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Volatiles of the Inflorescences of the Madeiran Orchids, *Goodyera macrophylla* Lowe and *Gennaria diphylla* (Link) Parl. and Their Role in Pollination

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This paper is dedicated to Professor Yoshinori Asakawa for his 65th birthday.

Goodyera macrophylla, considered a rare endemic, and *Gennaria diphylla* are two native terrestrial Orchidaceae species that can be found mainly in Madeiran *Laurisilva.* The volatile compounds contributing to the floral scent of these two Madeiran orchids were analysed and compared for the first time. The volatiles, isolated by distillation-extraction in a Likens-Nickerson-type apparatus, and those extracted by headspace sorption, were analysed by GC and GC-MS. The main volatile components isolated by distillation-extraction were γ-terpinene (13%) and *n*-nonanal (11%) in *G. macrophylla* and *n*-octacosane (19%) and *n*-heptacosane (13%) in *G. diphylla*. The main volatile components isolated by headspace sorption were *p*-cymene (21%), α-pinene (15%) and linalool (14%) in *G. macrophylla* and *cis*-arbusculone (28%) and lilac alcohol (26%) in *G. diphylla*. The importance of the volatiles in the pollination of these orchids is discussed.

Keywords: *Goodyera macrophylla* Lowe, *Gennaria diphylla* (Link) Parl., Orchidaceae, pollination, volatiles, distillation-extraction, headspace sorption, GC, GC-MS.

Five native terrestrial species of Orchidaceae can be found in Madeira, three of them being endemic to Madeira [1], namely *Goodyera macrophylla* Lowe, *Dactylorhiza foliosa* (Verm.) Soó, *Orchis scopulorum* Summerth. and two widespread species, *Neotinea maculata* (Desf.) Stearn and *Gennaria diphylla* (Link) Parl. Among these species, *G. macrophylla* and *G. diphylla*, have floral scent, which may play a role in the attraction of pollinators.

The genus *Goodyera* has a widespread distribution in North America and Eurasia and its species are pollinated by various *Bombus* species, halictid bees (Halictidae) and syrphid flies (Syrphidae) [2-4]. At Madeira, *G. macrophylla* Lowe is a terrestrial orchid that grows in the humid evergreen forest, *Laurisilva*, in the central and northern parts of the island, from 300 up to 1200m [5-7]. The inflorescence bears 40 to 60 white flowers with a glabrous fleshy labellum [5]. It is a rare endemic [5,6,8,9] with only five populations known to exist on the main island of Madeira, three of which have been recently discovered [6].

G. diphylla (Link) Parl. is a nectariferous Mediterranean orchid and the only species of the

genus present on Madeira [10]. This orchid is characterised by having only two cordate leaves at the base. The plant is 8 to 45 cm in height with a glabrous inflorescence bearing 8 to 46 flowers. In the Madeiran archipelago, this orchid can also be found in the higher parts of Porto Santo and Deserta Grande. On the main island, *G. diphylla* grows mainly in the *Laurisilva*, but it can also occur at lower altitudes. This species is also common in Portugal, Southern Spain and the Canaries Islands, and is considered rare in Sardinia, Corsica, and Tunisia [11]. In the Portuguese mainland it grows in small populations sheltered by *Corema album* along the coastal woods [12,13].

Plant species use different strategies to attract pollinators and increase their reproductive success. It is well established that floral odours are important signals for chemical communication between plants and pollinators, and namely in the pollination of the orchids [14, 15]. Volatile emission varies quantitatively over time in association with pollinator activity, strongly suggesting that scent plays a crucial role as an attractant for flower-visiting animals, including bees and lepidopterans [16]. Sometimes floral scent attracts pollinators, which reproduce within the flower they pollinate [17]. The role played by the unique combination of floral characters in *Ophrys* orchids which mimic female insects in appearance and odour is well known [15,18]. Among the various floral stimuli, odours play an important role in night pollination of flowers by insects, providing signals for both flower location and plant selection by visitors [19] and nocturnally pollinated plant species are known to have strong floral scents [20]. Lilac compounds and benzaldehyde are known to attract noctuids [see 21]. There are indications that odour learning influences the responses of moths to floral volatiles during foraging [22,23]. An associative olfactory learning in the moth *Manduca sexta* has been observed when odour was paired with sucrose. These cues can help pollinators find flowers during feeding at night [22]. Cunningham *et al.* [23] demonstrated the role of two volatiles, phenyl acetaldehyde and α-pinene, on innate and learnt behaviours of moth pollinators. Although differences between the corolla colour and its background are important for pollinators to detect flowers [24], recent studies with moths have revealed their capacity to learn the relationship between flower odours and sucrose reward by reinforcement [22].

