


Communication

Volatile Organic Compounds in *Dactylorhiza* Species

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Abstract: HS-SPME-GC-MS analysis of the scent of *Dactylorhiza viridis* revealed the presence of verbenone (28.86%), caryophyllene (25.67%), β -terpineol (9.48%), and δ -cadinene (6.94%). In the scent of *Dactylorhiza romana* β -ocimene (18.69%), pentadecane (18.40%), α -farnesene (14.65%), and isopropyl 14-methylpentadecanoate (14.32%) were found. *Dactylorhiza incarnata* contained tetradecane (11.07%), pentadecane (28.40%), hexadecane (19.53%), heptadecane (17.33%), and α -cubenene (11.48%). Analysis of *Dactylorhiza saccifera* showed the presence of caryophyllene (17.38%), pentadecane (6.43%), hexadecane (6.13%), and heptadecane (5.08%). Finally, the aroma components found in *Dactylorhiza sambucina* were caryophyllene (12.90%), β -sesquiphellandrene (32.16%), 4,5-di-*epi*-aristolochene (10.18%).

Keywords: *Dactylorhiza*; volatile organic compounds; solid phase microextraction; gas chromatography; mass spectrometry



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1. Introduction

The taxonomy of the genus *Dactylorhiza* Necker ex Nevski is one of the most studied in the Orchidaceae family [1–8], widespread in Eurasia, North Africa, Alaska [9,10], with a great variety of confused and difficult to classify forms in precise taxa. It includes a number of species that varies strongly between authors, from twelve [1] to seventy-five species [6], or to thirty-six species and forty-six subspecies [11]. Traditional classifications recognize four sections of the genus *Dactylorhiza*: Aristatae, Sambucinae, Iberanthus, and *Dactylorhiza* s.s. [6]. In the early 2000s, new genetic work led to the inclusion of *Coeloglossum viride*, the only species of the genus *Coeloglossum* Hartm. 1820, in the genus *Dactylorhiza* as *Dactylorhiza viridis* (L.) [12,13]. Several authors have used this new classification in recently published monographs [14–16], while others [17–19] have been reluctant to include *Coeloglossum viride* within *Dactylorhiza*, arguing that current evidence is insufficient.

In this work it was decided to consider *Coeloglossum* in *Dactylorhiza* recognizing *D. viride*, as reported in recent molecular biology [20–25] and molecular genetics studies [10,26–30]. The scents emitted by five species of *Dactylorhiza* present in Southern Italy, four present in Basilicata, *Dactylorhiza viridis* (L.) R. M. Bateman, Pridgeon, and M. W. Chase (Figure 1a), *Dactylorhiza romana* (Sebastiani) Soó (Figure 1b), *Dactylorhiza sambucina* (L.) Soó (Figure 2a), and one present in Campania, *Dactylorhiza saccifera* (Brongn.) Soó (Figure 2b), and *Dactylorhiza incarnata* (L.) Soó (Figure 2c) were analyzed.

D. viridis is an uncommon species in Basilicata; it always grows in small populations (from 1 to 10 plants) in open woods and pastures from 600 m a.s.l. at 2100 m a.s.l. It has a very variable morphology, due to its stature (plants more than 30 cm high in the open woods and meadows of the mid-hill, or a few centimeters low in altitude meadows), due to its color (from light green to reddish yellow), and the number of flowers. *D. viridis* is the only species of the group that has a rewarding strategy by rewarding pollinators with nectar,

even if in minimal quantities [27], while, on the contrary, the other species of *Dactylorhiza* do not produce nectar by pursuing a strategy of food deception. Food-deceptive orchids are more common than sexually deceptive ones; their flowers provide signals of food gratification but do not provide re-compensation [31–33]. In Basilicata the populations of *D. romana* and *D. sambucina* are characterized by the simultaneous presence of flowers colored from yellow to red with more or less frequent various intermediate forms, while the populations of *D. saccifera*, and *D. incarnata*, have flowers with pinkish, white, or fleshy pinks often faded and attenuated in their features.

