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Characterization of the Essential oil of the Bat-Pollinated *Passiflora mucronata*

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The genus *Passiflora* is an important source of food, therapeutic substances and for the horticultural economy. In the last decades, a detailed chemical composition of the essential oil of *Passiflora* species has been reported, but only for few species, mainly of agricultural interest, although little attention has been paid to chiropterophilous Passifloraceae, such as *P. mucronata*. The present study is focused on analyzing the essential oil composition of *P. mucronata*, a Brazilian bat-pollinated species. From GC/FID and GC/MS analyses of the volatile fraction from fresh flowers and leaves, hydrocarbons were quantified as 47.9% and 42.8% of the total volatiles of flowers and leaves, respectively, esters for 50.8% in flowers and 6.4% in leaves, and alcohols 38.2% and 0.3% of the total volatiles from leaves and flowers, respectively. Other classes of compounds, such as monoterpenes and aldehydes, together with phytol, were detected in higher concentration in leaves compared with flowers. The higher content of methyl and ethyl esters of long chain saturated and unsaturated fatty acids, i.e. ethyl linolenate (38.3%), methyl linolenate (7.0%) and ethyl palmitate (3.6%), were the most representative suggesting that esters might play a critical role for fertilization of *P. mucronata* acting as bat attractors.

Keywords: *Passiflora mucronata*, Essential Oil, Esters, Pollination, Bat.

The genus *Passiflora*, family Passifloraceae, consists of more than five hundreds species of vines, lianas and small trees widely distributed throughout tropical regions and divided into 4 subgenera, *sensu* Feuillet and MacDougal [1]. Passion flowers exhibit several typical floral features such as a series of brightly colored corona filaments, a prominent androgynophore and complex nectary structures. Due to their adaptation to habitats and pollinators *Passiflora* species show a high-level of co-evolution and a wide range of pollinators, mainly moths, bees, hummingbirds and even mammals, like bats [2]. Furthermore, *Passiflora* species are traditionally used as food for their sweet fruits and medicinal properties to treat insomnia and for soothing [3].

P. mucronata Lam. belongs to the subgenus *Passiflora*, Serie Simplicifoliae (Harms) Killip, with a chromosome number of 2n=18 [4]. This plant is found over a large area of NE and SE Brazil and blooms during the night from January to early summer [5]. Night-blooming is linked with its pollinators [6]; in fact, two species of phyllostomid bats were identified to be involved with *P. mucronata* fertilization, i.e. *Glossophaga soricina* and *Carollia perspicillata.* These unusual (at least for the Passifloraceae family) pollinators also entail some peculiarities in flower morphology. Passiflora flowers turn from an actinomorph type, typical of the genus *Passiflora*, to a zygomorphic one leading to an eccentric arrangement of anthers ready to accept pollen [6]. It is important to notice that *P. mucronata* is almost entirely self-incompatible, requiring cross-pollination with different genotypes [7,8]. Consequently, volatile substances present in flowers are considered as key attractors for chiropterophily and chiropterochory [9,10].

HS/GC-MS coupled with steam distillation are commonly employed methods to analyze the floral scent of several taxa, although there is a paucity of studies investigating the fresh tissue chemical composition Passifloraceae [11,12], especially *P. mucronata* [13,14]. The aim of the present work was to determine the flavor constituents of both fresh flowers and leaves of this species in order to assess their role in plant pollination.

The yields of *P. mucronata* essential oil obtained by steam distillation from fresh flowers and leaves were 0.3 mg/g and 0.2 mg/g, respectively. The GC analysis revealed the presence of 46 constituents, as reported in Table 1, in which compounds are listed in order of their elution on the Elite-5MS column and reported as percentages of the total oil. The main bulk of constituents of both volatile fractions were hydrocarbons, accounting for 47.9% and 42.8% in flowers and leaves, respectively. This class of compounds was mainly constituted of saturated linear chain hydrocarbons in the range C_{10}-C_{27} of which heneicosane (17.6%) and tricosane (14.9%) were the most abundant in flowers, and pentacosane (27.4%) and heptacosane (8.7%) in leaves.

These results are in agreement with the data previously reported by Calevo *et al*. [11], indicating that a higher content of odd linear long-chain hydrocarbons was detected in three new bee-pollinated *Passiflora* hybrids. The presence of hydrocarbons is associated with epicuticular wax chemistry playing an important role in plant-herbivore interactions, acting as allelochemicals [15]. Saturated alkanes (42.8% and 47.9% in leaves and flowers, respectively) exert an interesting, although limited, activity in pollinator deception in different floral species [16-18].

Figure 1: Flower and leaves of *Passiflora mucronata* cultivated in Sanremo (Italy).