Both studied Madeiran orchids, *G. diphylla* and *G. macrophylla*, use floral osmophores to produce fragrances that seem to play an important role in their pollination. These secretary structures are usually restricted to the labellum, but can be also spread on other plant parts, and in this case all inflorescence participates in the pollinators' attraction at distance, which seems to be the case of these species. In earlier studies, we have shown that both orchids produce nectar rewards and are pollinated by different insects [1, 10, 25]. Scent plays an important role in orchid pollination, having, therefore, an impact on reproduction and consequently a role in their long-term conservation.

This work reports, for the first time, a comparative evaluation of the volatiles, extracted by two different methodologies, from two scented Madeiran orchids, one of which is a rare endemic species. According to our best knowledge, the role of the scent in these orchids' pollination is unknown.

Both *G. macrophylla* and *G. diphylla* produce only minute amounts of volatiles and the first one has a restricted distribution in Madeira. Together, these factors impair obtaining high amounts of volatiles, thus the entire aerial parts were used in these experiments.

The volatiles of *G. macrophylla* and *G. diphylla*, isolated by distillation-extraction (D-E) or captured by headspace sorption (HS) and identified by GC-MS showed very different qualitative and quantitative compositions. Identified components on each sample and their percentages are listed in Tables 1 and 2, respectively, in the order of their elution from a DB-1 column.

Monoterpenes, sesquiterpenes and phenylpropanoids could be identified on the volatiles isolated from these orchids. A fraction designated as "others" in Tables 1 and 2, since components were neither terpenes nor phenylpropanoids, and which was mainly composed of non-aromatic alcohols, saturated and unsaturated non-aromatic aldehydes and hydrocarbons, was particularly important in the volatiles extracted by distillation-extraction from both orchids.

Goodyera macrophylla: Twenty-nine components were isolated by distillation-extraction and identified by GC-MS in the inflorescence volatiles. A limited number of components with relative amounts of 0.5-3% each and some trace components could not yet be identified; these are not included in Table 2.

Table 1: Percentage composition of the volatiles isolated, by distillation-extraction (D-E) and by headspace sorption (HS), from *Goodyera macrophylla* collected during the flowering phase.

Components	RI [a]	D-E	HS
n-Heptanal	897	3.9	
α-Thujene	924		1.1
α-Pinene	930	0.6	14.8
Camphene	938		t
Sabinene	958		4.6
Octen-3-ol	961	5.0	
β-Pinene	963	0.6	4.6
n-Octanal	973	0.8	
Myrcene	975	0.6	1.4
α-Phellandrene	995		t
Phenylacetaldehyde	1002	t	
α-Terpinene	1002	0.4	
ρ-Cymene	1003	2.6	20.9
β-Phellandrene	1005	0.3	
Limonene	1009	1.5	5.8
Z-β-Ocimene	1017	0.1	
E-β-Ocimene	1027	0.1	
γ-Terpinene	1035	13.2	4.7
p-Cresol*	1045	8.2	
n-Octanol	1045	0.9	
Terpinolene	1064	0.7	
n-Nonanal	1073	10.9	
Linalool	1074	t	14.4
Indol	1255	0.4	
n-Dodecanal	1397	0.1	
α-Cedrene	1400	3.5	4.2
β-Funebrene	1404	1.9	6.7
β-Caryophyllene	1414	1.0	
E-Methylisoeugenol	1471	1.4	
Tridecanal	1499	1.1	
Z-Isoelemicin*	1534	1.2	
β-Asaron	1579	1.6	
α-Asaron	1632	5.7	
% Identification		68.3	83.2
Grouped components			
Monoterpene hydrocarbons		20.7	57.9
Oxygen containing monoterpenes		t	14.4
Sesquiterpene hydrocarbons		6.4	10.9
Phenylpropanoids		9.9	
Others		31.3	

RI [a] = Retention index relative to C_8-C_{17} *n*-alkanes on the DB-1 column. t = trace (<0.05 %). * Based on mass spectra only. MS in Supplementary data.

Together they accounted for the lower percentage of identification in this extract. In contrast, only thirteen volatile compounds were captured by headspace sorption from the inflorescences (Table 1). These compounds represented a level of identification of 68% and 83% for D-E and HS, respectively. Four volatile components, α-thujene, camphene, sabinene and α-phellandrene, were detected in the orchid fragrance in very small amounts and are not isolated by distillation-extraction.

