

FRAGRANCE COMPOSITION OF *DENDROPHYLAX LINDENII* (ORCHIDACEAE) USING A NOVEL TECHNIQUE APPLIED *IN SITU*

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ABSTRACT

The ghost orchid, *Dendrophylax lindenii* (Lindley) Bentham *ex* Rolfe (Orchidaceae), is one of North America's rarest and well-known orchids. Native to Cuba and SW Florida where it frequents shaded swamps as an epiphyte, the species has experienced steady decline. Little information exists on *D. lindenii*'s biology *in situ*, raising conservation concerns. During the summer of 2009 at an undisclosed population in Collier County, FL, a substantial number (ca. 13) of plants initiated anthesis offering a unique opportunity to study this species *in situ*. We report a new technique aimed at capturing floral headspace of *D. lindenii* *in situ*, and identified volatile compounds using gas chromatography mass spectrometry (GC/MS). All components of the floral scent were identified as terpenoids with the exception of methyl salicylate. The most abundant compound was the sesquiterpene (*E,E*- α -farnesene (71%) followed by (*E*- β -ocimene (9%) and methyl salicylate (8%). Other compounds were: linalool (5%), sabinene (4%), (*E*- α -bergamotene (2%), α -pinene (1%), and 3-carene (1%). Interestingly, (*E,E*- α -farnesene has previously been associated with pestiferous insects (e.g., Hemiptera). The other compounds are common floral scent constituents in other angiosperms suggesting that our *in situ* technique was effective. Volatile capture was, therefore, possible without imposing physical harm (e.g., inflorescence detachment) to this rare orchid.

Keywords: ghost orchid, South Florida, (*E,E*- α -farnesene, volatiles, scent, conservation

Introduction

Few orchids native to North America have received as much widespread interest as the ghost orchid, *Dendrophylax lindenii* (Lindley) Bentham *ex* Rolfe (Orchidaceae) – the subject of best-selling books and a hit movie. The species is restricted to Cuba and the Big Cypress eco-region of S Florida (e.g., the Fakahatchee Strand; Brown 2005) where it frequents shaded swamps as an epiphyte. In SW Florida, the species has experienced steady decline due to habitat loss and poaching. The public's fascination with this species is attributed, in part, to its alluring floral display (Fig. 1). At maturity, *D. lindenii* is leafless and only a small percentage of plants in a population flower in a given year. Although advances are being made to propagate this species artificially (e.g., Davis 2009), the process has been slow and often unreliable. This fact, coupled with the orchid's rarity and inaccessibility, has resulted in its widespread removal (poaching) from protected and private areas by unscrupulous collectors. Currently, the species is listed as endangered in Florida (Brown 2005) where its natural range is mostly restricted to two counties undergoing rapid urban development.

To facilitate the sustainable conservation of *D. lindenii* and other orchids worldwide, habitat preservation must be augmented by understanding the species' ecological requirements *in situ*, including pollination and breeding systems (= integrated conservation; Stewart 2007;

Swarts 2007). Only ca. 36% of orchid species native to North America have been studied with respect to their pollination mechanisms (Catling and Catling 1991), and even less is known about the chemical attractants utilized



Fig. 1 The ghost orchid (*Dendrophylax lindenii*) in flower in southwest Florida (2009). The long nectar spur can be seen beneath the lower lip (labellum) of the flowers. Scale bar = 6 cm.

by these species to attract pollinators (floral scent). Currently, no published reports exist that describe natural pollination or floral fragrance chemistry of *D. lindenii*. The species' floral characteristics, namely the long nectar spur, white color, and sweet smelling evening fragrance (pers. observations), suggest pollination by sphinx moths (Faegri and van der Pijl 1979; Dressler 1981; Grant 1983).

