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A simple solid injection device for the analyses of *Bulbophyllum* (Orchidaceae) volatiles

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Abstract

The volatile components of three orchid species (*Bulbophyllum weddellii*, *B. ipanemense* and *B. involutum*) were detected using GC/MS. Due to the minute dimension and amount of flowers, special traps and extraction methods were applied and the analyses were finally successful using a special solid sample injector which was constructed in our own laboratory. The chemical composition comparison revealed the high similarity between the volatiles present in *B. weddellii* and *B. involutum*, which is coherent with the pollinator sharing. On the other hand, the volatiles of *B. ipanemense*, a species pollinated by other pollinator species, are different from the two previous species. © 1998 Published by Elsevier Science Ltd. All rights reserved.

Keywords: *Bulbophyllum weddellii*; *B. ipanemense*; *B. involutum*; Orchidaceae; GC/MS; Volatiles; Solid injector

1. Introduction

The orchid family is widespread across the world and over 25,000 species are known to exist (Dressler, 1993). The variety of shapes, colors and scents present in Orchidaceae is seldom found in other plant families. They are pollinated by moths, carrion-flies, bees, butterflies, birds, etc. and there is a definite relationship between the floral scent and the pollinators (van der Pijl & Dodson, 1966). Three Brazilian orchid species, *Bulbophyllum weddellii*, *B. involutum* and *B. ipanemense*, native of Minas Gerais state, Brazil, were extensively studied from the floral biology and phenology point of view, revealing that the three species are pollinated by females of *Pholeomyia* (Diptera: Milichiidae) with a high degree of specificity (Borba, 1997).

The above observations have attracted our attention as they might indicate that the orchid scents play a significant role in this orchid-pollinator relationship (Dressler, 1968; Janzen, 1971; Faegri & van der Pijl, 1979). We have thus focused on the chemical analysis of the volatile components of these three *Bulbophyllum* species.

2. Results and discussions

The first part of our investigations concerned the optimization of the flower scent trapping and we have used *B. weddellii*. Based on previous experiments (Kaiser), the headspace technique was our first choice. A glass vessel enclosing the orchid, a battery-operated pump and a trap containing Porapak Q was our major device. The trapped volatiles were desorbed from the trap with bidistilled solvent (dichloromethane and methanol 1.5 mL) and directly injected in the GC/MS, after reducing the volume of the solvent to 0.1 mL. After several attempts we came to the conclusion that this methodology was not efficient for these analyses due to loss of some rather volatile components and decomposition of others. High pressure Soxhlet extraction with CO_2 (J and W High Pressure Soxhlet Extractor) and microhydrodistillation (Ferracini et al., 1995) were also performed, but in the three methodologies we had to face analogous problems: the insignificant amount of extracted material, minute dimensions of the flowers and decomposition of the floral scent within a few hours. We came finally to the conclusion that our analyses would never be correct unless the use of solvents was avoided.

We have thus looked for a direct loading technique (Senanayake et al., 1976) which should be applicable

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to most gas chromatographs without requiring any instrument modification. Solid phase microextraction was one of our choices (Pawliszyn, 1997) but the results with a 30 μm polydimethylsiloxane coating fiber were not satisfactory and unfortunately no other fibers were available at the time of the analysis. Thus we turned our attention to the solid sample injectors. This technique has been used by several research groups, particularly applied to pheromone research (Attygalle & Morgan, 1988) and one solid sample injector kindly provided by Dr Vostrowsky (Institut fü Organische Chemie) was available in our laboratory. However this device required some modification of our HP gas-chromatograph injector which we were reluctant to apply. An alternative method was the use of appropriate needles from Dynatech which were not available for our analysis. We have therefore modified a B–D stainless steel syringe needle (80 × 0.8 mm or gauge 21) which allowed the injection of a small piece (*ca.* 3,0 × 0.3 mm) of the orchid flower in the GC/MS (splitless mode) and the analyses of the volatiles (Fig. 1). Our first experiments concerned the reliability of our analyses. Thus different parts of the *B. weddellii* flower were analyzed and we could observe that the chemical composition was analogous although the relative amount of the constituents would vary depending on the location of the flower piece.

