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CHEMICAL COMPOSITION OF FLORAL NECTAR COLLECTED FROM THE GHOST ORCHID, *DENDROPHYLAX LINDENII* (LINDL.) BENTH. EX ROLFE (ORCHIDACEAE: ANGRAECINAE), IN FLORIDA

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Abstract

Numerous studies have confirmed the importance of nectar for effective pollination and reproductive success in orchids, but the chemical composition of floral nectar has received surprisingly little attention. We report the chemical compounds present in the nectar of the well-known ghost orchid, *Dendrophylax lindenii* (Lindl.) Benth. ex Rolfe, using gas chromatography and mass spectrometry (GC/MS) analysis. This rare species exists as a leafless epiphyte in forests of South Florida and Western Cuba and yields a striking floral display consisting of fragrant white flowers with long (11-17 cm) nectar spurs. Nectar samples were obtained from naturally-occurring populations within the Florida Panther National Wildlife Refuge during July, 2021 and analyzed in the laboratory one wk later. Present results revealed the presence of three sugars (glucose, fructose, and sucrose), three acids (lactic, malic, and threonic), as well as 4-hydroxyl benzyl alcohol. In addition, all three sugars were detected on the upper surface of flower's labellum where moisture is known to collect due to its concave shape. This study supports the contention that sugars are an ubiquitous component of orchid floral nectar, but the presence of the other compounds (acids) deserve further investigation. Further knowledge about the compounds in *D. lindenii* nectar will provide more insight into how and why hawk moths visit and pollinate the flowers of this rare orchid in nature.

Introduction

ORCHIDS CONSTITUTE the most diverse family of flowering plants with 28,484 species (Govaerts *et al*., 2017) representing *ca*. 8% of all vascular plants worldwide (Dressler, 2005; Scotland and Wortley, 2003). The drivers behind this diversification appear to be linked to several factors especially co-evolution with specific insect pollinators needed for sexual reproduction. The majority (60-70%) of orchid species entice and reward pollinators within a blend of attractants that typically include oils, waxes, fragrance (aromatic compounds) and sugar-rich nectar (Ackerman, 1986; Brzosko and Mirski, 2021; Dressler, 1981; Tremblay *et al.,* 2005). About 35 million years ago, orchid speciation accelerated when the family adapted to an arboreal lifestyle aided by their ability to conserve water via crassulacean acid metabolism or CAM (Stokstad, 2015), and flowers targeted winged insects especially bees (Hymenoptera) and moths (Lepidoptera) for pollination. This timeframe coincides with the evolutionary appearance of nocturnal hawk moths (Sphingidae; Kawahara *et al*., 2019) that pollinate many members of the Epidendroideae, the largest orchid subfamily. This subfamily includes the well-known angraecoids of Africa and Madagascar (*Angraecum sesquipedale* Thouars; Pridgeon *et al*., 1999, 2014), and members of the genus *Dendrophylax* in the New World. Collectively, moth-pollinated orchids are characterized by their white flowers that emit a sweet-smelling evening fragrance, and long nectar spurs that often match the proboscis length of the pollinator (Dressler, 1981; Faegri and van der Pijl, 1979; Pal *et al*., 2019; Prakash and Pathak, 2020). These spurs are often filled with copious amount of sugar-rich nectar that serves to reward the pollinator with a carbohydrate source needed to power muscles for rapid flight over long distances.

Numerous studies have confirmed the importance of nectar for effective pollination and reproductive success, but the chemical composition of orchid nectar has received surprisingly little attention (Brzosko and Mirski, 2021). Based on a limited number of earlier studies (Jeffery *et al*., 1970; Percival, 1961), moths generally prefer nectar with lower sugar concentrations (Kim *et al*., 2011) with hawk moths preferring a sucrose concentration of 34% (Josens and Farina, 2001). Brzosko and Mirski (2021) compiled data on nectar attributes in different orchid species and concluded that orchids with longer spurs (>5 mm) tend to have more dilute nectar (*ca*. 18%) with a higher glucose/fructose ratio. They also noted that longer spurred orchids are pollinated by 1.7 different species, on an average, and tend to be specialists, whereas orchids, in general produce nectar catering to a wider range of unspecialized pollinators with different dietary needs (Brzosko and Mirski, 2021). Moreover, sugar composition, as well as nectar concentration, both play a role in pollinator selection (Brzosko and Mirski, 2021). In bromeliads,

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Krömer *et al*. (2008) determined that nectar sugars were reflective of pollinator preference, not phylogenic relationships, and it is conceivable that the same may hold true for orchids.