During the summer of 2009 at a restricted, undisclosed *D. lindenii* population in Collier Co., FL, a substantial number (ca. 13) of individual plants initiated anthesis. Several of these plants flowered < 2 m from the forest floor, offering a unique research opportunity to study this species *in situ*. We hypothesized that the ghost orchid would produce aromatic compounds that attract night-flying insects and would have a similar composition to other moth-pollinated orchids. If a similar composition, compared to other angiosperms and orchids, is achieved then the novel technique of headspace collection can be utilized for other epiphytic species. In this paper we report the primary floral fragrance compounds emitted by the ghost orchid using an adsorbent that serves to trap volatile compounds *in situ*, followed by laboratory analysis by means of GC/MS. The technique described herein (modified from Kaiser 1993) allows for volatile compound capture without imposing physical harm (e.g., inflorescence detachment) to this rare orchid.

Materials and methods

Study site

Samples were obtained from a restricted *Dendrophylax lindenii* (Lindley) Bentham *ex* Rolfe population on private property in Collier Co., FL. (The exact location of this site is withheld here to reduce the likelihood of subsequent poaching.) Compared to other ghost orchid populations throughout S Florida, this location harbored a sizable number of plants (ca. 50 individuals), 13 of which produced flowers in June 2009. In previous years (e.g., 2008), plants from this population produced mature seed capsules, suggesting that the flowers are naturally pollinated at this site. The majority of the orchids were affixed to the bark of pond-apple (*A. glabra* L.) on low hanging branches ca. 2 m above the sediment floor.

Volatile sampling

During a 2 day period (22–23 June 2009), three flowers from three individual plants were sampled in the early morning hours (100–600 hrs) when fragrance was most readily detected by the authors. Volatiles were collected using an apparatus modified from Kaiser (1993) that was modified for arboreal fragrance extraction (Figs. 2 and 3). Volatile adsorption was carried out using the procedure described by Cancino and Damon (2007). The adsorbent was washed with diethyl ether before use. After 2–3 hrs of

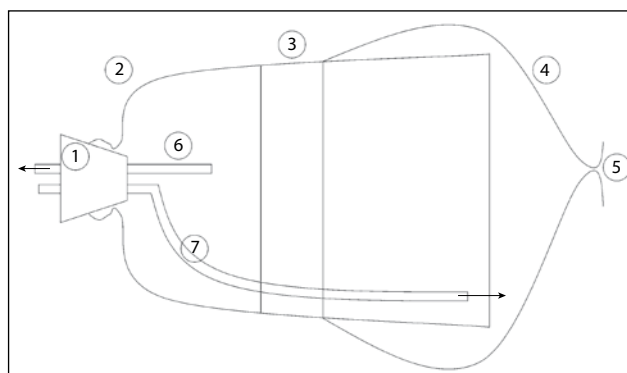


Fig. 2 Diagram of the apparatus (modified from Kaiser, 1993) used to absorb floral volatiles *in situ*. (1) rubber stopper; (2) glass bell flask; (3) Paraflim "M" (Pechiney Plastic Packaging, Menasha, WI) securing oven bag on outside of bell flask; (4) plastic-like oven bag (Reynolds Kitchens, Richmond, VA); (5) opening to allow flower in apparatus and to close around stem; (6) headspace pumped out of flask via mechanical pump through adsorbent; (7) activated charcoal-filtered air from environment.



Fig. 3 The apparatus (modified from Kaiser 1993) enclosing inflorescences *in situ* while suspended from the canopy of the host tree and a stand constructed from PVC pipe. The vacuum pump and charcoal adsorbent can be seen at the lower right and lower left, respectively. The Porapak Q adsorbent is denoted by the arrow. Scale bar = 15 cm.

adsorption, the volatiles were eluted with ca. 1200 μ L of diethyl ether into glass vials and stored at -70 °C prior to laboratory analysis (2 days). The samples were briefly (6 hrs) removed from storage when they were transported by car to Gainesville, FL for analysis. During transport, vials were placed into a portable cooler containing dry ice.

