Phytochemistry, Vol. 26, No. 6, pp. 1603–1606, 1987.
Printed in Great Britain.

0031-9422/87 $3.00+0.00

FLORAL FRAGRANCES IN *ACTAEA*, USING DIFFERENTIAL CHROMATOGRAMS TO DISCERN BETWEEN FLORAL AND VEGETATIVE VOLATILES

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(*Received* 29 *September* 1986)

Key Word Index—*Actaea*; *Ranunculaceae*; floral fragrance; plant volatiles; pollination; monoterpenes; gas chromatography-mass spectrometry.

Abstract—Floral fragrances in four species of *Actaea* (Ranunculaceae) and a hybrid were studied by GC–MS. They consist primarily of 10–15 closely related monoterpenes, and differ quantitatively between species. In order to separate the volatiles actually produced by the flower, parallel collections were made from inflorescence and vegetative parts, respectively, and the volatiles of the latter were subtracted from the inflorescence sample. This method proved particularly effective when tested with a single plant individual and a long collection period.

INTRODUCTION

Pollinator attraction in angiosperms rests primarily on visual and olfactory cues [1]. For visual cues, studies of the evolutionary patterns of floral pigments, such as anthocyanins [2] and other flavonoids [3, 4], have a long tradition. Meanwhile, the study of floral fragrance evolution has only become possible in recent years through advances in analytical techniques. This field is interesting, since olfactory cues play vital roles in the attraction of beetles [5], moths [6, 7] and bees [8]. It is widely believed that speciation can occur in, e.g. orchids as a result of single mutations changing the floral fragrance [9, 10], in a fashion unknown from species relying on visual cues.

It is therefore important to study the composition of fragrance in closely related species to look for patterns of radiation: what biogenetic pathways are utilized? What changes carry adaptive significance? Such questions are already under address for highly advanced tropical orchids [8], and have been the subject of an isolated study in the Magnoliaceae [11].

Here we present data on fragrance composition in an ancestral genus of herbs, and utilize a simple method allowing determination of the spatial origin within the plant of the collected volatiles. This is important, as we assume that compounds active in pollinator attraction should emanate precisely from the flower, rather than from the vegetative parts. The flower is a highly differentiated organ for the purpose of pollinator attraction and manipulation, and localization to vegetative parts of fragrance production not only removes precise spatial attraction of the pollinator by the flower but also requires differentiation to allow the exact temporal production patterns utilized in pollinator attraction. Vegetative parts, on the other hand, produce different volatile substances in deterrence against herbivores. It is unlikely that these should follow a cyclic pattern of production, or at least one similar to that of pollinator-attracting components, since the risk of herbivore damage only changes over longer periods of time. We thus expect to find relatively small quantities of a broad spectrum of deterrent substances collected around vegetative parts, while those around active inflorescences should contain high concentrations of true floral fragrances and small amounts of deterrents from the vegetative parts of the inflorescence.

In order to discern substances originating from floral and vegetative parts, respectively, volatiles from leaves and stems of several species were collected in parallel with fragrances from the inflorescences. The chromatograms of inflorescences and vegetative parts were compared, and compounds shared between the two were considered as emanating from vegetative parts. Exceptions were made when disproportionate amounts appeared in the inflorescence sample (compared to other shared compounds), in which case they were conservatively considered as produced in all plant parts. The applicability of this technique will be further discussed below.

The genus *Actaea* (Ranunculaceae) comprises four species, growing in temperate regions of the northern hemisphere [12]. All species have rosy or citrus-like fragrance, produced in minute staminodia. In the Eurasian *A. spicata* the fragrance modifies the close-range behaviour of its primary pollinator, the beetle *Byturus ochraceus* [13]. North American *A. pachypoda* and *A. rubra* are pollinated by a variety of beetles and small bees, presumably attracted by the fragrance [14]. The Eurasian *A. erythrocarpa*, previously described as a separate species, was shown to be synonomous with *A. rubra* [14]. Its fragrance was analysed from eastern Soviet Union specimens in 1984. In one of its westernmost outposts, however, it is pollinated by flies and all fragrance production has ceased [14]. *A. asiatica* grows in Japan and

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adjacent parts of continental Asia, and nothing is known about its pollinators. In addition to these, a spontaneous hybrid between *A. asiatica* and *A. rubra* was analysed.

RESULTS

Subtraction of volatiles from vegetative parts proved particularly successful in the analyses of *Actaea pachypoda*. Figure 1A shows the chromatograms of two parallel scent collections. With the exception of 10 substances, all other peaks were also present in the leaf sample. These peaks showed strong intra-sample proportionality between the two samples, with a few interesting exceptions: 6-methyl-5-hepten-2-one was over-represented in the flower + stems sample, and that compound was included in the differential chromatogram (Fig. 1B). A series of alkanes (marked with ↓ in the figure), ranging between 12 and 19 carbon atoms, were more or less strongly over-represented in the vegetative sample. Alkanes are typical components in leaf waxes [15], and are thus likely to be over-represented because of the very large leaf surface area in that sample. More prominent shared classes of substance in the leaf sample were common aldehydes, acetates, alcohols and the monoterpene pinenes.