We report the chemical compounds present in the nectar of the well-known ghost orchid of the New World, *Dendrophylax lindenii* (Lindl.) Benth. ex Rolfe, using gas chromatography and mass spectrometry (GC/MS) analysis. This rare species exists as a leafless epiphyte in forests of South Florida and Western Cuba and yields a striking floral display consisting of fragrant white flowers with long (11-17 cm) nectar spurs. Recent studies have confirmed that *D. lindenii* is pollinated by hawk moths (Danaher *et al*., 2019; Houlihan *et al*., 2019) consistent with many other members of its subtribe (Angraecinae). Accordingly, we hypothesized that the sugars present in the nectar of *D. lindenii* would be the same as those reported for other hawk moth-pollinated orchids, but we also wanted to determine if other chemical compounds may be present other than just carbohydrates. In addition, we also document the chemical compounds present on the upper surface of the ghost orchid's labellum where moisture is known to accumulate due to its concave shape.

Material and Methods

Field Site

Nectar was sampled from floral spurs of *Dendrophylax lindenii* at the 10,684 ha Florida Panther National Wildlife Refuge (FPNWR) within the Big Cypress-Basin Ecoregion of Southern Florida. The site was chosen because it encompassed the Northern portion of the Fakahatchee Strand known for harbouring the highest density of ghost orchids in the United States. Field sampling took place during July 3-7, 2021 at a time when flowering was well underway. Nectar samples were obtained from 10 different *D. lindenii* flowers on 7 individuals, specifically from those that appeared to have opened recently and had no visible signs of prior pollinator activity (*i.e*. pollinia remained intact on the column). Additionally, from 8 of these 10 flowers, we collected extra floral samples from the labellum (lip).

Nectar Collection

Nectar from the spur (Fig. 1) was collected using the micro-rinse method (Power *et al*., 2018). Type I water (5 µl) was introduced into the nectar spur with a clean, unused, graduated Drummond® PCR pipette (Drummond® #5-000-1001-X10; Fig. 2). After 15 sec, the liquid was removed with the same pipette, quantified visually using the pipette's gradations, and transferred to a clean 0.5 ml, Eppendorf™ Safe-Lock Tube (Eppendorf™ #022363611). Liquid from the outer labellum was collected directly via capillary action (Fig. 3) and quantified with a graduated Drummond® PCR pipette (Item #5-000-1001-X10). All samples were subsequently stored at or below 0°C until ready for analysis.

Nectar Analysis

The nectar samples were analyzed by gas chromatography mass spectrometry (GC/MS) after trimethyl silyl (TMS) derivatization (Pais and Chaves Das Neves, 1980). Water was removed from the nectar sample by placing the opened Eppendorf™ tube in a

