* emitted an intense, distinct odour with balsamic, musky and somewhat bitter and rancid notes. Chromatograms from freshly opened flowers were dominated by a single volatile compound, trans-4,8-dimethyl-nona-1,3,7-triene (DMNT). This compound accounted for 99.82–99.99% of the total fragrance detected. The only other compound detected consistently was its isomer (cis-4,8-dimethyl-nona-1,3,7-triene; Fig. 3). Trace amounts (<0.0002% of total floral odour) of limonene were present in flowers and vegetation. In addition, some samples contained traces of unidentified compounds characterised by ion fragment m/z 107.

Pollinators

Flowers of *C. elatus* were only pollinated by halictid bees. Pollinaria become attached to the halictid proboscis when they introduce their head into the flower to gather nectar. During 2005, we recorded 22 halictid bees (18 *Augochlora nausicaa* and four *Pseudoagapostemon jensenii*) and during 2006 we only observed five *A. nausicaa* individuals. *Apis mellifera* bees were observed visiting the flowers during 2005 but did not effectively pollinate them.

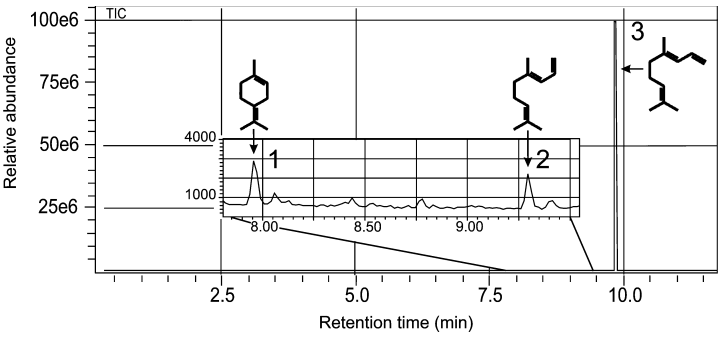

Fig. 3. Representative gas chromatogram of floral scent from opened flowers of *Cyclopogon elatus*. The inserted panel shows a magnified baseline between 7.8 and 9.8 min. Numbered peaks are identified as follows: 1. limonene (7.99 min); 2. cis-4,8-dimethyl-1,3,7-nonatriene (9.19 min) and 3. trans-4,8-dimethyl-1,3,7-nonatriene (9.71 min).

Pollinator behaviour was observed during spring 2007. We recorded a total of 28 halictid bees (27 *A. nausicaa* and one *P. jensenii*), one colletid bee, and 31 individuals of *A. mellifera*. Empty chimney tubes did not receive any bee visits, whereas enclosed flower spikes were visited by seven individuals of *A. nausicaa*, which approached in an upwind zig-zag fashion and entered at the openings of the tubes. This indicates that these bees can find *C. elatus* flower spikes utilising odour cues alone. Bees visiting uncovered inflorescences also approached in the same fashion and, once near the spike, landed directly on an open flower. Considering only solitary bees, we did not find significant differences between the number of visitors to the enclosed and the open spikes (Wilcoxon test: $n = 40$; $W = 128.50$; $P = 0.219$). No *A. mellifera* bees were observed to visit the enclosed inflorescences.

DISCUSSION

This study of flowers of *C. elatus* reveals a scent gland that is unusual for plants, both in its localisation and anatomical features, as well as a fragrance that is interesting for the simplicity of its composition and the chemical nature of its major component.

Osmophore localisation and anatomy

The presence of two sharply delimited patches of cells on the abaxial surface of the labellum is unique to this species and among orchids studied thus far. Although the most widespread location of scent emission in orchids is the labellum, it is never restricted to the abaxial surface. Osmophores localised on the abaxial surface of the perianth have been recorded for three exceptional orchid species (Vogel 1990). To our knowledge, among angiosperms in general, scent emission is rarely localised on the outer surface of the perianth (Effmert *et al.* 2006).