Esters were detected in higher amount in flowers, accounting for 50.8% of the total volatiles, compared with leaves, in which they formed 6.4% of the total oil. These compounds were mainly characterized by methyl and ethyl esters of fatty acids such as ethyl linolenate (38.3% in flowers and 0.1% in leaves), methyl linolenate (7.0% in flowers and 0.1% in leaves) and ethyl palmitate (3.6% in flowers and 0.3% in leaves).

Compared with flowers, leaves were instead characterized by the presence of a higher amount of methyl pentanoate (2.6% in leaves and 0.2% in flowers) and methyl salicylate (2.1% in leaves and 0.1% in flowers). The main odoriferous compounds of *P. mucronata* flowers, i.e. ethyl linolenate (38.3%), methyl linolenate (7.0%) and ethyl palmitate (3.6%), are an important part of this lipid-based scent acting as an attractor for the nectariferous phyllostomid bats *Cynopterus brachyotis*, *C. sphinx* and *Mystacina tuberculata* when searching for *Ficus hispida* and *F. scortechinii* ripened fruit, and *Dactylanthus taylorii* flowers, respectively [19-21].

Alcohols represented 38.2% of the total oil from leaves, but only 0.3% of the total oil from flowers. These differences were predominantly ascribed to phytol (25.7% in leaves, 0.1% in flowers) and to the two aromatic alcohols, benzyl alcohol (9.4% in leaves, 0.1% in flowers) and 2-phenylethanol (1.8% in leaves, trace amount in flowers).

Flower and fruit-typical esters are clearly identified by the frugivorous bat *C. perspicillata* and a significant correlation between threshold values of detection and carbon chain length of the odorous molecule(s) has been observed also for acetic esters and aliphatic alcohols [10].

Phytol was found to be ubiquitous and highly abundant in leaves, being a constituent of chlorophyll, and its presence is probably due to its antimicrobial activity as part of *P. mucronata* defense mechanisms [22]. A lower amount of aldehydes and monoterpenes compared with the other classes of constituents was detected in the volatile fractions of *P. mucronata* (see Table 1); both these classes of constituents were found to be higher in leaves (2.0% and 4.0% for aldehydes and monoterpenes, respectively) than in flowers (0.1% for both classes). The low levels of these compounds in the volatile fraction of *P. mucronata* here investigated was previously reported for other *Passiflora* hybrids [11].

Table 1: Volatile composition of flowers and leaves of *P. mucronata*.

	Compound[a]	Al Tab[b]	Al[c]	Leaves %	Flowers %
1	*cis*-3-Hexenal	797	798	0.4	Tr
2	2-Methyl-2-pentenal	821	820	0.5	0.1
3	Methyl pentanoate	829	830	2.6	0.2
4	*cis*-3-Hexenol	855	855	0.9	Tr
5	Benzaldheyde	960	958	0.4	-
6	Decane	1000	1000	0.2	-
7	Benzyl alcohol	1040	1034	9.4	0.1
8	γ-Terpinene	1059	1059	1.0	0.1
9	Guaiacol	1090	1088	0.4	0.1
10	Undecane	1100	1100	0.5	0.2
11	2-Phenylethanol	1119	1115	1.8	Tr
12	Methyl salicylate	1190	1193	2.1	0.1
13	Dodecane	1200	1200	0.7	-
14	Cinnamaldehyde	1235	1218	0.7	Tr
15	Geraniol	1258	1262	0.7	Tr
16	Furfuryl valerate	1272	1271	0.5	-
17	Allyl octanoate	1280	1281	0.5	-
18	Tetradecane	1400	1400	0.7	0.1
19	β-Ionone	1487	1488	0.3	-
20	Nerolidol	1527	1533	2.0	Tr
21	Ethyl dodecanoate	1579	1593	0.2	0.1
22	Hexadecane	1600	1600	0.3	0.1
23	Pentadecan-2-one	1698	1699	-	0.2
24	Unidentified MW=266	-	1724	0.3	-
25	1-Octadecene	1791	1793	0.3	0.2
26	Unidentified MW=256	-	1866	-	0.1
27	Ethyl pentadecanoate	1890	1895	-	0.3
28	Heptadecan-2-one	1903	1902	-	0.1
29	Methyl palmitate	1927	1921	-	0.3
30	Palmitic acid	1968	1963	1.0	0.1
31	Ethyl palmitate	1992	1993	0.3	3.6
32	Eicosane	2000	2000	-	0.1
33	Methyl linolenate	2078	2051	-	0.2
34	Methyl linoleate	2093	2096	0.1	7.0
35	Heneicosane	2100	2100	1.9	17.6
36	Phytol	2113	2116	25.7	0.1
37	Ethyl linolenate	2169	2171	0.1	38.3
38	Heneicosane-3-methyl	2172	2174	Tr	7.6
39	1-Docosene	2190	2194	0.2	Tr
40	Ethyl octadecanoate	2193	2196	-	0.7
41	Docosane	2200	2200	0.4	2.4
42	Tricosane	2300	2300	0.1	14.9
43	Tetracosane	2400	2400	0.7	0.5
44	Pentacosane	2500	2503	27.4	3.5
45	Hexacosane	2600	2600	0.7	0.1
46	Heptacosane	2700	2701	8.7	0.6
	Hydrocarbons			**42.8**	**47.9**
	Esters			**6.4**	**50.8**
	Alcohols			**38.2**	**0.3**
	Monoterpenes			**4.0**	**0.1**
	Aldehydes			**2.0**	**0.1**
	Miscellaneous			**1.0**	**0.4**
	Unidentified			**0.3**	**0.1**
	Total			**94.7**	**99.7**