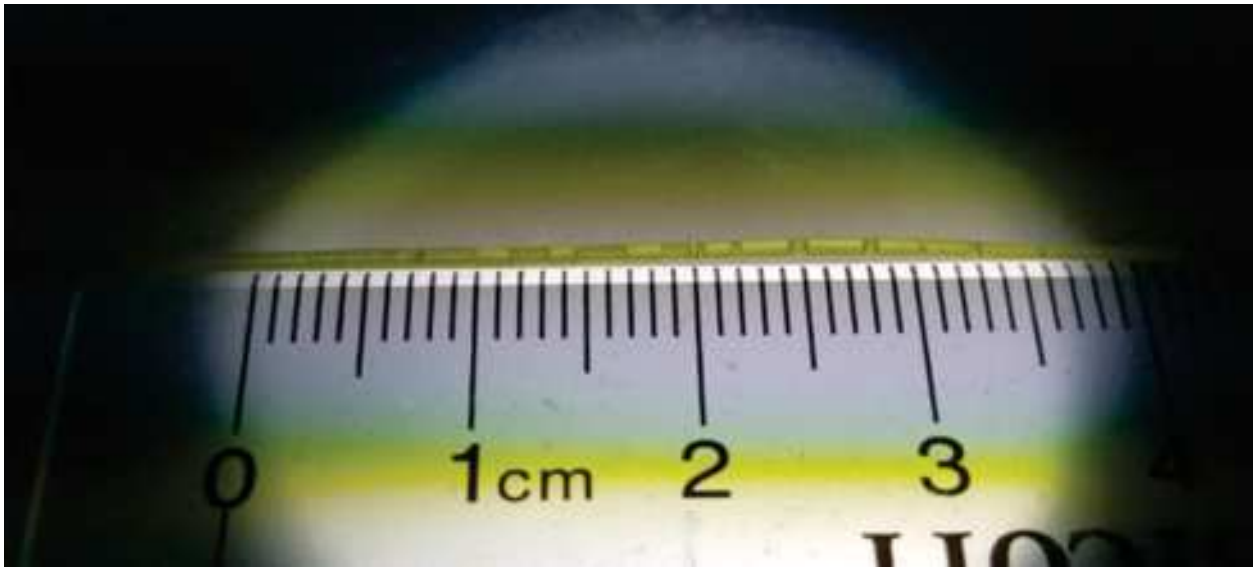

Fig. 1. Nectar spur of *Dendrophylax lindenii* placed above a metric ruler for scale. The spur was illuminated from below revealing bands of nectar broken up by airspaces instead of a continuum of nectar. This photo was taken a year prior to the current study when rainfall in the region was below average.

Fig. 2. *Dendrophylax lindenii* with three open flowers, one in the act of being probed for nectar using a micro-pipette inserted into the nectar spur. Note the leafless form of the orchid, typical of this species.

vacuum chamber with Drierite® under high vacuum at ambient temperature for 17 hrs. The remaining residue was dissolved with anhydrous N,N-dimethylformamide (10 µl), the solution was vortexed for 10 sec, and then transferred to a 250 µl glass insert with polymer feet (Agilent #5181-1270) situated in a 2 ml A-Line screw top vial (Agilent 5190-9589) with a 12 mm cap (Agilent #5182-0720). N,O-(bistrimethylsilyl) trifluoroacetamide (BSTFA) (20 µl) was added via micro-syringe, the capped vial was vortexed for 10 sec, and then heated at 65°C for 15 min.

The derivatized samples were analyzed on an Agilent 6890/5973 coupled GC/MS. Sample (1 µl) was introduced using a 10:1 split injection at 230°C. A 30 m DB-5MS + 10 m Duraguard Agilent J and W GC column

Fig. 3. Liquid is shown being collected from the concave labellum of a *Dendrophylax lindenii* flower, extracted into a pipette by means of capillary action.

(Agilent #122-5532G) with 0.25 mm ID and a film thickness of 0.25 µm was used with helium carrier gas at constant flow with an average velocity of 37 cm sec^{-1}. The GC oven was programmed with an initial temperature of 70°C held for 4 min followed by a temperature ramp at a rate of 10°C/min to a final temperature of 325°C. The final temperature was maintained for 16 min. The injector and MS transfer line temperature were maintained at 230°C. The ion source temperature was 220°C with EI ionization at 70 eV and the quadrupole scan range set to m/z 50 to 600 with a scan rate of 2.7 $scansec^{-1}$. The GC/MS data were processed using Agilent Chemstation version 3.02. Compounds were identified by comparison with NIST mass spectral library Version 2.0.

Results and Discussion

Nectar was extracted from the floral spurs of *Dendrophylax lindenii* from a naturally occurring population in Florida and subsequently analyzed by GC/MS; it revealed the presence of three sugars typically found in nectar of other orchids: glucose, fructose, and sucrose (Fig. 4). These three sugars were also present on the upper surface of the flower's labellum. In addition, three different types of acids (lactic acid, malic acid, and threonic acid) and 4-hydroxy benzyl alcohol were also detected (Fig. 4). To the best of our knowledge, this is the first report documenting the chemical constituents of nectar in the well-known rare ghost orchid, *D. lindenii.*