The osmophore of *C. elatus* is also unusual for its anatomical structure. Unlike most osmophores, this orchid has trichomes instead of a secretory epithelium consisting of more or less uniform epidermal cells (Stern *et al.* 1986; Vogel 1990; Stpiczyńska 2001). Trichome osmophores have seldom been recorded on flowers of other angiosperms (Mattern & Vogel 1994). Among orchids, trichome-derived osmophores in flowers have only been recorded in four other species (Swanson *et al.* 1980; Vogel 1990; Teixeira *et al.* 2004). Osmophore cells of *C. elatus* contain lipid droplets but no starch, in contrast to most studied orchids (but see Stpiczyńska 2001). Although starch was abundant in the labella of buds, it was restricted to parenchymatic cells surrounding the bundle sheath.

The glandular nature of the trichomes is supported by their uptake of neutral red, the immediate release of odour when they are rubbed, morphological and anatomical features observed under SEM and LM, and by the presence of lipid droplets in their cytoplasm. A reduction of the cytoplasm in cells constituting the osmophore, *i.e.* trichomes, epidermal cells proper and parenchymatic cells in withered flowers, as well as the consumption of starch, provide further evidence of the glandular activity of this structure.

The anatomical features of the scent gland and underlying tissues are consistent with the idea of a functional layering of the osmophore structure into storage, production and emission layers as found in many structured osmophores (Vogel 1990). In *C. elatus*, the storage layer is represented by parenchyma cells surrounding the vascular bundles, which are rich in starch grains that are consumed during flower anthesis. The production layer is constituted by the upper parenchyma and epidermal cells. There is an apparently intense flux of metabolites from this layer to the trichomes, as suggested by conspicuous pit fields. Finally, the trichomes function as the emission layer, as suggested by the following evidence: (i) accumulation of lipid-rich substances, probably precursors or the fragrance itself, as documented in osmophores of other orchids (Swanson *et al.* 1980; Pridgeon & Stern 1983); and (ii) lack of cell walls at the trichome top. Apparently, fragrance compounds accumulate beneath the cuticle and then diffuse through it, as suggested by the detachment of cytoplasm from the cell wall. Trichomes maintain their integrity after odour release, in contrast to the secretion mode described for *Cyphomandra* species, in which the cuticular blisters burst (Sazima *et al.* 1993).

Fragrance

Flower fragrances are usually moderately to highly complex mixtures, consisting of tens to hundreds of volatile

organic compounds (reviewed by Knudsen *et al.* 2006). Thus, the fact that the fragrance of *C. elatus* flowers is simple and dominated by one compound is unusual. The literature supports at least two models of odour specificity in pollinator attraction: (i) species-specific ratios of commonplace compounds such as in euglossine bee-pollinated orchids (Dressler 1982; Eltz *et al.* 1999), *Ophrys* (Borg-Karlson 1990; Schiestl *et al.* 1999) and *Ficus* (Gibernau *et al.* 1997), or (ii) simple presence of unique or unusual odours such as in the sexually deceptive *Chiloglottis* (Schiestl *et al.* 2003), *Epichlöe* endophyte fungi (Schiestl *et al.* 2006) and carrion mimicry in *Helicodiceros* (Stensmyr *et al.* 2002). Depending on the information content of specific volatile compounds, pollinator specificity could be enhanced by increased (*e.g.* euglossine-pollinated orchids; Dodson *et al.* 1969; Williams & Whitten 1983) or decreased odour complexity (*e.g.* sexual and brood site deception; Raguso 2004). Previous pollination studies in *Cyclopogon* reported a highly specific relationship with halictid bees, with a given orchid species pollinated by only one or two bee species (Singer & Cocucci 1999; Singer & Sazima 1999; Benitez-Vieyra *et al.* 2006). In the case of *C. elatus*, specialised flowers are associated with simple scent composition. The specificity of this plant–pollination interaction may hinge upon the spatial (microhabitat, background, light quality) and temporal (bee and plant phenology, presence of competitors) contexts in which *C. elatus* blooms, as well as the composition of its floral signals (Vázquez & Aizen 2003).

