

Why Does an Obligate Autogamous Orchid Produce Insect Attractants in Nectar? – A Case Study on *Epipactis Albensis* (Orchidaceae)

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Abstract

Background

The flowers of some species of orchids produce nectar as a reward for pollination, the process of transferring pollen from flower to flower. *Epipactis albensis* is an obligatory autogamous species, does not require the presence of insects for pollination, nevertheless, it has not lost the ability to produce nectar, the chemical composition of which we examined by gas chromatography-mass spectrometry (GC-MS) method for identification of potential insect attractants.

Results

During five years of field research, we did not observe any true pollinating insects visiting the flowers of this species, only accidental insects as ants and aphids. As a result of our studies we find that this self-pollinating orchid produces in nectar *inter alia* aliphatic saturated and unsaturated aldehydes such as i.e. nonanal (pelargonal) and 2-pentenal as well as aromatic ones (i.e. syringaldehyde, hyacinthin). The nectar is low in alkenes, which may explain the absence of pollinating insects. Moreover, vanillin and eugenol derivatives, well-known as important scent compounds were also identified, but the list of chemical compounds is much poorer compared with a closely related species, insect-pollinating *E. helleborine*.

Conclusion

Autogamy is a reproductive mechanism employed by many flowering plants, including the orchid genus *Epipactis*, as adaptation to grow in habitats where pollinating insects are rare observed due to the lack of nectar-producing plants they feed on. The production of numerous chemical attractants by self-pollinated *E. albensis* confirms the evolutionary secondary process, i.e. transition from ancestral insect-pollinating species to obligatory autogamous.

Background

Epipactis albensis Nováková et Rydlo was formally described as separate taxon in 1978 based on plants found in the flood plain forests adjoining the river Elbe in Central Bohemia [1]. Since that time, numerous localities of this species were reported from the Czech Republic [2], Slovakia [3], Romania, Austria, France, Germany, Poland [4, 5, 6] and Ukraine [7]. Two varieties (intraspecific taxa) are distinguished within the species, i.e. *Epipactis albensis* var. *albensis* and *E. albensis* var. *fibri* (Scappat. & Robatsch) P. Delforge [8]. Some researchers, based on morphological characters, distinguish a subspecies *Epipactis albensis* subsp. *lusatia* [9].

Epipactis albensis is an obligate autogamous taxon [6], which means that pollen is transferred to the stigma of the same flower, but without the involvement of pollinating insects. The gynostemium of this species does not produce viscidium, its rostellum is non-functional, clinandrium is reduced, anther sessile and pollinia are powdery [10, 11], therefore, theoretically it is not suitable for pollination by visiting insects. Interestingly, our observations showed that inflorescences of *E. albensis* are occasionally visited by insects searching for new food. Because the species does not produce viscidium, even when the flowers are penetrated by insects, the pollinia do not attach to their bodies and therefore remain inside the flowers. Nevertheless, this species produces floral nectar. This is surprising since orchids commonly use nectar to entice their pollinators.

The pollination biology, including chemical composition of the nectar of allogamous *Epipactis* species is well known [12–18], however, the composition of nectar produced by self-pollinating species has not been studied so far.

In this study, for the first time, we characterize the composition of the nectar produced by the autogamous *E. albensis*, and we try to explain why this taxon produces attractants and food for potential visiting insects while, in theory, it is not pollinated by them.

Results

Field observations

Observations carried out in field conditions confirmed nectar secretion between 9:00 a.m. and 6:00 p.m. in some specimens of *Epipactis albensis* (Figure 1A). We observed visible droplets of floral nectar that accumulated inside the basal portion of the labella named hypochile (Figure 1B).

Observation Of Insect Visitors

During the five-year observation, we did not find any insects that could be considered as true or accidental pollinators of *Epipactis albensis*. Importantly, we did not observe the activity of both diurnal and nocturnal insects visiting or pollinating this orchid in the studied habitats. Sporadically we observed accidental visitors as male and female mosquitoes (*Culex* sp.) feeding on nectar, as well as *Myrmica rubra* (Hymenoptera, Formicidae, Myrmicinae), (Figure 2A,B). Aphids were observed on seventeen plants (~ 4% of all plants) of *E. albensis* in all studied populations. These small insects feed on plant juices mainly from the orchids' upper part of shoots, feeding in colonies (Figure 2A).