The results of the analyses of the volatile components of the three *Bulbophyllum* species (*B. weddellii*, *B. involutum* and *B. ipanemense*) are in Table 1. Comparison of the chemical composition of the *Bulbophyllum* flower scents with those of other fly pollinated orchids (*Bulbophyllum gracillium*, *Dracula chestertonii* etc. (Kaiser) revealed that the classes of compounds present in these orchid scents seem to follow a chemical pattern related to their pollinator.

Thus fly-orchids have similar flower scent composition concerning the compound classes (*n*-alkyl-ketones, *n*-alkyl-aldehydes, *n*-alkyl-alcohols, aromatic, few terpenes) while the chemical composition of orchids pollinated by bees reveal an abundance of terpenes (mono and sesquiterpenes). Comparison of the scent constituents (RI up to 1600) revealed a higher similarity between *B. weddellii* and *B. involutum* (Jaccard index = 0.4667 (Dunn & Everitt, 1982)) than between *B. ipanemense* and these species (Jaccard index for *B. weddellii* and *B. ipanemense* = 0.2500; *B. ipanemense* and *B. involutum* = 0.1875). These results are absolutely compatible with the fact that both *B. weddellii* and *B. involutum* share the same pollinator species (two *Pholeomyia* species) but with a different hierarchy, i.e. the main pollinator of *B. weddellii* is secondary to *B. involutum* and vice versa. The unique pollinator species of *B. ipanemense* is a different species of *Pholeomyia* (Milichiidae). From these results one would expect to find several natural hybrids between *B. weddellii* and *B. involutum*, since they are sympatric

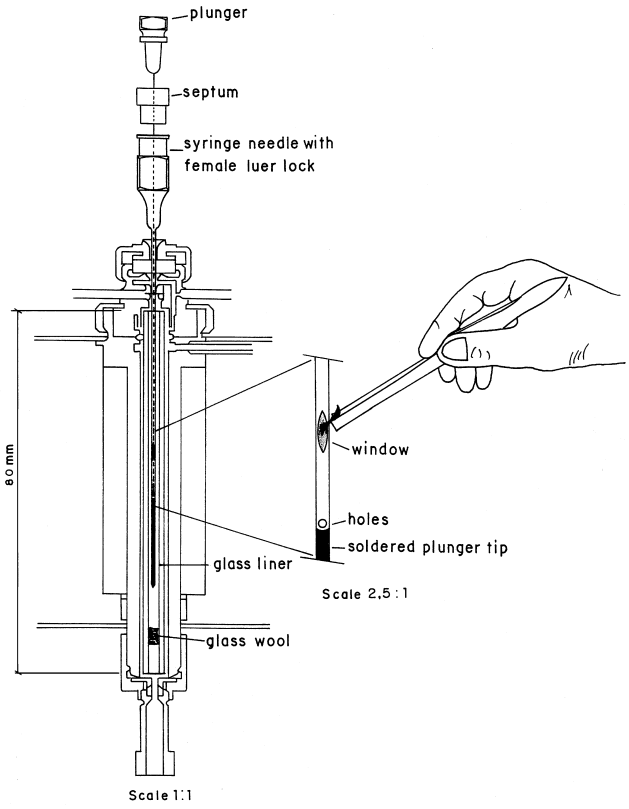

Fig. 1. Solid sample injection device in the splitless capillary inlet system. and the enlarged details showing the window to insert the solid sample, the holes and the soldered plunger tip.

and flower at same time. But this is not true because there are several other natural barriers operating between these two species that allow the species identities to prevail. One should mention that only one natural hybrid of *B. weddellii* and *B. involutum* has been identified which is apparently sterile. *B. involutum* and *B. ipanemense* are closely related species, and these are unrelated to *B. weddellii*. These results agree with Dressler (Dressler, 1968), who stated that when similar orchid species are found growing in proximity, and with phenological events synchronized, there is a selection pressure for producing different volatile compounds attracting different pollinator species. On the other hand, due to the presence of other interspecific barriers, unrelated species could produce similar compounds attracting the same pollinators, and in spite of that maintain their integrity.