The major D-E volatile components of *G. macrophylla* were γ-terpinene (13%) and *n*-nonanal

Table 2: Percentage composition of the volatiles isolated, by distillation-extraction (D-E) and by headspace sorption (HS), from *Gennaria diphylla* collected during the flowering phase.

Components	RI [a]	D-E	HS
n-Heptanal	897	0.9	
n-Nonane	900	t	1.1
Benzaldehyde	927	t	t
α-Pinene	930	t	4.7
Sabinene	958		10.2
β-Pinene	963		t
n-Octanal	973	1.4	
Myrcene	975	t	0.2
n-Decane	1000	t	3.2
p-Cymene	1003		t
Limonene	1009	1.8	2.7
Z-β-Ocimene	1017	t	
Z-Arbusculone*	1017		28.3
E-β-Ocimene	1027	0.3	
γ-Terpinene	1035	0.7	
E-Arbusculone*	1036		1.0
p-Cresol*	1045	7.6	
Terpinolene	1064	2.2	
n-Nonanal	1073	6.9	
Linalool	1074	t	5.7
n-Undecane	1100	t	2.3
Lilac alcohol 1*	1132		2.7
Lilac alcohol 2*	1134		3.2
n-Decanal	1180	t	t
Lilac alcohol 3*	1186	1.5	26.1
Lilac alcohol 4*	1191	0.4	t
n-Dodecanal	1397	t	
n-Tetradecane	1400	t	
n-Pentadecanal	1688	t	
n-Heptadecane	1700	t	
n-Uncosane	2100	6.4	
n-Docosane	2200	1.6	
n-Tricosane	2300	8.9	
n-Tetracosane	2400	1.7	
n-Pentacosane	2500	8.1	
n-Hexacosane	2600	0.8	
n-Heptacosane	2700	13.4	
n-Octacosane	2800	19.3	
n-Nonacosane	2900	2.0	
% Identification		85.9	91.4
Grouped components			
Monoterpene hydrocarbons		5.0	17.8
Oxygen containing monoterpenes		1.9	67.0
Others		79.0	6.6

RI [a] = Retention index relative to C_8-C_{29} *n*-alkanes on the DB-1 column. t = trace (<0.05 %). * Based on mass spectra only. MS in Supplementary data.

(11%). However, their relative amount in the D-E volatiles had no correspondence in the HS volatiles, γ-terpinene accounting for less than 5% of the headspace sorption volatiles and *n*-nonanal not detected at all. The major volatile components of *G. macrophylla* fragrance were *p*-cymene (21%), α-pinene (15%) and linalool (14%). This composition represented a modification of fragrance pattern when compared with the composition of the D-E volatiles, because *p*-cymene and α-pinene attained 3% and

0.6%, respectively, whereas linalool was detected only in trace amounts (<0.05%), in the latter case.

Gennaria diphylla: Thirty-two components were isolated by distillation-extraction and identified by GC-MS in the inflorescence volatiles, whereas only eighteen volatile compounds were captured by headspace sorption (Table 2). These compounds represented a level of identification of 86% and 91%, respectively. Seven volatile components detected in the orchid fragrance were not isolated and detected in the inflorescence D-E volatiles. These components were sabinene, β-pinene, *p*-cymene, *Z*-arbusculone, *E*-arbusculone, lilac alcochol 1 and lilac alcochol 2.

Non-terpenoid compounds were the main components (79%) of the D-E volatiles of *G. diphylla*, whereas monoterpenes represented only 7% of the total fraction. The major D-E volatiles were *n*-octacosane (19%) and *n*-heptacosane (13%). However, they were not present in the corresponding *G. diphylla* volatiles isolated by headspace sorption. Oxygen-containing monoterpenes were the major compounds of the volatiles isolated by headspace sorption, corresponding to 67% of the total fraction. Among those, 5-methyl-5-vinyl-tetrahydrofuranyl derivatives were the dominant components (61%) with *cis*-arbusculone (28%) and lilac alcohol (26%), as the main volatile components of this fraction.