The substracted floral fragrance chromatograms of the other species and hybrids gave the results shown in Table 1. The composition is qualitatively uniform in all members of the genus, with 10–15 monoterpenoids present in each species. In *A. asiatica* × *rubra* one sample differed from the other by having much less linalool and a large proportion of citronellyl acetate. *trans*-β-Ocimene was found to originate from vegetative parts in *A. pachypoda*, but this could not be conclusively shown in *A. rubra* and *A. spicata*. The situation with 6-methyl-5-hepten-2-one is also unclear in these cases.

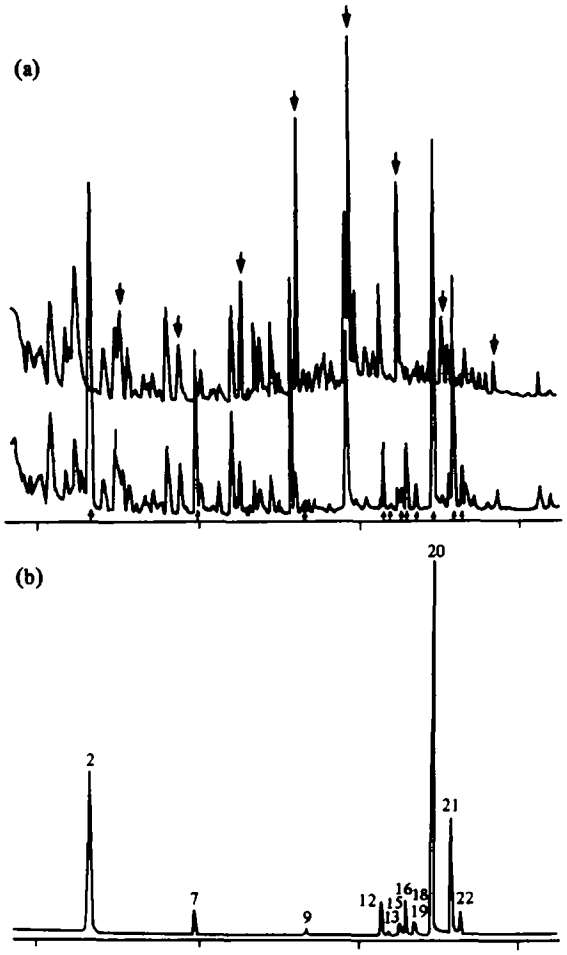

Fig. 1. (A) Gas chromatograms of parallel samples of *Actaea pachypoda* leaf + peduncle (above) and inflorescence (flowers, pedicels + peduncle) (below). Lower chromatogram has been magnified 2 × along the *y*-axis (highest peak cut at 50 %) to facilitate comparison between samples. Compounds that only exist in floral fragrance (marked with arrows in A: lower) are shown as the differential chromatogram in B, while disproportionately larger, shared substances in the leaf sample are marked with an arrow in A: upper. They are alkanes, and are believed to originate from leaf waxes.

DISCUSSION

The fragrance analyses show that the four species of *Actaea* and the hybrid have remarkably similar chemical composition. Composed of monoterpenes, a sesquiterpene, and the monoterpenoid 6-methyl-5-hepten-2-one, differences are mainly quantitative. It is interesting to notice that the rosy fragrance of, e.g. *A. rubra* sometimes appears to be more citrus-like (Pellmyr, unpublished data). It could well be that the proportions of the geraniol or nerol like substances fluctuate, and longitudinal analyses of single individuals are necessary to resolve this matter. As pointed out in a previous study [16], the substances that we here identify as florally produced are very closely related from a biogenetic point of view; they can be derived by a few synthetic steps from geraniol or nerol.

The subtraction technique allows a considerable improvement over the method used in previous analyses [16]. This is best shown in the analysis of *A. pachypoda*, where more than 80 compounds were present in the flower + stem sample, but subtraction allowed the conclusion that at most 12 originated from the floral staminodia. Similarly, we reported more than 40 substances from complex samples of *A. spicata* and *A. erythrocarpa* in our previous study [16]. We now show that only 16 originate in the flowers.

The effectiveness of the subtraction technique relies on the replicability of the analyses. It is excellent in the case of *A. pachypoda*, while in some of the others there are 'suspicious compounds' that we think originate from the vegetative parts, even though they failed to appear in the respective backgrounds. It seems likely, however, that the analytical method *per se* is accurate, and that the differences originated in the material analysed. First, cutting of the flowers may release intracellular substances and change the physiological status of the plants. Second, use of plant parts from several individuals can muddle the picture through individual variation. In the light of our results, it seems strongly advisable to collect fragrance samples from single individuals for a longer time than from several individuals for a shorter period. Also, collection should be made under as natural conditions as possible; collection at a natural growth site is probably best, while potted plants are superior to cut flowers.