Gc-ms Analyses Of Nectar Composition

The extract of *Epipactis albensis* labella contained total of 48 compounds, most of which were identified (Table 1, Fig. 3). The extract mainly consisted of long-chain hydrocarbons and fatty acid (c.a. 74% of

total amount). Among them heptacosane was the most prominent (21, Figure 3). Only one n-alkene, i.e. 1-nonacosene (24) was identified in the sample (2%, Table 1). Oxygen-containing compounds were less abundant accounting for only 15% of total amount of the nectar. Among them aromatic alcohols namely monosubstituted phenols, e.g. 4-ethylphenol and derivatives of benzyl alcohols such as vanillyl alcohol (15) and methoxyeugenol (16) were identified in greater amounts than the aliphatic ones (7% vs 0.7%). The extract also contained unbranched and branched aliphatic saturated aldehydes C5-C28 (5%) as well as aromatic ones (2%) e.g. hyacinthin (10). Detailed analysis of the spectrum indicated also various plant sterols, e.g. campesterol, stigmasterol, gamma-sitosterol etc. among discussed compounds (Fig. 3B). Phytosterols are a group of naturally occurring compounds found in plant cell membranes that can also modulate the activity of membrane-bound enzymes [19]. These compounds are also linked to plant adaptation to temperature and plant immunity against pathogens [20]. However due to their low importance as insect attractants their occurrence in the flowers has not been discussed. In the sample trans-phytol (*E*-3,7,11,15-tetramethyl-2-hexadecen-1-ol), a diterpene alcohol obtained from the degradation of chlorophyll was also identified.

Table 1
List of organic compounds identified in the nectar of *Epipactis albensis*.

	Compound	CAS Number	Relative amount (%)
Oxygen-containing compounds			
1	methyl isobutyl ketone (4-methyl-pentanone)	108-10-1	0.23
2	2-pentenal	1576-87-0	0.15
3	2,3-dimethylpentanal (2,3-dimethyl valeraldehyde)	32749-94-3	1.80
4	heptanal	111-71-7	0.43
5	nonanal (pelargonal)	124-19-6	0.16
6	octadecanal	638-66-4	1.66
7	heptacosanal	72934-03-3	0.44
8	octacosanal	22725-64-0	0.66
9	octacosanol	557-61-9	0.70
10	phenylacetaldehyde (hyacinthin)	122-78-1	1.33
11	4-methylphenol (<i>p</i> -cresol)	106-44-5	0.79
12	4-ethylphenol	123-07-9	5.09
13	4-hydroxybenzaldehyde	123-08-0 ₂	0.63
14	4-(hydroxymethyl)phenol	623-05-2	0.23
15	4-hydroxy-3-methoxybenzyl alcohol (vanillyl alcohol)	498-00-0	0.42
16	4-allyl-2-(methoxymethoxy)-phenol (methoxyeugenol)	6627-88-9	0.35
17	3,5-dimethoxy-4-hydroxybenzaldehyde (syringaldehyde)	134-96-3 ₃	0.22
Long-chain hydrocarbons and fatty acids			
18	tricosane	638-67-5	0.41
19	pentacosane	629-99-2	1.63

	Compound	CAS Number	Relative amount (%)
20	hexacosane	630-01-3	6.36
21	heptacosane	593-49-7	40.92
22	octacosane	630-02-4	8.06
23	nonacosane	630-03-5	13.98
24	1-nonacosene	18835-35-3	2.09
25	triacontane	638-68-6	0.82
26	palmitic acid (hexadecanoic acid)	57-10-3	0.47
Identified compounds			total amount: 90.03%
Unidentified compounds (14 substances)			total amount: 9.97%

The composition of the nectar of *E. albensis*, as compared to the insect-pollinated species of the genus, is poorer especially in long-chain carboxylic acids and its esters (Table 2).