3. Experimental

3.1. Plant material

Individuals of *Bulbophyllum weddellii* and *B. involutum* species were collected at Serra do Cipó, municipality of Santana do Riacho, and *B. ipanemense* at Serra de Camargos, municipality of Nazareno, both localities

in Minas Gerais state, southeastern Brazil. These individuals were cultivated in the greenhouse of Departamento de Botânica of the UNICAMP until flowering when the flowers were collected for the analysis. Voucher specimens of the species are deposited in the Herbarium UEC (Departamento de Botânica, Universidade Estadual de Campinas): *Bulbophyllum weddellii* (Lindl.) Rchb. f. (voucher # E.L. Borba 151), *Bulbophyllum involutum* Borba, Semir and F. Barros, (voucher # E.L. Borba 150), *Bulbophyllum ipanemense* Hoehne (voucher # E.L. Borba 177).

3.2. GC and GC/MS

Analyses were carried out using a HP-5890/5970 system equipped with J and W Scientific DB-5 fused silica capillary column (30 m × 0.25 mm × 0.25 μm); column temperatures were programmed from 50° to 290° at 4°/min. **GC**: Injector and detector temperatures were 290° and 300° respectively. Hydrogen was used as carrier gas, flow rate 2 mL/min, split/splitless mode 0.5 min. Injection volume, 1 μl of solution. **GC/MS**: The carrier gas was helium and the temperature program was the same as that for the GC experiments. Injector and interface temperatures were 290° and 285° respectively. The MS were taken at 70 eV. Scanning speed was 0.84 scan/s from m/z 40 to 550.

The volatiles were analyzed by GC and GC/MS and identification was made on the basis of standard compound coinjection and comparison of retention indices (Van den Dool & Kratz, 1963) as well as by computerized matching of the acquired mass spectra with those stored in the Wiley/NBS mass spectral library of the GC/MS data system and other published mass spectra (Adams, 1995).

3.3. High pressure Soxhlet extraction with CO_2

In a J and W Scientific extractor 15–20 fresh flowers (1–1.2 g) were introduced together with dry ice (165 g). The apparatus was closed and the extractions were performed at 35–45° and the pressure was maintained between 700 and 900 psi, for three hours. The apparatus temperature was then decreased to −70° (dry-ice/ethyl alcohol bath) and the CO_2 was liberated through the escape valve. Dichloromethane (1 mL) was inserted in the round bottom flask and the mixture was transferred to a vial. The GC/MS analyses were performed after the volume of the solvent was reduced to 0.1 mL. No coherent GC/MS analyses were obtained.

Table 1
Volatile components detected in *Bubophyllum weddellii*, *B. involutum* and *B. ipanemense*