In general, the volatiles obtained by distillation-extraction, both from *G. macrophylla* and *G. diphylla*, yielded a larger number of compounds when compared with the headspace sorption. For each of the species, the considerable variation between compounds isolated by distillation-extraction and headspace sorption can be explained by the use of the different isolation procedures. However, some of the differences in the relative composition of both fractions must be determined by selective emission of fragrance components by orchids. The large amount of hydrocarbons detected in the distillation-extraction oil from *G. diphylla*, is probably due to the degradation of the leaf cuticle waxes. According to Dobson [26] a greater uptake of high boiling-low volatility compounds is obtained using distillation methodology, which can lower the capacity of the method to provide an accurate measure of the floral fragrance. Nevertheless, distillation-extraction is one of the most common methodologies used to study plant volatiles composition and it can be used as an additional way of species scent characterisation. On the other hand, although headspace sorption is considered a particularly efficient technique for the capture of volatiles produced by flowers, it may be ineffective to trap the full range of compounds of the floral fragrance and its relative composition, mostly because trapping time influences the relative amounts. In addition, some compounds, such as lilac aldehydes can suffer degradation processes on charcoal filters, during trapping and storage, as has been demonstrated during the study of fragrances from other orchid species [27].

Role of floral fragrances in pollination of Goodyera macrophylla and Gennaria diphylla: Orchids have evolved a remarkable variety of flower shapes and colours in their interactions with pollinators, but the diversification of scents has also been a crucial evolutionary step in this process [28]. Fragrance compounds are now widely implicated in attraction of insect pollinators [29]. In addition, recent studies have shown that some insects, such us *Apis mellifera* and *Bombus terrestris,* can be conditioned to learn a novel odour when it is associated with a sugar reward. Higher sucrose concentrations induce higher pollination performances and insect conditioned responses, when compared with lower sucrose concentrations [30]. Both *Apis mellifera* and *Bombus terrestris* can learn to use flower scent, colour or shape (or a combination of all three features) to identify plant species, which previously provided them with a sucrose reward [31].

Floral fragrances can often be the dominant means of pollinator attraction, especially in moth-pollinated orchids visited by insects during the night [32]. Flowers pollinated at night present a syndrome associated with moth pollination based on the production and emission of a complex fragrance mixture composed by acyclic tertiary terpene alcohols, monoterpenes and/or esters of benzoic acid [28].

The major compounds of the volatile fraction of *G. diphylla* are terpenes, which play an important role as pollinator attractants for several night pollinated species [28,33]. Oxygen-containing monoterpenes were dominant in *G. diphylla*. Lilac alcohol was the second most commonly occurring fragrance and this compound has been shown to guide the moth pollinator *Hadena bicruris* to flowers of *Silene latifolia* [34]. Additionally, Cunningham *et al.* [23] found an innate attraction of the moth *Helicoverpa armigera* to □-pinene and phenyl acetaldehyde. They

suggested also a learnt foraging behaviour when this compound is reinforced by the presence of sucrose, a feeding stimulus. This suggests that foraging decisions that occur during one night of feeding can influence behaviour on the following nights [23]. The presence of α-pinene in the fragrance could serve to attract moth pollinators from a long distance. The nectar [25] could maintain their fidelity, influencing moth behaviour in the following nights. Attraction by odour may have a high ecological value in this species, since in its natural habitat there are few nectar rewarding flowers of other species to maintain the pollinators and long distance attraction of pollinators may be critical. The most commonly occurring compounds isolated by distillation-extraction (D-E) were alkenes and alkanes, namely *n*-octacosane and *n*-nonacosane. Schiestl *et al.* [35] found that these compounds attracted males of *Andrena nigroaenea* (Kirby) as pollinators of *Ophrys sphegodes* Mill., a sexually deceptive orchid. These compounds could be implicated in the attraction of *Chrysodeixis chalcites* Esper, *Phlogophora wollastoni* Baker and *Mythimna unipuncta* Haworth, nocturnal insects, which seem to be the potential pollinators of the *G. diphylla*. All nocturnal insect specimens collected and identified were male, indicating possible sexual attraction [1]. However, additional data concerning pollinators of this orchid are needed to test this hypothesis.

The volatile fractions of *G. macrophylla* also showed two major monoterpenes and, like *G. diphylla*, this orchid produced α-pinene, a compound that could be involved in the attraction of moths, as suggested by [23]. Nocturnal pollinators of *Goodyera* have not previously been reported, but the presence of α-pinene suggests that this topic should be further investigated. However, *Bombus maderensis* Erlandsson regularly visits the flowers of *G. macrophylla* to obtain nectar [1,25]. Laloi *et al.* [30] have shown that *Bombus terrestris* has an ability to associate the presence of a sugar solution with a scent and can respond to linalool when it occurs associated with a sugar reward. The high percentage of linalool in the scent of *G. macrophylla* and their association with nectar production could explain the attraction and attendance of *B. maderensis* at its flowers. As in *G. diphylla*, the attraction by odour could have a high ecological value for this orchid, providing long distance attraction. The flowering period of *G. macrophylla* in Madeira is between October and December, which coincides with the end of the flowering season of most species in the habitat. Consequently, long distance advertisement and white flowers contrasting with the dark forest background may be important to pollination success without, or with only a few, flowering species in the habitat. The important presence of α-pinene in the floral fragrance may provide additional long distance attraction, as postulated for *G. diphylla*.

