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ARE VOLATILE ORGANIC COMPOUNDS THE SECRET TO VISITATIONS BY POLLINATORS IN THE FOOD DECEPTIVE ORCHID *Vanda tessellata* (ROXB.) HOOK. EX G. DON. (ORCHIDACEAE)?

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Abstract
Vanda tessellata is an indigenous, endangered, and protected orchid species with a wide range of flower variations in Sri Lanka. The plant has high ornamental and medicinal values and therefore faces serious threats due to over-exploitation and habitat destruction. Thus, sound knowledge of pollination biology is important in the conservation and propagation of the species. The current study investigates the diurnal and nocturnal pattern of pollinators' behaviour with the volatile fragrance profile of *V. tessellata*. Gas chromatography-mass spectrometry (GC-MS) analysis was carried out to identify the odour profile. The detailed GC-MS results produce 65 peaks of 36 volatile compounds. The diurnal pollinator, *Xylocopa tenuiscapa* (order Hymenoptera) and the nocturnal pollinator, *Daphnis nerii* (order Lepidoptera) visitations to *V. tessellata* flowers and the intensity of floral odours were well correlated proving the ability of *V. Tesselatta* flowers to attract both diurnal and nocturnal pollinators for successful cross-pollination.

Keywords: Orchid, fragrance, odoriferous compounds, pollination

Introduction

Vanda tessellata (Roxb) Hook. f. ex D. Don. is an orchid indigenous to Sri Lanka, consisting of over 344 floral variations (Dasanayake & Fosberg 1981, Gunasekara 2016). It is a food-deceptive orchid (Gunasekara *et al*. 2019a), distributed in the dry and intermediate zones of Sri Lanka (Dasanayake & Fosberg 1981). *Vanda tessellata* has high ornamental and medicinal values resulting in serious threats due to overexploitation (Gunasekara & Wijeundara 2008, Gunsekara *et al*. 2019a,b), which is compounded by habitat destruction (Gunsekara *et al*. 2019a,b). The species is protected under the national legislation of the Fauna and Flora Protection Ordinance (FFPO; Anon 1937, 2009) and listed as threatened under the Endangered (EN) category of The National Red List 2020 of Sri Lanka (MoE 2020). Proper knowledge of pollination biology is important in conserving and propagating this species. When a food-deceptive orchid species shows such a wide range of floral variations, a question arises about the complexities it may create for pollinators. Pollinators associate rewards with a variety of floral signals such as colour, shape, size, and fragrance (Wright 1943, 1978; Kay 1978, Mogford 1978, Faegri & Van der Pigl 1979, Waser & Price 1981, Cresswell & Galen 1991, Galen 1999, Levin & Brack 1995, Steiner 1998, Verdú & Gleiser 2005, Salzmann & Schiestl 2007, Spaethe *et al*. 2007). Approximately one–third of all orchid species achieve pollination through food deception: that is flower contains no nectar or other rewards but resembles or mimics floral signals of rewarding plants to attract pollinators (Dafni 1983, Nilsson 1992, Tremblay *et al*. 2005, Jersakova *et al*. 2006). Consequently, variations in floral traits are expected to be high in food-deceptive orchids, because pollinators will learn to avoid common unrewarding floral phenotypes (Heinrich 1975, Schiestl 2005, Jersakova *et al*. 2006, Laloi *et al*. 2009).

According to researchers, pollinator insects have been observed to fly greater distances or to switch to flowers with different forms or colour pollination, after visiting flowers that do not offer nectar rewards. This rare morph advantage and negative frequency-dependent selection (NFDS) has been hypothesized to explain the maintenance of floral polymorphism in reward-less orchids, at least for colour traits (Smithson & MacNair 1997, Gigord *et al*. 2001). A study on *Tolumnia variegata* in Puerto Rico shows that common forms of reward-less flowers may be readily learned and avoided by pollinators, but unusual floral forms may take a longer time for recognition and would be visited more frequently. Such frequency-dependent visitation can maintain high levels of floral variation (Ackerman *et al*. 1997).