Analysis of volatiles

The samples were analyzed on an Agilent 6890/5973 coupled GC/MS. One μ l of the samples were introduced using splitless injection at 220 °C. A 30 m DB1 methyl silicone GC-column with 0.25 mm ID and a film thickness of 0.25 μ m was used with helium carrier gas at constant

flow with an average velocity of 30 cm/sec. The GC oven had an initial temperature of 35 °C for 0.5 min and then programmed 10 °C per minute to a final temperature of 230 °C and maintained at this temperature for 5 min. The injector and MS transfer line temperature was 240 °C. The ion source temperature was 220 °C with EI ionization at 70 eV and the quadruple scan range set to m/z 30 to 400 with a 2 scan averaging giving a scan rate of 3.85 scan⁻¹. The GC/MS data were processed using Agilent Chemstation version 3.02. Compounds were identified by comparison with commercially available NIST05 and Adams EO libraries and a floral scent library created at the department of Chemical Ecology, Göteborg University, Sweden and when possible by utilizing mass spectra of commercial standards.

Results and Discussion

GC/MS analysis revealed 8 compounds associated with the floral fragrance of *D. lindenii* (Fig. 4). Samples obtained from three different plants during a two day period yielded similar results. These compounds consisted

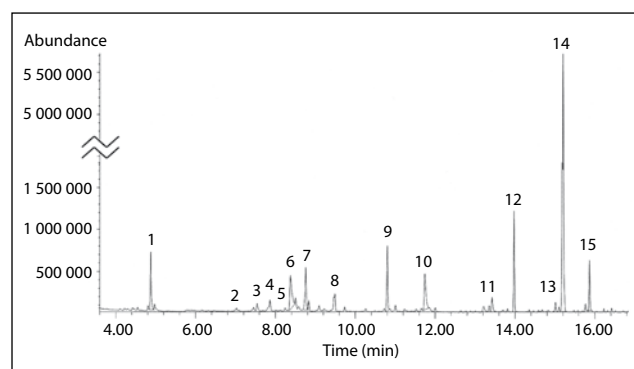


Fig. 4 GC spectra analysis of 15 peaks: (1) solvent background; (2) *α-pinene*; (3) 6-methyl-5-hepten-2-one; (4) myrcene; (5) *3-carene*; (6) *sabinene*; (7) (*E*)-*β-ocimene*; (8) *linalool*; (9) *methyl salicylate*; (10) geraniol; (11) phenylbenzene; (12) tetradecane; (13) (*E*)-*α-bergamotene*; (14) (*E,E*)-*α-farnesene*; (15) Diethyl phthalate. Compound names that are italicized indicate confidence that these were from the ghost orchid.

Table 1 The percent composition of the primary volatile compounds collected from the ghost orchid's scent. Notice the percents add to 100 to ease comparison between the eight compounds of the four trials. Trial 1 is displayed in Fig. 4. Four replicates were done during the same time period between different orchid flowers.

Rt (min)	Compound	Floral Scent Percent Composition				Mean	Standard Deviation
		Trial 1	Trial 2	Trial 3	Trial 4		
7.054	<i>α-pinene</i>	0.672	1.410	0.668	1.245	0.999	0.386
8.266	<i>3-carene</i>	0.704	0.822	0.694	1.228	0.862	0.251
8.517	<i>sabinene</i>	1.718	2.457	4.384	8.601	4.290	3.086
8.773	(<i>E</i>)- <i>β-ocimene</i>	7.922	7.052	5.337	16.139	9.112	4.806
9.515	<i>linalool</i>	2.607	7.559	2.022	6.647	4.709	2.800
10.822	<i>methyl salicylate</i>	9.281	7.353	9.592	4.906	7.783	2.159
15.037	(<i>E</i>)- <i>α-bergamotene</i>	1.543	1.653	1.726	1.250	1.543	0.209
15.249	(<i>E,E</i>)- <i>α-farnesene</i>	75.553	71.693	75.577	59.984	70.702	7.375