Table 2

Selected chemical compounds identified in the nectar of autogamous *Epipactis albensis* and allogamous *Epipactis helleborine*. * Data based on [12, 14, 17].

Chemical compound	<i>Epipactis albensis</i>	<i>Epipactis helleborine</i> *
benzoic acid	-	+
benzylalcohol	-	+
pentadecanol	-	+
heptadecanol	-	+
eicosanol	-	+
2-pentenal	+	+
heptanal	+	+
nonanal	+	+
hexadecanal	-	+
octadecanal	+	+
nonadecanal	-	+
4-hydroxybenzaldehyde	+	+
phenylacetaldehyde	+	+
4-hydroxy-3-methoxy-benzaldehyde (vanillin)	-	+
4-hydroxy-3-methoxy-benzylalcohol (vanillyl alcohol)	+	+
2-methoxy-4-(2-propenyl)-phenol (eugenol)	-	+
2,6-dimethoxy-4-(2-propenyl)-phenol (methoxyeugenol)	+	+
4-methylphenol	+	+
2,6-dimethoxy-phenol (syringol)	-	+
3,5-dimethoxy-4-hydroxybenzaldehyde (syringaldehyde)	+	-
4-(hydroxymethyl)phenol	+	+
eicosane	-	+
heneicosane	-	+

Chemical compound	<i>Epipactis albensis</i>	<i>Epipactis helleborine*</i>
tricosane	+	+
pentacosane	+	+
hexacosane	+	+
heptacosane	+	+
octacosane	+	+
heneicosane	+	+
hexadecanoic acid	+	-
tetraeicosanoic acid	-	+
oleic acid	-	+
octadecenoic acid	-	+
pentadecenoic acid	-	+
heptadecenoic acid	-	+
hexadecenoic acid	-	+
eicosanoic acid methyl ester	-	+
tetracosanoic acid methyl ester	-	+
pentadecenoic acid methyl ester	-	+
hexadecenoic acid methyl ester	-	+

Discussion

The evolutionary shift from outcrossing to self-fertilization is one of the most frequent evolutionary transitions in plants [21]. As early as the nineteenth century, Darwin argued that outcrossed progeny of plants is usually more vigorous than those produced by self-fertilization [22, 23]. This observation led him to interpret many features of flowering plants as adaptations for outcrossing [24]. It is now believed that about 10-15% of flowering plants are predominantly self-fertilizing [21]. In flowering plants, autogamy has the disadvantage of producing low genetic diversity in the species that use it as the predominant mode of reproduction [25].

The self-pollination has also been found in other orchid genera, including *Cephalanthera* and *Epipactis* [26]. Autogamous species of the *Epipactis* genus arose secondarily as a result of adaptation to colonization of poor habitats in terms of the presence of potential pollinators. From a pollinator's perspective, a flower provides food, typically in the form of nectar and pollen. The production of nectar by

the self-pollinating *E. albensis*, with a chemical composition similar to the nectar of allogamous species, confirms that autogamy is a secondary process that is the adaptation of plants to the absence of pollinating insects in their habitats. This is exactly the situation we have observed in three surveyed populations. It is worth noting that in none of the populations we studied, we found no other associated species that produce nectar-rich or pollen-rich flowers. In dark habitats, environments poor in nectar-rich species of herbaceous plants, insects will not find food, which explains the lack of observation of flower visits. The studied plants do not produce *viscidium*, which distinguishes them from allogamic *Epipactis* taxa, but they have not yet blocked the attractant synthesis pathway, which may suggest that *E. albensis* is a relatively young species in its evolutionary history. This is also confirmed by phylogenetic research [11].

The chemical composition of *E. albensis* nectar confirms the assumption that autogamy in *Epipactis* is a secondary process. Thus, the differences in gynostemium structure that we observe in self-pollinating taxa evolved earlier than nectar synthesis blockade.