While our discussion so far has dealt with refined

Table 1. Relative amounts of floral volatiles in analysed *Actaea* species and hybrids. tr = trace amount, indicated by selective ion monitoring

Taxon	*Rubra USA*	*Rubra Asia*	*Asiatica*	*Asiatica × rubra*	*Asiatica × rubra*	*Spicata*	*Pachypoda*
Mevalogenins							
Monoterpene hydrocarbons							
1. Δ^3-Carene	tr	0.2	tr	tr	< 0.1	3.0	0.9
2. Myrcene	44.7	13.9	7.5	4.6	4.2	23.9	33.5
4. Limonene	2.5	0.8	0.6	0.4	0.3	5.6	1.4
5. *cis*-β-Ocimene	tr	0.1	0.1	0.1	< 0.1	0.6	—
6. *trans*-β-Ocimene	4.1	3.6	1.2	0.7	0.1	12.2	—
Monoterpene alcohols							
9. Linalool	1.1*	6.0	32.8	33.6	0.3	2.1	0.4
19. Citronellol	—	—	5.3*	tr	18.4*	—	1.2*
20. Nerol	11.4	16.1	16.7	13.2	22.5	1.8	35.9
21. Geraniol	6.8	14.0	14.0	17.4	9.7	13.8	10.7
Monoterpene keto compounds							
12. Neral	0.8	—	0.2	0.1	0.4	2.9	3.1
16. Geranial	1.2	tr	0.4	0.4	0.4	9.2	2.8
22. Geranyl acetone	—	—	—	—	—	—	1.6
Monoterpene ether							
8. Linalool oxide I	—	0.2	0.2	0.2	< 0.1	—	—
Monoterpene esters							
11. Citronellyl acetate	0.3	1.5	1.7	0.7	19.0	—	—
13. Methyl geranate	tr	0.6	0.3	0.6	tr	0.6	0.2
15. Neryl acetate	1.7	3.8	6.6	5.4	15.2	—	1.4
18. Geranyl acetate	7.8	10.8	10.5*	18.1	9.2*	7.9	0.6*
Sesquiterpenes							
14. Bicyclic hydrocarbon	—	—	—	—	—	2.9	—
17. α-Farnesene	2.4	6.4	—	0.8	—	1.6	—
Acetogenins							
24. Nonadecane	—	5.7	0.1	0.2	tr	—	—
3. 3-Methyl-1-butanol	tr	2.0	tr	tr	—	—	—
7. 6-Methyl-5-heptern-2-one	0.8	0.4	0.1	0.3	0.1	11.7	6.6
Benzenoids							
10. Benzaldehyde	1.9*	—	—	—	tr	0.2	tr
23. Benzyl alcohol	12.6	13.4	1.8	3.1	0.1	tr	—

Pairs of compounds within species marked with * did not separate completely; quantities were estimated by integrating relative quantities of characteristic fragments. Substance numbers are the same as in Fig. 1.

spatial identification of fragrance production, the subtraction technique should also be applicable in temporal differentiation. As an example, Nilsson [7] analysed diurnal and nocturnal emission of volatiles from the moth-pollinated *Platanthera chlorantha* (Orchidaceae). Eleven compounds were found, and they were identical for both samples with the exception of strong emission of methyl benzoate and some monoterpenes during the night. It seems quite plausible that the dominant substance in day-time collection, caryophyllene, as well as the other substances with constant emission originated from the peduncular and pedicellar parts of the inflorescence.

A possible complication with subtraction would be that floral volatiles interact with vegetative-part volatiles in pollinator attraction. This is theoretically possible, but unlikely, because of lowered spatial definition of the odor source which reduces directability of the pollinators. In the case of *Actaea*, the geraniol or nerol type compounds are indeed responsible for behaviour modification of the pollinators [13], while in *P. chlorantha* the methyl benzoate is necessary to elicit search behaviour in potential pollinators [7].

EXPERIMENTAL

All species except *A. spicata* were analysed from specimens growing in the Botanical Garden in Uppsala. For *A. rubra* (6), *A. 'erythrocarpa'* (15), *A. asiatica* (3), and *A. asiatica × rubra* (2, 2, 4), inflorescences containing 10–20 flowers each were cut and put in vases. Numbers in parentheses give number of inflorescences for each trial. For *A. spicata* the same procedure was used, except that the 12 inflorescences used were brought in from a wild population on Öland (southern Sweden). In *A. pachypoda*, a potted specimen with a single inflorescence was analysed intact.

The inflorescences were placed in either a 1 l. E-flask (*erythrocarpa, spicata*) or in a polyacetate bag (others). Purified air was pumped over the flowers at 125 ml/min for *ca* 24 hr (53 hr for *A. pachypoda*), and existing volatiles were adsorbed on a Porapak Q 80–100 plug (150 mg). Each plug was subsequently rinsed with 2 ml of pentane (*spicata, erythrocarpa*) or ether (others).

Capillary GC–MS (Finnigan 4021) was used for separation and identification of the volatile compounds. A 25 m fused silica capillary column coated with Superox FA as stationary phase was used. Column temperature programming was: 60° for 4 min, then heated to 220° at a rate of 5°/min. See also [17] and [16].

Acknowledgments—We thank Dr. Örjan Nilsson, director of the Botanical Garden in Uppsala, for permission to collect floral fragrances from plant material. Financial support from the Swedish Natural Science Research Council is gratefully acknowledged.

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