a) Compounds are listed in order of elution from an Elite-5 column. b) Retention Indices according to Adams [26] c) Retention index determined on an Elite-5 column using a homologous series of *n*-hydrocarbons.

No sulfur compounds were detected in the essential oil of either flowers or leaves of *P. mucronata*, indicating that these may not be the main bat attractors, as demonstrated also for other bats like *C. sphix* [23].

To our knowledge, this is the first report characterizing the volatile components of *P. mucronata*. Hydrocarbons are well represented in the essential oil from both inflorescence (47.9%) and leaves (42.8%), probably acting as crucial agents in *P. mucronata* defense strategies. Moreover, long chain methyl and ethyl esters, accounting for 50.8% of the essential oil from flowers, seem to be key attractors for bats such as *Carollia perspicillata* and *Glossophaga soricina*, driven by the olfactory stimuli of these compounds for foraging when visiting *P. mucronata.*

Experimental

Plant material: *Passiflora mucronata* Lam. plants (Figure 1) are part of a collection funded by RGV-FAO project that is aimed at

maintenance and valorization of germplasm. They were grown in 30 cm pots supplied with plastic supports to allow climbing of the tendrils and were maintained in a greenhouse heated during winter and shaded during summer time, with temperatures ranging from 10°C (min) to 32°C (max), in Sanremo (Italy), UTM WGS84, Zone 32T North: Long 400142.51 Lat 4852298.79. At flowering stage, flowers and young leaves produced in the current year of the same phenological stage and without any defect were collected at about 9.30 am, put into Pyrex 250 mL sealed bottles with CH_2Cl_2 as preservative and stored at 4°C until analysed. A voucher specimen is deposited at CREA-OF with code PASS012 (Sanremo, Italy). The specimen has been fully characterized by morphological characters and by the ISSR markers [24].

Isolation of volatile fraction: Plant material (about 7.0 g fresh flowers and 6.6 g fresh leaves) was steam distilled with odor-free water in a Clevenger-type apparatus for 1 h. The distillate was saturated with NaCl, extracted with freshly distilled Et_2O (3 x 100 ml), dried over anhydrous Na_2SO_4 concentrated at first with a rotary evaporator and finally using a stream of nitrogen and then analyzed by GC/FID and GC/MS.

Analysis of essential oils: GC/FID analyses were carried out using a Perkin Elmer model 8500 GC, using Elite-5MS (5% phenyl methylpolysiloxane) capillary column of 30 m × 0.32 mm i.d., film 0.32 μm thick. Samples (0.5 μL) were injected in "split" mode (1:30) with a column temperature program of 40°C for 5 min, then increased to 260°C at 4°C/min and finally held at this last temperature for 10 min. Injector and detector were set at 250 and 280°C, respectively; the carrier gas was He with a head pressure of 12.0 psi. GC-MS analyses were carried out using a GC Model 6890 N, coupled to a bench top MS Agilent 5973 Network, equipped with the same capillary column in the same GC/FID chromatographic conditions. The carrier gas was He at a constant flow of 1.0 mL min. The sample (1.0 μL) was manually injected into the GC system with a split ratio of 30:1. The ion source temperature was set at 200°C, while the transfer line was at 300°C. The acquisition range was 40-500 amu in electron-impact (EI) positive ionization mode using an ionization voltage of 70 eV.

Identification and quantification of the volatile oil components: Identification of the volatile oil components was made from their retention indices (RI) and mass spectra, and by comparison with a NIST database mass spectral library [25], as well as with literature data [26-27]. Retention indices were calculated by Elite-5MS capillary columns using an *n*-alkane series (C_6-C_{35}) under the same GC conditions as for samples. The relative amount of each individual component of the oil was expressed as percent peak area relative to the total peak area from GC/FID analyses of the whole extracts.

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