Although the presence of these sugars was expected, the three acids detected in the nectar samples were not expected. Lactic acid (2-hydroxypropanoic acid), malic acid (hydroxybutanedioic acid or 2-hydroxysuccinic acid), and threonic acid (2,3,4-trihydroxybutanoic acid) are natural derivatives, but why they were present in nectar, is unknown. Threonic acid has been reported from male scent organs of African milkweed butterflies (Lepidoptera: Danainae) leading to speculation that they may be used in chemical communication or speciation in these mimetic species (Schulz *et al*., 1993). The role of lactic acid on insects has not been fully explored, but it has been used as a constituent of baits used in light traps for phlebotomine sand flies (Diptera: Psychodidae; Andrade *et al*., 2008). Malic acid is not a known attractant, and its presence in nectar may be a byproduct in the synthesis pathway of another compound (A. Jakubska-Busse, pers. com.). The presence of these acids may also be explained by other factors that deserve further inquisition. Given that the spurs were sampled after the flowers had already fully opened, the possibility exists that visiting insects may have introduced microbes into the nectar spur via

probing, and these microbes would then be capable of altering the nectar chemistry through fermentation or some other biochemical process. This scenario seems unlikely, however, considering that all flowers sampled by hand appeared to have intact pollinia on the flower's column. If moths had introduced microbes through feeding, we would have expected at least some of the flowers to have had missing pollinia. In future studies, bagging of flowers prior to their opening would be one way to rule out fermentation by introduced microbes. A second related explanation for why acids were detected in the nectar may be linked to fermentation that took place in the Eppendorf tubes between field collection and analysis in the laboratory *ca*. one wk later. However, this possibility seems unlikely considering that the pipettes and storage tubes were purchased new and were assumed to be clean at the time they were used for experimentation.

The last compound detected, 4-hydroxybenzyl alcohol (known commonly as gastrodigenin) has also been found in other orchid species. It was first identified in the rhizome of *Galeola faberi* (Li *et al*., 1993) and subsequently in *Gastrodia elata* (Hayashi *et al*., 2002). It may be a stand alone species or a component in the synthesis of a derivative such as vanillin. Derivatives of vanillin are produced by orchids in the genus *Epipactis,* but they are not known to be pollinated by Lepidoptera (A. Jakubska-Busse, pers. com.). It may also be a component in a compound such as the antioxidant, gastrodin [4-(beta-D-glucopyarnosyloxy) benzyl alcohol] found in *Gastrodia elata* (Hayashi *et al*., 2002) or the anti-herbivory compound, habenariol, isolated from *Habenaria repens* (Johnson *et al*., 1999; Wilson *et al*., 1999). We plan to identify whether more complex derivatives of 4-hydroxy benzyl alcohol are present in nectar in the future using different methodology (CC/MS with dichloromethane as a solvent; A. Jakubska-Busse, pers. com.).

The present study supports the contention that sugars are an ubiquitous component of orchid floral nectar. While we assume that the carbon source used in their biosynthesis originated from photosynthesis, orchids are also known to supplement photosynthesis through mycotrophy (Rasmussen, 1995). Thus, it is conceivable that the sugars, and perhaps the other compounds present in *D. lindenii* nectar reported herein, were derived from the mycorrhizal fungi present in the roots of the orchid. It seems plausible that carbon compounds released from the digestion (lysis) of intracellular hyphae (pelotons) in the root cortex may be capable of translocation up the raceme. Sadler *et al*. (2011) reported the presence of eight volatile compounds present in the floral fragrance emitted by *D. lindenii in situ* and raised the possibility that some of these compounds may have been synthesized from carbon sources provided by mycorrhizal fungi. As speculative as this may be, the possibility of a biochemical link between the fungi present in roots and compounds in flowers deserves further inquiry, not just for *D. lindenii* but for other members of the Orchidaceae, as well. Considering that *D. lindenii* can be propagated with and without mycorrhizal fungi (Hoang *et al*., 2017), the opportunity exists for future researchers to determine if, and to