Species-specific floral scent composition, when recognised by an insect, is a prerequisite of flower constancy, which is in turn required for successful pollination and by repeated visit of pollinators [36]. The highly diverse volatile fraction of *G. diphylla*, when compared with that of *G. macrophylla* suggests a potential strategy for pollinator fidelity. *Bombus* sp. were not observed visiting *G. diphylla*, despite its widespread distribution across the island.

Earlier studies showed that both orchids grow in habitats poor in nectar producing species. In addition, both *G. macrophylla* and *G. diphylla* show low frequencies of pollinator visits per hour [1]. Given that oceanic islands have a relatively low number of insect pollinators compared to mainland areas [37], the production of floral scent may have evolved specifically to provide both long distance attraction as well as pollinator fidelity in these two species.

Experimental

Plant Material: Aerial parts of *G. macrophylla* and *G. diphylla* were collected during the flowering phase at Montado dos Pessegueiros for *G. macrophylla* (1/10/2005) and Montado do Sabugal for *G. diphylla* (1/4/2005). One voucher specimen of each species has been deposited in the Herbarium of the Botanical Garden of Madeira, *G. macrophylla* (MADJ 10180) and *G. diphylla* (MADJ 10155).

Isolation Procedure: The volatiles were isolated from deep-frozen (-20 °C) fresh plant material, by distillation-extraction, with *n*-pentane as the organic solvent, during 3 h using a Likens-Nickerson-type apparatus [38]. In the field, volatile substances emitted by orchid inflorescences were trapped by headspace sorption using a glass vessel with 5 mg charcoal activated filter (SIGMA, C-3014, 20-60 mesh), embedded between glass wool. Prior to use, the charcoal filter was cleaned and conditioned by rinsing with diethyl ether and heating at 200 °C. The volatile sampling was undertaken during 48 h. The volatile compounds were desorbed through elution with 2 mL diethyl ether.

Gas Chromatography: Analyses were performed using a Perkin Elmer 8700 Gas Chromatograph, with two FIDs, a data handling system and a vaporizing injector port into which two columns of different polarities were installed: a DB-1 fused-silica (30 m x 0.25 mm i.d., film thickness 0.25 μm; J & W Scientific Inc., Rancho Cordova, CA, USA) and a DB-17HT fused-silica (30 m x 0.25 mm i.d., film thickness 0.15 μm; J & W Scientific Inc.). Gas chromatography (GC) analyses were performed using a temperature regime of 45-175°C, at 3°C/min, and subsequently at 15°C/min up to 300°C, and then held isothermal for 10 min. Injector and detector temperatures were set at 280 and 290°C, respectively. Hydrogen carrier flow adjusted to a linear velocity of 30 cm/s. The samples were injected using the split sampling technique, with a ratio 1:50. The percentage composition of the volatiles was computed by the normalization method, using GC compound peak areas, which were calculated as mean values of two injections per sample, without using response factors.

Gas Chromatography-Mass Spectrometry: The components of orchids' volatiles were identified by mass spectrometry, using a Carlo Erba 6000 Vega Gas Chromatograph, with a DB-1 fused-silica column (30 m x 0.25 mm i.d., film thickness 0.25 μm; J & W Scientific Inc.) and a Finnigan MAT 800 Ion Trap Detector (ITD; software version 4.1). Injection volumes and oven temperature were as above in GC separations. Transfer line temperature and ion trap temperature were 280 and 220°C, respectively. The helium carrier gas flow was adjusted to a linear velocity of 30 cm/s. Remaining Gas Chromatography-Mass Spectrometry (GC-MS) parameters were: split ratio of 1:40; ionization energy, 70 eV; ionisation current, 60 μA; scan range, 40-300 u; scan time of 1 s.

The oil fraction component identity was assigned by comparison of their retention indices with the C_9-C_{21} *n*-alkane indices and GC-MS spectra from a home-made library, constructed based on the analyses of reference oils, laboratory-synthesised components and commercially available standards.

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