Our research has shown that *E. albensis* is also a species with very interesting relationships with visiting insects. We observed the male and female mosquitoes, though rarely, feed on nectar, aphid honeydew, and plant juices [27]. Observed by us on flowers of *E. albensis* representatives of Aphididae are not pollinators, they are connected with orchid vegetative sprouts because of the fact of feeding with their juices. Insects from this group, e.g. *Aphis ilicis* have been early observed on *E. helleborine* [13] and *Epipactis atrorubens* [17]. Similarly, the *Myrmica rubra* ants observed rarely transfer the pollen of *Epipactis* orchids, they readily collect honeydew from black aphids and feed on flower nectar [17].

In general, insect-pollinated plants communicate with their pollinators through a number of floral signals, such as unique flower shape, coloration and odour [28]. Floral scents are among the key signals in many plant-pollinator systems for attracting pollinators from both short and long distances [28]. If a plant is not pollinated by insects, as *E. albensis* we studied, why it is producing strong chemical attractants? It is possible, and it is an evolutionary legacy of ancestors. There is a noticeable similarity in the scent bouquets of the *E. albensis* and the *E. helleborine* studied earlier [12, 14, 16, 17] (Figure 2 and Table 2). The likeness of the scent profiles of both species certainly confirms that this two taxa are closely related. It should be noted, however, that the list of semiochemicals identified in the investigated nectar is much poorer compared to *E. helleborine* (Table 1). This might be explained that unlike *Epipactis* described here *E. helleborine* is well-known as insect-pollinated plant [12, 13, 15, 17, 18]. Our earlier studies have shown that the main pollinators of *E. helleborine* include representative of Hymenoptera, e.g. *Bombus* sp., *Apis mellifera*, *Vespa vulgaris* and *V. germanica* and well as Diptera, i.e. *Episyrphus balteatus* [13, 15, 17].

In the nectar sample of the *E. albensis* we mainly identified various long-chain alkanes and only one fatty acid (Table 1 and Figure 3). As for long-chain alkanes their presence in the sample is not surprising since these types of compounds are ubiquitous in the wax layer of the flower of various plants [29]. In *E. albensis* flowers, the inner part of the lip (hypochil) in which the nectar is produced has such a waxy layer. Similar alkanes were identified in the nectar's species we compare (Table 2). Some of them, i.e.

heneicosane and hexacosane were identified as Hymenoptera attractants, substances luring bumblebees (*Bombus* sp., Hymenoptera, Apidae). Interestingly, heneicosane is used as a pheromone by the queen or king termites in the species *Reticulitermes flavipes* [30]. These hydrocarbons (heneicosane and hexacosane) have also been found in *Serapias*, another genus of orchids [31, 32]. However, in contrast to the high number of fatty acids and its esters identified in the *E. helleborine* lack of these compounds in the sample could support the absence of pollinating insects. It's well known, that saturated and unsaturated fatty acids are strongly associated with the biosynthesis of alkenes with different double-bond position, the key components of pollinators' sex pheromones. [33].

Surprisingly, we found in flower extract of *E. albensis* long-chain aliphatic saturated aldehydes described in the literature as insects attractants, e.g. nonanal and octadecanal (Table 1). Peach-specific aldehyde nonanal (pelargonal) is considered an insect attractant or pheromone. This semiochemical attracts numerous species of butterflies, i.e. female oriental fruit moths, *Grapholita molesta* (Lepidoptera: Tortricidae) [34]. This compound is also attractive to the Africanised honey bee *Apis mellifera scutellata* (Hymenoptera, Apidae), as well as its lure species from different insects groups, order Formicidae, both the ant subfamilies Formicinae and Myrmicinae [35]. It is therefore possible that the activity of *Myrmica rubra* on *E. albensis* we observed was due to the insects reaction to this compound and/or results of synergistic effect of others chemicals. Octadecanal is another example of semiochemicals which attracts different species of an ants, e.g. *Apterostigma pilosum* (Myrmicinae, Attini) [36] *Polyergus rufescens* (Formicinae, Formicini) [37], or *Cerapachys jacobsoni* (Cerapachyinae, Cerapachyini) [38]. This chemical compound is also a potential attractant for, e.g. *Psithyrus vestalis* (Apinae, Bombini) [39] and *Hypotrigena ruspollii* (Meliponinae, Meliponini) [40]. Interestingly, the representatives of Lepidoptera also react to it, e.g. *Andraca bipunctata* (Bombycidae, Oberthuerinae) [41], *Earias vittella* (Noctuidae, Chloephorinae [42], *Cerconota anonella* (Oecophoridae, Stenomatinae) [43], *Heliconius melpomene plesseni* (Nymphalidae, Heliconiinae, Heliconiini) [44], or *Manduca sexta* (Sphingidae, Sphinginae, Sphingini) [45].