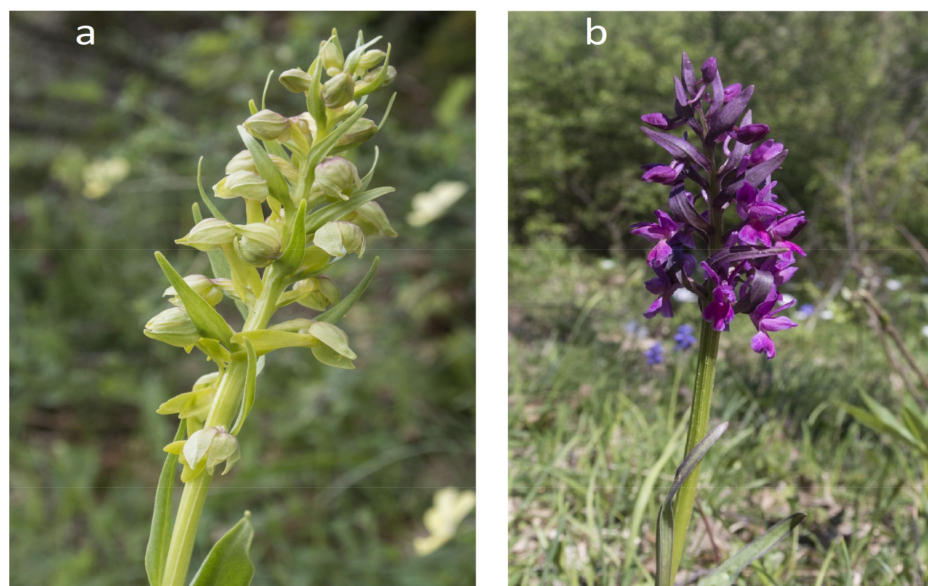


Figure 1. (a): *Dactylorhiza viridis*, Moliterno (Pz), 23 May 2018; (b): *Dactylorhiza romana*, Rifreddo (Pz), 10 April 2017. Photos of V. A. Romano.

The color, smell, size, and shape of flowers are key signals that attract pollinators in search of rewards [34–36]. Floral color is often included in studies on the evolutionary models of pollination systems while floral scents, whose characterization requires sophisticated and complex tools, have been little studied, even if they are often considered the main attraction for pollinators [37–40]. Different types of floral scent variations can be distinguished. The floral odor can vary in composition (when the flowers emit different compounds or distinct ratios of the same compounds) and/or emission speed (that is, when the flowers emit the same bouquet but in different quantities). In many cases, it has been reported that the chemical profiles of floral scents vary both between individuals of the same population and between different populations, through an almost infinite number of combinations of volatile compounds [41–47].

Comparative analysis of the emission of scents revealed a greater variation of the compounds among the same individuals in the deceptive orchids compared to the gratifying orchids [48,49]. The flower bouquet varies significantly between food deceptive orchids, even within the same genus, and can affect pollinator species and their behaviors in multiple ways [31,41]. The analysis of floral odors in orchids has been mainly carried out to characterize olfactory signals presumably attractive to pollinators, but only in a few cases have these studies been coupled, with the analysis of the pollinator response, to the volatile compounds emitted using electrophysiological techniques [48,50,51] and behavioral tests [52–55]. Therefore, there is an increasing need for a better understanding of the evolution of floral scents in order to obtain a more complete view of the mechanisms and patterns of evolution of pollination systems.

Food-deceptive orchids are pollinated by generalist pollinators [56], mainly bumblebee queens and various other bees [57–60], just emerged from the soil, without experience which, after the first attempts at pollination, abandon the plant, moving away in search

of other inflorescences [61]. It is thought that the wide variability of the morphology of flowers and scents in deceptive species for food favors cross-pollination by reducing the learning in the recognition of flowers by newly emerged, inexperienced pollinators, thus limiting self-pollination and geitonogamy and favoring outbreeding.



Figure 2. (a): *Dactylorhiza sambucina*, Moliterno (Pz), 21 May 2018; (b): *Dactylorhiza saccifera* Abriola (Pz), 30 June 2017; (c): *Dactylorhiza incarnata*, Mandranello, Padula (Sa), 6 June 2018. Photos of V. A. Romano.

While *D. romana*, *sambucina*, and *saccifera* are widespread in the territory of Basilicata, also forming large populations, *D. incarnata* has been identified in a single station on the Maddalena Mountains (Padula, Salerno) on the border between Basilicata and Campania where it forms a large population mixed with *D. saccifera* and where it is possible to find many hybrid forms between the two species and also many plants of *D. incarnata* var. *ochroleuca*. This population of *D. incarnata* is the southernmost station reported in Italy.