In addition to colour, various chemical compounds emitted at different times of the day are known to assist orchids in attracting pollinators (Gries *et al*. 2002). Flower scent, therefore, is an important attribute in the evolution of flowering plants, playing an important role in driving diversification, especially so in the evolutionary history of orchids (Salzman *et al.* 2007). Previous studies have found that orchid species show variability in emitted odour compounds within their populations (Olsen & Knudsen 1994, Knudsen & Stahl 1994, Giurfa *et al*. 1999, Gries *et al*. 2002, Schiestl 2002, Dotterl *et al*. 2005, Gasket *et al*. 2005, Salzman *et al*. 2007, Dudareva & Pichersky 2008, Shi *et al*. 2008; Fenster *et al*. 2009, Peakall *et al*. 2010, Nielsen & Moller 2015, Ramya *et al*. 2020, Ahmad *et al*. 2022).

Recent studies have indicated that the chemical compound emittance is more intense towards full bloom with the petal open than during the closed immature stage (Mohd-Hairul *et al*. 2010). Furthermore, within a flower, parts such as petals and sepals are known to release volatile compounds separately (Toh *et al*. 2017). The fragrance emitted by volatile chemical compounds, together with colour, shape, surface structure, and nectar, plays a crucial role in attracting pollinators (Kessler & Halitschke 2009, Kessler *et al*. 2013). This study was designed to investigate volatile chemical compounds that act as floral scents and their role in attracting pollinators to *V. tessellata*. Considering the floral polymorphism of *V. tessellata*, the current study investigated the chemical compounds associated with flowers at or close to full bloom. Concurrently, the key pollinators were also identified.

Material and Methods

Sample collection. Flower samples were collected from 80 plants of *V. tesellata*, from a total of 670 plants maintained in a selected home garden nursery (7º30′26″N, 80º23′72″E) in the Kurunegala District within the intermediate zone of Sri Lanka, from 1 January 2017 to 31 December 2020. The selected plants exhibited a variety of floral variations such as sepal, petal, and labellum colour, etc. (Gunasekara 2016). To ensure that all sampled flowers were mature, all samples were collected between 3 to 4 days after the emergence of flower spikes, when the flowers were in full bloom (Fonseca *et al*. 2015).

Samples were collected at the following eight time points: 12 a.m., 3 a.m., 6 a.m., 9 a.m., 12 p.m., 3 p.m., 6 p.m., and 9 p.m. and each lot of samples consisted of eight flowers. Each lot of samples was immediately inserted into a poly bag, sealed, and then stored in a refrigerator at a temperature of 4°C for 4 hours. A further poly bag without flowers was also sealed and stored to serve as a control.

Identification of volatile compounds. The method described by Stökl *et al*. (2005) with minor modifications was used to identify volatile compounds in *V. tesellata* flowers. In brief, eight *V. tesellata* flowers were collected into 250 mL of hexane and then shaken for 12 hrs at 200 rpm to obtain an extract containing volatile compounds. The resulting extract was filtered and vacuum evaporated at 60 ^{o}C to obtain a final yield of 0.5 mL. From the final yield, 0.2 µL was injected splitless into a Hewlett-Packard 6890 series Gas chromatography-Mass Spectrometer (GC-MS) (Agilent USA), fitted with a fused silica HP-5MS capillary column (30 m × 0.25 mm; film thickness 0.25 µm). The oven temperature was programmed to increase from 60–280 ^{o}C at a rate of 4 ^{o}C/min using Helium as the carrier gas at a flow rate of 2mL/min. The gas chromatograph was coupled to a Hewlett-Packard 6890 mass selective detector. The MS operating parameters of an ionization voltage of 70eV and an ion source temperature of 200 ^{o}C, were maintained. The compounds present in the extract were identified by comparison of their retention indices (RI) and mass spectra fragmentation, with references stored on the Wiley W9N08 database and the NIST (National Institute of Standards and Technology) database. The same experimental procedure was followed to determine the number of volatile compounds at various times of the day (at three hours intervals for the period of 24 hrs) and variations in their occurrences as percentages were investigated. Triplicates were maintained where necessary. and variations in their occurrences as percentages were investigated.