Another important compound identified in the nectar of *E. albensis* is 2-pentenal. We previously identified both this compound and its derivatives, e.g. 2-pentanol in *E. helleborine* (Jakubska-Busse, unpublished data). This semiochemical is attractive to, among others, for jewel bugs *Chrysocoris stollii* (Heteroptera, Scutelleridae, Scutellerinae) [46] and the scuttle fly genus *Megaselia* (Diptera, Phoridae, Metopiinae, Metopinini) [47].

E. albensis also produces phenylacetaldehyde, an aroma organic compound known as hyacinthin or benzeneacetaldehyde. This semiochemical was identified in the nectar of *E. helleborine* and is emitted by other orchids, such as moth-pollinated *Gymnadenia odoratissima* [48]. The aroma of this pure compound can be characterized as honey-like, sweet, rose, green and grassy and it is widely used in perfumery as fragrance to impart hyacinth, daffodil, or rose nuances [49]. It is notable for being a floral attractant for numerous species of Lepidoptera from e.g. orders Noctuidae, Sphingidae, Geometridae, Danaidae, Crambidae or Pyralidae [35, 50]. Hyacinthin is also an attractant of other insect groups, including e.g. the western honey bee *Apis mellifera* (Hymenoptera, Apidae) [51] or a species of march fly

Plecia nearctica (Diptera, Bibionidae) [52]. Although the plants of *E. albensis* produce hyacinthin, we were unable to observe any moths visiting the flowers, even those active at night. Interestingly, this compound is also attractive to Formicidae (Hymenoptera), which may explain our observation of ants visiting flowers of *E. albensis*.

Another compounds worth mentioning are vanillin and syringaldehyde, well-known attractants for many different groups of insects, e.g. different species of bark beetles of the genus *Scolytus* (Coleoptera, Scolytidae), i.e. *Scolytus multistriatus* [53], as well as for a species of leaf-footed bugs *Leptoglossus phyllopus* (Heteroptera, Coreidae) [54] and/or for neotropical butterfly *Heliconius melpomene rosina* (Lepidoptera, Nymphalidae) [55]. The presence of numerous vanillin and eugenol derivatives in flower nectar was previously found in other insect-pollinated *Epipactis* species [12, 14, 16, 17]. Eugenol is a widespread and important scent compound, which has also been identified in floral scent emitted by another orchids, i.e. *Cypripedium calceolus* [56] and/or *Gymnadenia* species [57]. Although we couldn't identify vanillin in the nectar of *E. albensis*, the presence of vanillyl alcohol and 4-hydroxybenzaldehyde, compounds considered as intermediates of vanillin biosynthetic pathway [58], might confirm the ability of this species to synthesize vanillin.

An interesting question that have so far remained unresolved is can *E. albensis* become an insect-pollinated species in the situation of changes in their habitats, which will result in the appearance of pollinating insects? If the production of nectar or its chemical composition remains unchanged or the process of its secretion is not blocked, theoretically such a variant is also possible. Independently, there would also have to be an evolutionary change in the structure of the gynostemium resulting in the production of viscidium, a sticky pad in the flowers of insects pollinated orchids, which adheres to the pollinator when contact is made.

Conclusions

To conclude, the evolution of plant breeding systems is particularly evident at the individual level as the adaptation to changes in environmental conditions [59, 60]. Our research unquestionably confirmed that lack of pollinators in habitats can stimulate a transition to autonomous selfing as reproductive assurance [61, 62]. The production of numerous chemical attractants by self-pollinated *E. albensis* confirms the evolutionary transition from ancestral insect-pollinating species to obligatory autogamous. From an evolutionary perspective, it is likely that the next step in the evolution of *E. albensis* will be to gradually block the synthesis of substances luring insects, since the production of insect attractants, which are not crucial for pollination biology of this orchid, is redundant.