The purpose of this work is to complete the collection of data on the various perfumes emitted by the species of orchids in Basilicata by using the same technique, solid phase microextraction performed on intact plants followed by gas chromatography–mass spec-

trometry. Until now, the volatile organic compounds constituents of the scent of the orchid species have been determined on several orchid species [62–69].

2. Materials and Methods

2.1. Plant Material

The sample of *D. viridis* was collected at Moliterno (Pz), 1100 m a.s.l., on 23 May 2018. The sample of *D. romana* was collected at Rifreddo (Pz), 1120 m a.s.l., on 10 April 2017. The sample of *D. sambucina* (yellow color) was collected at Moliterno (Pz), 1200 m a.s.l., on 21 May 2018. The sample of *D. saccifera* was collected at Abriola (Pz), 1200 m a.s.l., on 30 June 2017. The sample of *D. incarnata* was collected at Mandranello, Padula (Sa), 1200 m a.s.l., on 3 June 2018. The plants were collected by Vito Antonio Romano.

The plants were harvested about two weeks before flowering by taking all the clod of earth, taking care not to damage the root system, planted in special pots in the gardens of the University of Basilicata (Potenza 650 m a.s.l.), in waiting for their full bloom. Two days before the tests the plants were transferred to an air-conditioned room at 22 °C. The plants were tested whole without being damaged under a cylindrical glass bell (12 cm × 45 cm) in which only the inflorescence and the SPME probe were inserted.

To avoid contamination, the interior of the bell was isolated from the external environment with appropriate closing and sealing systems during the 24 h of the test (from 8 in the morning to 8 the following day).

In order to be sure that the internal environment of the bell was isolated from the external environment, various blank tests were carried out.

The plants were successively used for further studies on pollination, fertility, and germination of the plants. After these studies the plants were not in condition to be collected in an herbarium. However, these species can be recognized without ambiguities on the basis of their properties, well documented by the Figures 1 and 2. In view of the fact that the investigated taxa are rare wild plants, in order to preserve the species, we have chosen to use a single plant for our analysis.

2.2. Analysis of Volatile Organic Compounds

The SPME analysis of five different samples of *Dactylorhiza* has been performed. This way, the identified plants were collected and inserted in glass jars for 24 h where the fiber (DVB/CAR/PDMS) and SPME syringe were also placed. After this time the fiber was desorbed in a gas chromatographic apparatus equipped with a quadrupole mass spectrometer detector. A 50/30- μ m DVB/CAR/PDMS module with 1 cm fiber (57328-U, Supelco, Milan, Italy) was employed to determine VOCs. SPME fiber was maintained in the bell jar for 24 h. The analytes were desorbed in the splitless injector at 250 °C for 2 min. Analyses were accomplished with an HP 6890 Plus gas chromatograph equipped with a Phenomenex Zebron ZB-5 MS capillary column (30-m × 0.25-mm i.d. × 0.25 μ m FT) (Agilent, Milan, Italy). An HP 5973 mass selective detector (Agilent, Milan, Italy) in the range 0–800 m/z (Agilent) was utilized with helium at 0.8 mL/min as the carrier gas. The EI source was used at 70 eV. The analyses were performed by using a splitless injector. The splitless injector was maintained at 250 °C and the detector at 230 °C. The oven was held at 40 °C for 2 min, then gradually warmed, 8 °C/min, up to 250 °C and held for 10 min. Tentative identification of aroma components was based on mass spectra and Wiley 11 and NIST 14 library comparison. Single VOC peak was considered as identified when its experimental spectrum matched with a score over 90% that present in the library. All the analyses were performed in triplicate.