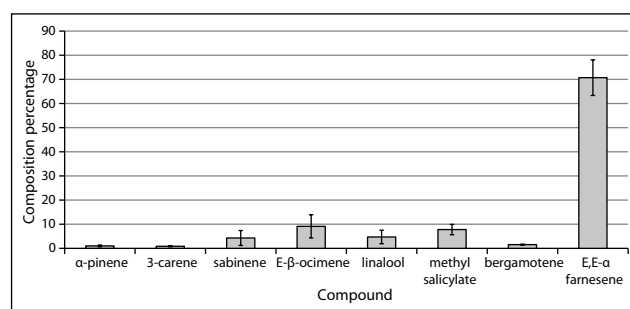


Fig. 5 Composition as a percentage for the eight volatile compounds that comprise the floral scent of *Dendrophylax lindenii*. Error bars reflect \pm standard deviation.

mostly of monoterpenes, along with sesquiterpenes and benzenoids. The most prevalent compound was the sesquiterpene (*E,E*)-*α-farnesene* (Figs. 4 and 5, Table 1). The other 7 compounds detected were *α-pinene*, *3-carene*, *sabinene*, (*E*)-*β-ocimene*, *linalool*, *methyl salicylate*, and (*E*)-*α-bergamotene*. The other compounds presented in the chromatogram (Fig. 4) occasionally occur as background in GC/MS analyses of volatile collections.

This is the first report describing the chemical composition of *D. lindenii*'s floral scent. Although the sample size was low, the results were reproducible. With the exception of (*E,E*)-*α-farnesene*, all of the compounds were common floral scent constituents, verifying that our *in situ* technique was effective. Of the 90 plant families investigated for floral fragrance compounds worldwide, orchids have received the most attention (417 spp./subsp.), followed by aroids (Araceae, 55 spp./subsp.) and palms (Arecaceae, 40 spp./subsp.) (Knudsen et al. 2006). While this total might appear sizable, the vast majority of the Orchidaceae (> 97%) remains unstudied considering that the family contains an estimated 17,000–35,000 species (Dressler 1993). Other plant families have been studied with respect to floral scent (e.g., Magnoliaceae, Rosaceae, Cactaceae, Rutaceae, Solanaceae, and Nyctaginaceae), but this aspect of angiosperm biology remains poorly understood (Knudsen et al. 2006). Nevertheless, a pattern of floral scent compounds is starting to emerge. For example,

more than two-thirds of the families investigated to date (> 66%) share a common profile consisting of five compounds: limonene (71%), (*E*)- β -ocimene (71%), myrcene (70%), linalool (70%), and α -pinene (67%) (Knudsen et al. 2006). With the exception of limonene and myrcene, these five basic compounds are also present in the floral scent of *D. lindenii*. The other compounds (3-carene, sabinene, methyl salicylate, (*E*)- α -bergamotene, (*E,E*)- α -farnesene) appear to be less prevalent among the angiosperm families studied so far; however, all but one (3-carene) have been reported from moth-pollinated Orchidaceae (Knudson et al. 2006).

From an ecological perspective, the link (specificity) between an orchid's fragrance and its pollinator is determined by the proportion (blend) of the compounds emitted, not just by the kinds of chemicals that are produced (J.A. Yoder, pers. comm.). In this case, the blend released by *D. lindenii* consisted of the sesquiterpene (*E,E*)- α -farnesene (71%) followed by (*E*)- β -ocimene (9%) and methyl salicylate (8%). Other compounds were linalool (5%), sabinene (4%), (*E*)- α -bergamotene (2%), α -pinene (1%), and 3-carene (1%) (Fig. 3). At close range (ca. 5 cm), the floral scent of *D. lindenii* was readily detectable to the authors and seemed to intensify at sunset (pers. observations). The scent can best be described as sweet-smelling and somewhat fruity. Of particular interest is (*E,E*)- α -farnesene – the most abundant constituent. This compound is odorless to humans and has been linked with insects and insect-damaged plants as well as intact plants (Himanen et al. 2005). Similarly, aphids (Hemiptera: Aphidae) release trans- β -farnesene as an alarm pheromone from the tips of their cornicles when attacked or disturbed (Yang and Zettler 1975). Indeed, pestiferous

scale insects (Hemiptera) were observed on ovaries of inflorescences at the time of sampling (Fig. 6). During a follow-up visit (24 July 2009), some of these insects were collected and later identified as members of the Coccidae and Pseudococcidae (J. A. Zettler, L. W. Zettler, L. W. Richardson, unpubl. data). Whether or not the presence of these insects triggered the release of (*E,E*)- α -farnesene captured in the sample is not known, but cannot be ruled out. Additional studies should explore whether the insects, the plant, or both are releasing this compound.