Pollinator observations. Pollinators and other visitors to *V. tessellata* were observed at the same location as the flower collection. Observations were carried out covering 24 hours of the day during the same period as the collection. Initial observations were carried out to determine the characteristics and behaviours of visitors vs randomly landed faunal species. Once familiar with visitors, overnight observations were carried out covering a total of 300 hours. Representative samples of visitors were collected using a hand net and the specimens were transferred to a killing jar and then mounted and curated following standard entomological techniques. Pollinator identification was performed using the keys provided by Karunaratna & Edirisinghe (2008).

Results

A total of 65 peaks corresponding to 36 volatile compounds, over trace amounts, were detected using GC-MS. The highest numbers of volatile compounds were detected from 3 a.m. to 9 a.m. Detection peaked at 9 a.m. with 15 volatile compounds (Fig. 1).

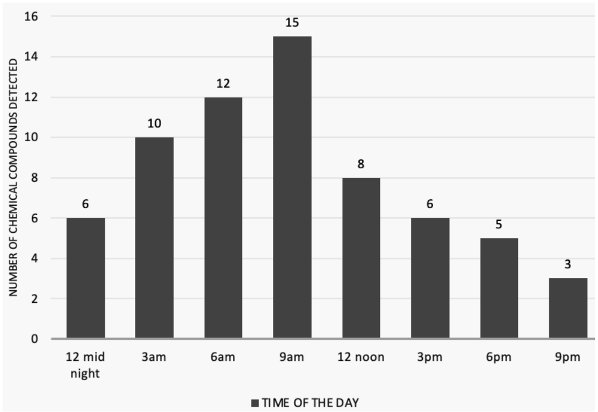

Figure 1. Number of floral scent compounds detected at different times of the day in *V. tessellata* flowers

As shown in Sup. Table 1, volatile compounds exhibited variations in their time of occurrence and concentration. Many compounds were exclusively detected at certain time periods. Piperitone, 2- methyl-octadecyne, 3-ethyl-3methylheptane, 1,4, Alpha-morphinan, Cyclo hexasiloxane, Heptacosane, Cycloheptasiloxane, Octacosane, Cysteine, Heneicosane, and Tetra decamethylcycloheptasiloxane, were detected only at night (9 p.m. to 3 a.m.). Dodecane, 4-vinyl phenol, 4-ethoxymethylphenol, Decane, Methyleugenol, N-dimethylhydrazine, Alpha-hexyl-cinnamaldehyde, Benzyl benzoate, Phyenylmethylenecyclopropane, Cyclopenta-2-benzopyran, Triacontane, Tripropargylamine, Phthalic acid, Methyl pentacyclic dodecane-8-carboxylate were detected only during the morning (6 a.m. to 9 a.m.). There were no volatile compounds specific to noon (12 p.m.). Linalool, Decamethyl pentasiloxane, Octadeamethyl-cyclononasiloxan, Tetracosa methyl-cyclododecasiloxan, Tricosamethyl-cyclododecasiloxan, Hexadecamethylhepta siloxane were detected exclusively in the evening (3 p.m. to 6 p.m.). Certain volatile compounds such as Linalool, Eugenol, Docosane, and Eicosane were detected across the day from the morning to the evening (6 a.m. to 6 p.m.).

Some volatile compounds were emitted at a higher concentration than others. Sup. Table 2 shows volatile compounds emitted with high concentrations (*i.e*., concentrations greater than 1%) by *V. tessellata* flowers during the morning hours (6 a.m. to 9 a.m.) and at noon (12 p.m.), together with the properties of those compounds. At 6 a.m. a total of eight volatile compounds were detected with a concentration greater than 1%. Only two compounds were found at noon (12 p.m.) with concentrations greater than 1%. However, the concentrations of those two compounds were relatively low compared to their concentrations in the morning. At 6 a.m., 4-ethoxymethylphenol had the highest concentration of 39.6%. Of the 15 compounds recorded at 9 a.m. only two compounds were detected in concentrations greater than 1%: 1,2-Benzenedicarboxylic acid (15.8%) and Cyclohexasiloxane (20%).