3. Results and Discussion

The scent of *Dactylorhiza* orchids has been the object of some studies in the past. Analysis of *D. sambucina* showed the presence of limonene, β -myrcene, α -pinene, α -bergamotene, β -bisabolene, caryophyllene, and β -selinene [70]. Dichloromethane extracts of flowers of *D. incarnata* showed the presence of 4-hydroxybenzaldehyde (16.88%), 4-hydroxybenzyl

alcohol (30.60%), and methyl 4-hydroxyphenylacetate (34.37%) [71]. Pentane, diethyl ether extraction of flowers of *D. incarnata* showed the presence of nonanal, 9-(*Z*)-heptacosene, 9-(*Z*)-nonacosene, tricosane, pentacosane, and heptacosane [72]. The analysis of the scent of *D. romana* (red flower), obtained by head space analysis of the scent absorbed on Porapak Q, showed the presence of several components, including nonanal (9.19%), sabinene (10.89%), (*E*)-ocimene (15.63%), and linalool (7.99%) [31]. Such different results, obtained by using different analytical techniques, allowed us to analyze the scent of these species by using SPME (solid phase microextraction) technique. The results are reported in Table 1.

The never studied before *D. viridis* contains, as its main components, verbenone (28.86%) and caryophyllene (25.67%), while other compounds present in relevant amounts are β -terpineol (9.48%) and δ -cadinene (6.94%) (Table 1 and Figure 3).

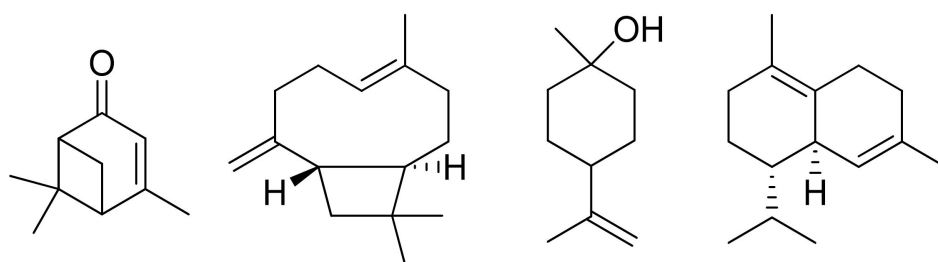


Figure 3. Main components of the scent of *D. viridis*.

In the scent of *D. romana* several compounds were found: β -ocimene (18.69%, found also in [31]), pentadecane (18.40%), α -farnesene (14.65%), and isopropyl 14-methylpentadecanoate (14.32%) (Table 1). Previous works published on the aroma of *D. incarnata* found phenolic compounds, in one case, while long chain alkanes and alkenes, were found in another case [71,72]. SPME analysis of the scent detected the presence of a mixture of hydrocarbons, but with lower molecular weight than those found in [72]. Tetradecane (11.07%), pentadecane (28.40%), hexadecane (19.53%), and heptadecane (17.33%) were the main components, together with α -cubenene (11.48%) (Table 1).

D. saccifera contained, as a main component, caryophyllene (17.38%), while other significant compounds were hydrocarbons, pentadecane (6.43%), hexadecane (6.13%), and heptadecane (5.08%) (Table 1). Finally, the aroma components found in *D. sambucina* were caryophyllene (12.90%, found also in [70]), β -sesquiphellandrene (32.16%, Figure 4), 4,5-di-*epi*-aristolochene (10.18%, Figure 4) (Table 1).

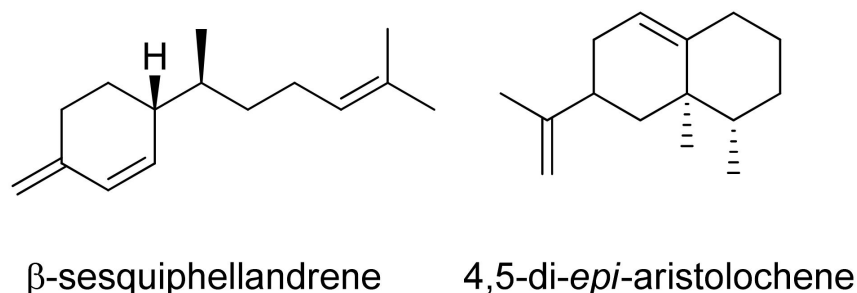


Figure 4. Main components of the scent of *D. sambucina*.

Table 1. SPME-GC–MS analysis of *Dactylorhiza* species.