Compounds	RI	*B. weddellii*	*B. involutum*	*B. ipanemense*
1. Hexanal	826	–	–	3.90
2. Furfural	850	1.77	1.02	–
3. 2-Furanomethanol	865	4.57	3.30	0.57
4. 5-Methyl-2(3H)-furanone	920	0.66	–	–
5. 5-Methyl-furfural	946	t	0.82	
6. 2-Pentyl-furan	972	–	–	0.65
7. 2,3-Dihydro-4-hydroxy-2,5-dimethyl-3-furanone	1043	t	1.23	–
8. 2-Methoxy-phenol	1074	9.19	4.57	1.51
9. Maltol	1098	t	–	–
10. 2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyrene-4-one	1134	1.75	15.55	–
11. 1,2-benzenediol	1197	t	3.53	1.28
12. 5-hydroxymethyl-2-furan-carboxyaldehyde	1224	–	2.71	–
13. 2,4-Decadienal-(*E*,*Z*)	1298	–	–	4.04
14. 2,3,5,6-Tetramethylphenol	1319	0.93	–	t
15. 2,4-Decadienal-(*E*,*E*)	1321	–	–	6.03
16. 2,6-Dimethoxyphenol	1357	0.96	–	t
17. 4-Hydroxy methyl benzoate	1465	2.84	–	–
18. Tridecanone	1505	–	–	1.14
19. Tridecanol	1512	–	–	3.42
20. 4-Hydroxy-3-methoxy methyl benzoate	1526	0.75	–	–
21. 2,6-Dimethyl-3-methoxymethyl-*p*-benzoquinone	1573	17.57	–	–
22. Methyl tetradecanoate	1719	–	–	0.72
23. Tetradecanoic acid	1751	1.83	1.34	5.27
24. Pentadecanoic acid	1833	0.89	2.90	1.75
25. 14-Methyl methyl pentadecanoate	1884	–	0.68	–
26. 9-hexadecenoic acid	1898	–	4.65	–
27. Hexadecanoic acid	1919	9.60	31.32	54.50
28. Ethyl hexadecanoate	1939	–	–	1.53
Total		53.31	73.62	86.31

3.4. Hydrodistillation

A round bottom flask (100 mL) equipped with a micro distillation head, containing 15 to 20 orchid flowers and water (50 mL) was heated and 30–35 mL of water were distilled. The distilled aqueous solution was extracted with dichloromethane (3 × 30 mL). The organic layers were dried over anhydrous Na_2SO_4. After filtration the solvent was reduced under vacuum to 0.2 mL and 1 μl of the mixture was injected in the GC/MS. Some volatile components were detected but the analyses were not reproducible after some hours indicating decomposition. Thus this methodology was considered unreliable.

3.5. Headspace

A glass vessel of (20 cm long and 4 cm of internal diameter) was specially constructed to fit the orchid flower with an opening to insert the flower and an outlet in the opposite side connected to an absorption trap (glass tube 5 cm long and 0.5 cm internal diameter) containing Porapak Q 80–100 mesh (100–200 mg) and glass wool at both ends (previously treated with solvent and activated at 150° under vacuum). The scented air was drawn through the trap by means of a battery-operated pump (500 mL/min) over a period of 24 h. The volatiles were extracted with dichloromethane and methanol. The volatiles concentration was below the detection threshold for the GC/MS analyses.

3.6. Solid sampling analyses

The solid sample injector, was used for introducing a sample of orchid flower volatiles into the capillary chromatography column of the GC/MS equipment. The solid sample injector containing 3 pieces of the orchid cepals (3 × 0.3 mm) were introduced in the GC/MS with the split vent closed for 30 s. The retention indices were obtained by coinjection of a cepal piece contaminated with a C_{11}–C_{24} normal hydrocarbon mixture.

3.7. Solid phase microextraction

A septum closed vial containing 15 fresh flowers was heated to 60° for 30 min. A fiber (30 mm of polydimethylsiloxane coating) was introduced through the septum in the saturated atmosphere above the flower (static headspace) for 30 min.

3.8. Solid sampler construction

This injector has the advantage of being inexpensive and adaptable to all GC equipments. The terminal part of the plunger of a stainless steel syringe needle (9 cm long and gauge 21) was cut (1 cm) and soldered inside the tip of the syringe. A window (3.5 mm long and 0.5 mm wide) was cut into the needle wall 27 mm from the tip and two holes (0.5 mm) were drilled at 21 mm from the tip of the needle to allow the volatile components to be carried into the column. A small septum was adapted into the female luer-lock and the plunger was introduced through it. For the analyses the sample was introduced into the window and gently pushed with the plunger into the needle, taking care not to crush the sample at the close end of the needle. The needle was then introduced in the normal GC injector, the needle plunger is then rapidly pushed down moving the solid sample to the bottom of the needle whereas the volatile components escape through the two holes (Fig. 1).

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