The highlight of this study was the technique (modified from Kaiser 1993) used to collect headspace volatiles from *D. lindenii* *in situ* without imposing physical harm to the donor plants (Figs. 2 and 3). This was achieved by suspending the adsorption apparatus from rope affixed to branches within the immediate area (canopy). The use of a lightweight, battery-powered vacuum permitted suitable airflow and subsequent capture of volatiles within glass vials containing Porapak Q adsorbent (Chromatography Research Supplies, Louisville, KY). Our technique fundamentally differs from the methods used in previous studies (e.g., Dutra et al. 2009) because in the latter, inflorescences are detached from donor plants and analyzed. For critically rare species in peril, the removal of inflorescences for this purpose may not be desirable or legally possible. Our sampling technique makes it possible for flowers to be receptive to pollination after sampling, increasing the likelihood of seed set. Clearly, this method would be practical for conservation programs involving other rare epiphytic orchids, as well as non-orchids (e.g., Bromeliaceae, Cactaceae, Piperaceae).

Other useful applications await researchers that adopt this technique, both *in situ* and *in vivo*. For example, intact plants may be sampled during an extended period of time (e.g., weeks or months). This might serve a useful purpose for those interested in documenting changes in the proportion (blend) of the compounds emitted over time. Yang et al. (2009) linked floral scent in a non-orchid (*Delphinium elatum* L., Ranunculaceae) to its substrate, and it is conceivable that this same phenomenon might also be observed in the Orchidaceae. Orchids would be of special interest given that these plants obtain all or part of their carbon from mycorrhizal fungi (via mycotrophy) present in the substrate (Rasmussen 1995). This technique might make it possible to monitor long-term changes in floral scent through the manipulation of substrate, fungi or both. In the short-term, we urge other researchers to document floral fragrance composition in other rare native orchids as part of a holistic (integrated) approach to conservation.

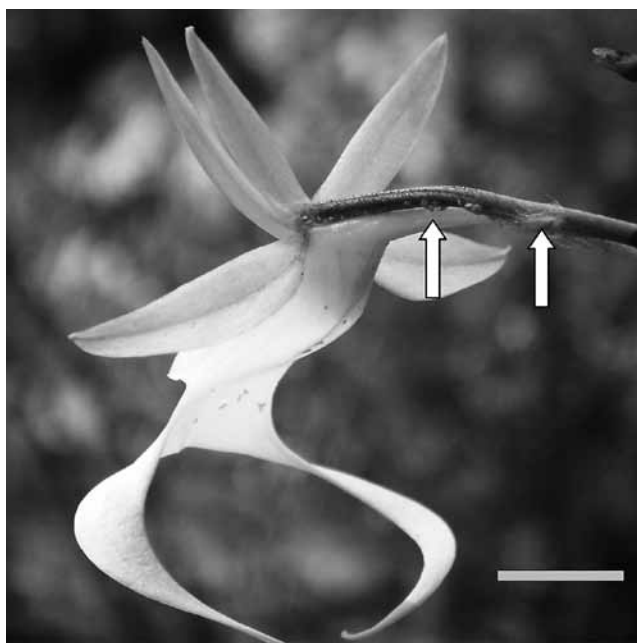


Fig. 6 A *Dendrophylax lindenii* flower photographed during one of the two sampling days (22–23 June 2009). Pestiferous insects (Hemiptera) can be seen on the developing ovary and are denoted by the arrows. Scale bar = 3 cm.

Conclusions

Eight primary volatile compounds comprise the floral scent of the ghost orchid, *Dendrophylax lindenii*. The technique described herein permitted the collection of

floral scent compounds *in situ* with minimal or no disturbance to this rare orchid. This technique may have practical merit for similar studies aimed at other epiphytic species in peril.

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