Compound	r.t. ^(a) (min.)	KI ^(b)	<i>D. viridis</i>	<i>D. romana</i>	<i>D. incarnata</i>	<i>D. saccifera</i>	<i>D. sambucina</i>
			Area % ± 0.03				
Mesityl oxide	4.94	782				2.03	
α-Pinene	7.78	933				0.20	0.30
Sabinene	8.77	972	2.27				2.44
β-Pinene	8.86	979					0.79
β-Myrcene	9.12	989	2.42				
2,2,4,6,6-pentamethyl-3-heptene	9.29	1018				0.60	
Limonene	9.79	1028				1.88	
Eucalyptol	10.00	1032	2.65				
β-Ocimene	10.33	1044		18.69			
β-Terpineol	10.74	1085	9.48				
Linalool	11.22	1100				0.85	
Lilac aldehyde A	12.09	1145				0.20	
Lilac aldehyde B	12.25	1154				0.90	
Dodecane	13.08	1200				0.45	
α-Terpineol	13.16	1209	2.07				
Lilac alcohol A	13.52	1221				2.08	
Verbenone	13.55	1223	28.86				
Lilac alcohol B	13.84	1235				4.00	
Citral	14.55	1265	4.21				
Tridecane	14.88	1300				2.88	0.85
2,4,4,6,6,8,8-Heptamethyl-2-nonene	16.02	1343				1.48	
α-Cubebene	16.42	1360			11.48		0.34
Tetradecane	16.64	1400			11.07	3.90	2.13
β-Elemene	16.69	1403					
Caryophyllene	17.22	1420	25.67			17.38	12.90
Methyl 2-phenylcyclopropanecarboxylate	17.31	1435				0.90	
cis-α-Bergamotene	17.38	1440					7.61
β-Farnesene	17.48	1458					0.68
Geranylacetone	17.61	1468		6.62			
Humulene	17.76	1473					0.42
4,5-Di- <i>epi</i> -aristolochene	18.23	1485					10.18

Table 1. Cont.

Compound	r.t. ^(a) (min.)	KI ^(b)	<i>D. viridis</i>	<i>D. romana</i>	<i>D. incarnata</i>	<i>D. saccifera</i>	<i>D. sambucina</i>
			Area % ± 0.03				
Pentadecane	18.25	1500	2.09	18.40	28.40	6.43	
β-Selinene	18.40	1511					1.28
α-Farnesene	18.45	1518		14.65		3.30	
β-Bisabolene	18.50	1523					2.64
δ-Cadinene	18.78	1530	6.94				
7-Hexadecenal	18.64	1555				1.00	
β-Sesquiphellandrene	18.76	1557					32.16
Elemicin	19.07	1566				3.30	
Hexadecane	19.71	1600			19.53	6.13	1.06
Methyl dihydrojasmonate	20.32	1650	2.55				1.23
Cyclotetradecane	20.47	1673				1.58	
Heptadecane	21.14	1700			17.33	5.08	0.64
3,5-Di- <i>t</i> -butyl-4-hydroxybenzaldehyde	22.13	1772				4.38	
Octadecane	22.48	1800			8.24	3.40	0.25
Phytone	23.14	1848					0.58
Nonadecane	23.70	1900				2.93	
Isopropyl 14-methylpentadecanoate	25.35	1915		14.32			1.94
7,9-Di- <i>t</i> -butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione	24.11	1929				1.88	
Eicosane	24.93	2000				1.83	
13- <i>epi</i> -Manoyl oxide	25.49	2015			3.42		
Docosane	27.22	2200				1.93	
Tricosane	28.31	2300				2.28	
Bis(1-phenylethyl)phenol	30.98	2426				2.08	

^(a) R.T. retention time; ^(b) KI Kovats index.

4. Conclusions

The results described above can give us some useful information. First, the results of our analysis, performed by using SPME technique, are not in agreement with previous described scent composition [31,70–72]. This difference may depend on several factors: first of all, the different location of the plants under study. The different environmental conditions could induce plants to adopt different strategies for pollination. Second, the different analysis methodology could play a significant role. Furthermore, we can observe that every species adopts a different strategy. *D. viridis* has a scent where terpenes are the main components. This statement is applicable also to *D. romana*; however, it is noteworthy that the terpenes involved in the scent are different from those observed in the other species. The scent of *D. incarnata* only included hydrocarbons as components; hydrocarbons are present in the scent of *D. saccifera* but, in this case, caryophyllene is also present in relevant amount. Finally, terpenes were detected in the scent of *D. sambucina*, as in *D. viridis* and in *D. romana*, but the compounds involved in the scent, with the exception of caryophyllene, are different from those observed in the other species.

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