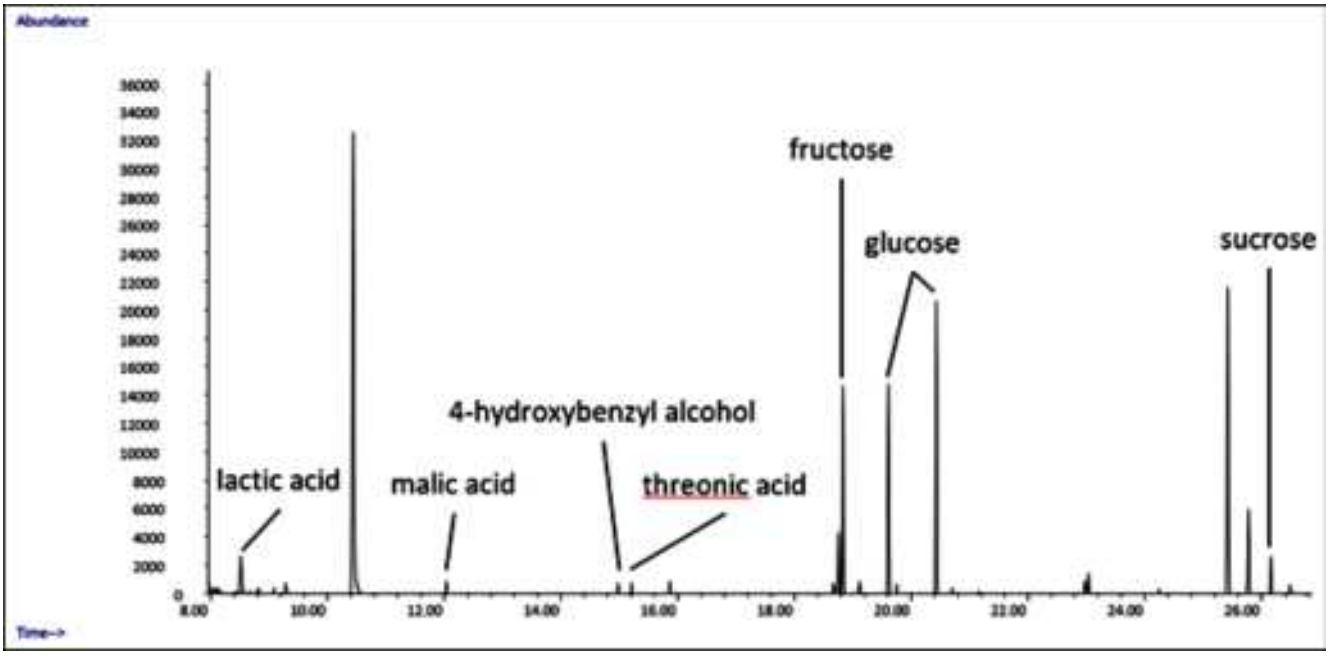

Fig. 4. Results of GC/MS showing the seven compounds detected in the nectar of *Dendrophylax lindenii*.

what extent, mycorrhizal fungi contribute carbon compounds found in both floral fragrance as well as nectar through carefully crafted experiments.

As a preliminary study, our results provide a baseline for more work. Plans are currently underway to modify our existing technique which will allow us to determine the per cent concentration of these sugars, their ratios, and also explain why the other compounds were present. For a more thorough analysis to be carried out, a higher quantity of nectar would be required which could be obtained by sampling more flowers and/or using pipettes with a narrower aperture. Mujica *et al*. (2021) noted that nectar volume levels measured were noticeably less during a prolonged dry period in 2020 (Fig. 1) as compared to the year before when conditions were wetter, in *D. lindenii*. Thus, nectar sampling may be more problematic during drought years. Although this may be viewed as a hardship, comparing sugar percentages and ratios in nectar obtained during wet years as compared to samples collected in dry years, this could reveal an important connection between weather patterns, nectar quality, and pollination in this age of climate change.

Further knowledge about the compounds in *D. lindenii* nectar may provide more insight into how and why hawk moths visit and pollinate the flowers of this rare orchid in nature. Our detection of sugars on the surface of the labellum is a curious finding and may explain why smaller lepidopterans (geometrid moths, skippers) were observed probing with distended proboscides while resting on this floral structure as Danaher *et al*. (2019) reported. Hawk moths generally hover in flight while probing for nectar, and it is conceivable that small traces of sugars on the labellum could entice the insects to probe deeper into the flower once the tip of their proboscis makes physical contact. Thus, the labellum sugars could serve as an appetizer of sorts helping to guide the moth deeper into the spur allowing for the insect's head (*e.g*. base of the proboscis) to contact the pollinia housed within the column. Considering that *D. lindenii* is not autogamous, relying instead on out crossing by a selected group of hawk moths for successful seed set, the long-term conservation of this rare orchid hinges on the survival of its pollinators. Meeting the dietary needs of its hawk moth pollinators, which is primarily floral nectar for the adult stage, is an important component of a larger holistic picture.

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