The main fragrance compound found in the flowers of *C. elatus* (DMNT) has been specifically associated in other plants with indirect defence against herbivores (Turlings *et al.* 1990, 1991; Boland *et al.* 1992; Dicke 1994; Pichersky & Gershenzon 2002). This unusual acyclic C_{11} homoterpene, commonly released from the foliage of herbivore-damaged plants among a suite of other induced volatile compounds, has been linked to indirect plant defence because of its attractiveness to herbivore enemies such as parasitoid hymenopterans (Boland *et al.* 1992; Paré & Tumlinson 1998). DMNT is thought to be derived from the sesquiterpene alcohol nerolidol (Degenhardt & Gershenzon 2000), a common constituent of many floral fragrances (Knudsen *et al.* 2006). In addition to its role in induced plant defences, DMNT has also been identified as a floral scent component in species from several angiosperm families (see Knudsen *et al.* 2006 and literature cited therein). It is chiefly detected in low quantities as part of complex mixtures (*e.g.* Azuma *et al.* 1999; Levin *et al.* 2001), with the exception of highly specialised fly-pollinated trap flowers of *Alocasia odoura* (Miyake & Yafuso 2005) and obligately moth-pollinated *Yucca filamentosa* (Svensson *et al.* 2005), in which it is a dominant but not exclusive scent component. Other minor compounds detected in the flower scent of *C. elatus* were the isomer *cis*-4,8-dimethyl-nona-1,3,7-triene and unidentified compounds whose partial mass spectrum was characterised by ion fragment m/z 107. The latter compounds were not present in sufficient quantity to determine chemical structure or possible biosynthetic affinity with DMNT. It is possible that other volatile components are present below the threshold of detection, but our methods have been sufficient to characterise behaviourally active blend components from other plant species with very weak emissions (*e.g. Fragaria virginiana*; Ashman *et al.* 2005).

The fact that this volatile associated with tri-trophic interactions and indirect plant defence is also distributed widely in floral fragrances (Kaiser 1993) is not surprising in view of hypotheses on the evolutionary origin of flower scents, suggesting that wound volatiles were utilised as kairomonal attractants by florivores whose activities resulted in pollination (Pellmyr & Thien 1986; Knudsen *et al.* 2006). Despite large gaps in our knowledge of the evolution and phylogenetic distribution of floral scent, the following evidence is consistent with such a hypothesis. First, similar volatiles are known from the leaves and flowers of basal angiosperms (Azuma *et al.* 1999; Bernhardt *et al.* 2003). Second, their production represents a simple and probably cost-efficient way to attract pollinators. In an evolutionary scenario, the initial floral scents might have been dominated by terpenoids that are common in damaged leaves, which may have changed to include oxygenated compounds from other pathways to recruit specific insect pollinators (Boland & Gäbler 1989; Azuma *et al.* 1999). Alternatively, DMNT may be unusually easy to learn, either by parasitic wasps hunting insect hosts or, in our system, by solitary bees searching for nectar; or its attractiveness to different insects might depend strongly on the context (height above ground, flower colour, presence of other volatiles, time of emission) in which it is perceived (*e.g.* Raguso 2004).

Pollinators

Our findings on *C. elatus* pollinators are consistent with previous reports (Benitez-Vieyra *et al.* 2006) and those observed for another two *Cyclopogon* species (Singer & Cocucci 1999; Singer & Sazima 1999), for which halictid bees were the only pollinators. Two aspects of pollinator behaviour were notable. First, floral fragrance, when isolated from visual floral cues, was attractive only to *C. elatus* pollinators (halictid bees) and not to nectar robbers (*A. mellifera*) (see Benitez-Vieyra *et al.* 2006). One possible explanation is that the floral odour of *C. elatus* is not attractive to *A. mellifera*; another is that honeybees require both visual and olfactory signals to approach the flowers. Second, floral fragrances are attractive for sweat bees in the absence of visual signals. Little is known about use of olfactory floral signals by halictid bees. Roy & Raguso (1997) reported that individuals of *Dialictus* species are equally attracted to the pseudoflowers produced by a fungus and to a blend containing the same chemical compounds as the pseudoflower fragrance. Also, in a recent study, Theis (2006) showed that halictid bees (*Lasioglossum* sp.) were attracted to scent traps containing the same chemical compounds as those emitted by

Cirsium arvense flower heads. Further research will be needed to determine whether sweat bee responses to the odour of *C. elatus* is innate or learned in association with the nectar reward offered by the orchid.

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