Volatile compounds found in the afternoon and at night with a concentration greater than 1% are shown together with their known properties in Sup. Table 3. At 3 p.m., six volatile compounds were found and five of them had concentrations greater than 1%. At 3 p.m., two chemical compounds (Tricosamethyl-cyclododecasiloxan and Octadeamethyl-cyclononasiloxan) jointly had the highest concentrations at 16.3% each. At 6 p.m., 3-ethyl-3methylheptane was the most dominant (33. 2%). At night 19 volatile compounds were found with five of them having concentrations greater than 1%. At 9 p.m., three volatile compounds had a concentration greater than 10%. At midnight two volatile compounds were detected with both having concentrations slightly over 1%. At 3 a.m. ten volatile compounds were detected, with all 10 compounds having a concentration of less than 1%.

Visitation times and the role of the insects that visited *V. tessellata* flowers are shown in Sup. Table 4. The role of each visitor observed during the study period is also given. *Xylocopa tenuiscapa* was the diurnal pollinator contributing the most significant share of pollination activities among the two detected pollinators. *Daphnis nerii* was detected visiting the flowers from dusk to night and engaging in active pollination. Although *Apis cerana* was observed visiting the flowers (between 7 a.m. and 3.30 p.m.), it did not engage in pollination.

Discussion

The current study confirmed that most volatile compounds are released during the onset of dawn (3 a.m.) and during morning hours (6 a.m. to 9 a.m.) in *V. tessellate*. However, the concentrations of the compounds emitted at night were comparatively higher than at other times. Particular compounds emitted during dawn and morning hours such as 4-vinylphenol, 4-ethyl methyl phenol, Methyleugenol, N-dimethyl hydrazine, Phyenylmethylenecyclo propane, Tripropargylamine, Cyclohexasiloxane, 1,2-Benzenedicarboxylic acid, are long-range, highly volatile and bear a sweet fragrance. In contrast, the main compounds emitted during the onset of dusk (6 p.m.) and night (9 p.m. to 3 a.m.) are heavy short-range volatile chemical compounds such as 2-methyl-octadecane, Octadeca Methyl-cyclononasiloxane, Tetradeca methyl-cyclopentasiloxane, 3-ethyl-3methyl heptane, Eugenol, Dococene, and Linalool. Interestingly, compounds such as linalool, docosane, piperitone, 2- methyl-octadecyne, and eugenol are also detected in insects including in some lepidopterans, as sex pheromones (Gries *et al*. 2002). Hence, it can be assumed that releasing similar compounds during dusk and night can facilitate the attraction of nocturnal visitors, such as hawk moths, tiger moths, or dipterans (Dodson *et al*. 1969, Gries *et al*. 2002).

Linalool and benzaldehyde are known to be important signal substances in plant-insect communications (Ramya *et al*. 2020). As some orchid species achieve pollination through food deception by mimicking floral signals of rewarding plants to attract pollinators (Dafni 1983, Nilsson 1992, Jersakova *et al*. 2006), the presence of these chemical compounds should be further investigated to identify their role in *V. tessellata*. The pollinator visitations to *V. tessellata* flowers and the intensity and the type of chemical compounds of floral odour correlated well. Visitations by the diurnal pollinator, *Xylocopa tenuiscapa* occurred between 7.30 a.m. and 11.30 a.m. It is a large solitary bee species that travels long distances to forage (Somanathan *et al*. 2017). Previous studies also have revealed that carpenter bees and other bee species visit as pollinators between 9 a.m. to 3 p.m. to collect pollen from orchid plants (Sugiura & Yumiyama 2016). Fragrant volatile chemical compounds such as 4-

ethoxymethylphenol were detected in a higher concentration around 6 a.m., while Cyclohexasiloxane, 1,2-Benzenedicarboxylic acid around 9 a.m., and 1,2-Benzenedicarboxylic acid were detected with a high concentration at 12 p.m. which could facilitate such visitors as these chemical compounds have molecular properties which allow for travel over long distances.