Methods

Plant materials

Fragments of ca. 300 fresh flowers with visible nectar secretion (hypochile) of *Epipactis albensis* used for the chemical analyses, were collected from three natural populations, i.e. 30 individuals of the populations located in the vicinity of Guzice near Polkowice, ca. 30 individuals from Wałkowa near Milicz (Lower Silesia, SW Poland), and from 32 individuals from Siechnice near Wrocław, between 24 July 2017 and 14 August 2021. Field observations and material sampling done with permission nos. WPN.6205.134.2017.IL, WPN.6400.33.2018.IL, WPN.6400.29.2019.AR, WPN.6205.87.2019.AR, WPN.6400.24.2020.MH, WPN.6205.113.2021.MR, and WPN.6400.20.2021.MR from the Regional Directors for Environmental Protection.

Field Observations Of Insects Activity

The observations were conducted during the peak of plant flowering period from 24 July to 17 August 2017-2020 and from 29 July to 13 August 2021 in the above mentioned populations, located in Lower Silesia (SW Poland). Due to the fact that *E. albensis* is a legally protected species in Poland and publishing its natural population coordinates of Global Positioning System (GPS) is an inappropriate protective procedure, we do not provide the exact location of the research sites. GPS coordinates are available from the authors upon request. The observations were carried out independently using two methods, i.e. a four cameras and direct observations were made over a span of 2-6 h per plant or the group of plants, covering daylight hours (9:00 a.m. - 6:00 p.m.). The visitors insects were photographed/documentated using a Nikon D50 camera with a Tamron 90 mm f/2.8 SP Di Macro lens, captured in the field conditions by AJ-B and identified by specialists. For recording night active visitors we used a four digital video cameras Sony HDR-CX450, equipped with a set of SD cards and batteries. In total, 728 h of directed observations were conducted.

Gc/ms Analyses Of Nectar Composition

Prepared samples of the basal part of the *E. albensis* labella (hypochile) containing nectar (0.22 g) were collected into the 2 mL glass vial followed by adding of 0.5 mL of dichloromethane (Sigma-Aldrich, 99.9%) at room temperature. The dichloromethane was used to extract foliar nectar drop. The extract was stored at -20°C until used for GC-MS analyses. GC-MS chromatography was performed on GCMS-QP2010SE SHIMADZU equipped with a mass selective detector (MS scan 17-550 m/z) and Zebron ZB-5ms (30 m 0,25 mm; Phenomenex) column operated at 40°C for 3 min, followed by heating to 320°C at rate of 30°C/min and then at 320°C for 7.7 min. Helium was used as a carrier gas.

Identification of the compounds was carried out using the NIST17 database. For identification of long-chain hydrocarbons, samples of C₉-C₂₄ and C₂₂-C₃₈ alkanes were analyzed by GC-MS using the same oven and column parameters and their spectra were compared with those obtained in the extract (Fig. 4-5). Relative amounts of compounds (%) in the sample were calculated from the MS detector response.

Abbreviations

Declarations

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Authors' contributions: AJ-B, IC, MJK and MH designed the study. AJ-B, IC, MJK and MH performed the experiment. AJ-B conducted a field study. AJ-B and IC wrote the first draft of the manuscript. AJ-B prepared the figures. AJ-B will handle correspondence at all stages of refereeing and publication, also post-publication. All authors contributed to the manuscript drafts and approved the manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Ethics approval and consent to participate

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Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