In contrast, the chemical compounds emitted during the onset of dusk (6 p.m.) and at night (9 p.m. to 3 a.m.) are heavy, waxy, aromatic, anti-oxidant, and anti-microbial, which make these compounds ideal for damp weather conditions. These compounds were emitted at comparatively higher concentrations when compared to those emitted in the morning (6 a.m. to 9 a.m.). Amongst them, 2- methyl-octadecyne, which was emitted at a high concentration of 28% at night, is a sex pheromone (Gries *et al*. 2002) of *Lepidopterans* (allomone). Therefore, those odoriferous compounds are ideal for the attraction of nocturnal pollinator moth species, *Daphnis nerii* (Olender Hawk moth), which was more frequent from 5.30 p.m. to 8.30 p.m. at *V. tessellata* flowers (Sup. Table 4).

The role of volatile compounds in *Ophyrs*-pollinator interactions has been explained by Bergstron (1978). Certain orchids lure their specific male insect pollinators to their flower by emitting “semiochemical” volatile compounds that mimic female-related sex pheromones (Schiestl *et al*. 1999, 2003; Wong *et al*. 2004, Mant *et al*. 2005, Stokl *et al*. 2007, Franke *et al*. 2009, Vereecken & Schiestl 2009). Studies carried out on *Ophrys iricolor* has shown that *Ophrys* flowers mimic the female sex pheromones of their pollinator species to attract males for pollination (Schiestl *et al*. 1999, Greis *et al*. 2002, Stokl *et al*. 2005, 2007, 2008, 2009, 2011; Spaethe *et al*. 2010; Streinzer *et al*. 2010, Ayasse *et al*. 2011, Bateman *et al*. 2011).

Substances detected in the current study such as 4-ethoxymethylphenol, Cyclohexasiloxane, 1,2-Benzenedicarboxylic acid, Decamethylpentasiloxane express a sweet odour (Gaytan *et al*. 2013, Cuna *et al*. 2021, Paudel *et al*. 2020, Wang *et al*. 2017), whilst p-vinylphenol is musty ((Nunez *et al*. 2016), Eugenol express the smell of clove oil (Dodson *et al*. 1969) and Methyl pentacyclododecane-8-carboxyloat expresses a ginger smell and is a long-range pheromone inhibitor (Mondragón & Theissen 2009). Some compounds such as 1,2-Benzenedicarboxylic acid are known solvents (Paudel *et al*. 2020), while Octadeamethyl-cyclononasiloxan, and 2- methyl-octadecyne are associated with antimicrobial properties (Hanif *et al*. 2022, Gries *et al*. 2002). Hence, this study reveals that the volatile compounds emitted by *V. tessellata* flowers are a very complex mixture with multiple purposes. *V. tessellata* may be attracting diurnal and nocturnal pollinators by emitting different odoriferous compounds at different times.

More focused studies on their chemical properties as well as the timing of their occurrence and the specific part of the flower that emits would be important. Also, as noticed in this paper, in addition to pollinators there were other visitors, who need to be further investigated. Previous studies have shown that *Vanda roxburghii (V. tessalata)* have useful properties as an aphrodisiac, antibacterial, antifungal, antiulcer, anticonvulsant, and antioxidant agent (Kumar *et al*. 2000, Subramoniam *et al*. 2013; Mukhtar & Kalsi 2017, 2018). The wealth of volatile chemical compounds with different chemical properties opens up the possibility of using *V. tessellata* for commercial use in the perfume industry.

This study exposed hitherto unknown facts regarding food deception and the role of specific compounds in *V. tessellata*. The pollinator visitations to *V. tessellata* flowers and the intensity and the type of chemical compounds of floral odour correlated well. *V. tessellata* exhibits a dual pollinator system where *Xylocopa tenuiscapa* is a diurnal pollinator and *Daphnis nerii* is a nocturnal pollinator in the successful cross-pollination of flowers. Therefore, the survival of the species, *V. tessellata* depends on the volatile organic chemical compounds and the presence of these pollinators. Further studies on those volatile chemical compounds are recommended. Protecting the pollinators of *V. tessellata* is also recommended.

Author contributions

All the authors contributed equally.

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