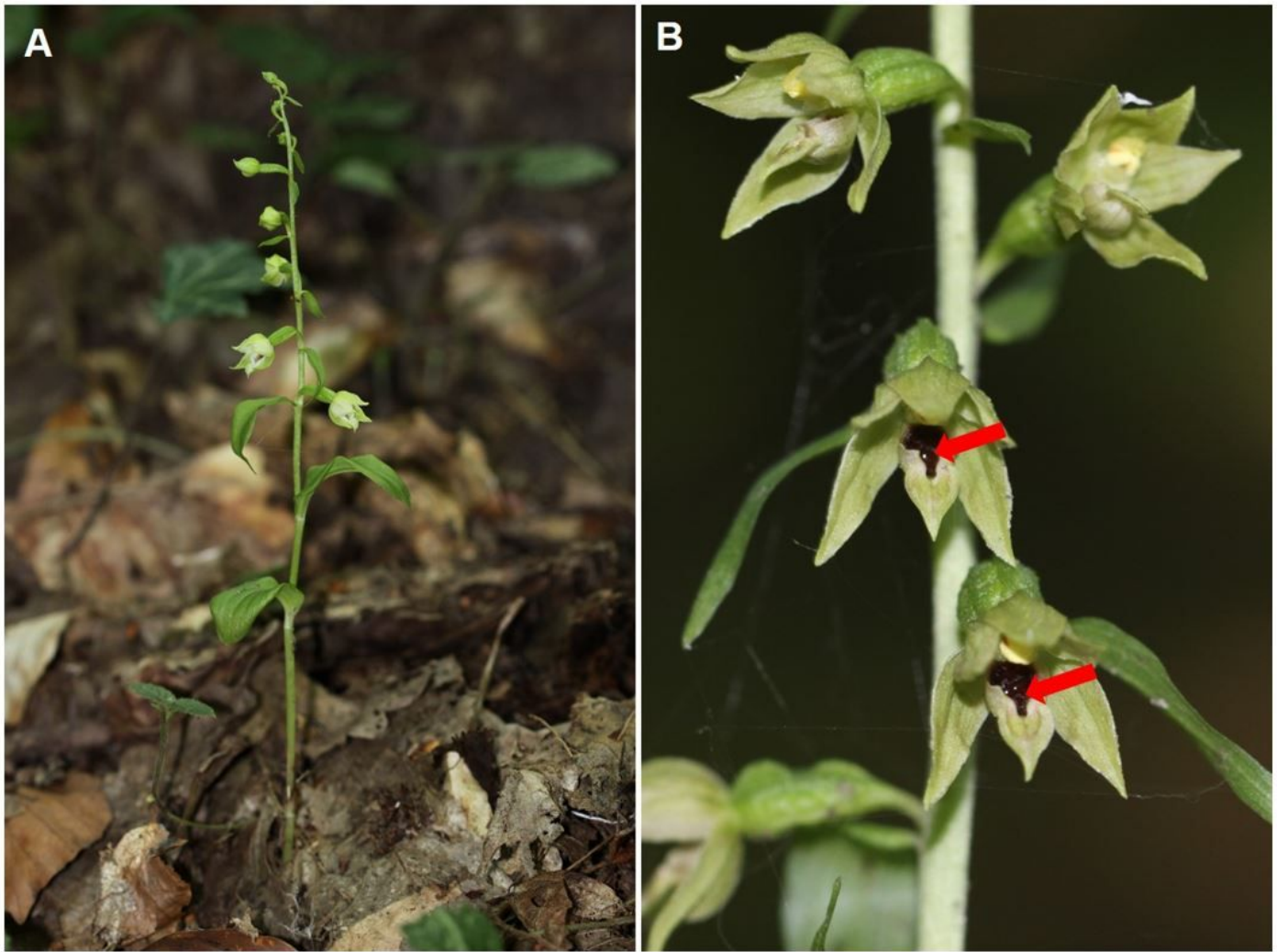


Figure 1

An obligate autogamous species. *Epipactis albensis*. (A) habit of plant; (B) details of flower secreted nectar. Hypochile (part of the lip) with nectar are marked with a red arrows.



Figure 2

(A) Workers of red ants (*Myrmica rubra*) tending black aphids. Above-ground aphid colonies producing honeydew very rich in sugar; (B) Visitor insects, *Myrmica rubra* (Hymenoptera) feeding nectar produced by *Epipactis albensis*, Guzice (SW Poland).

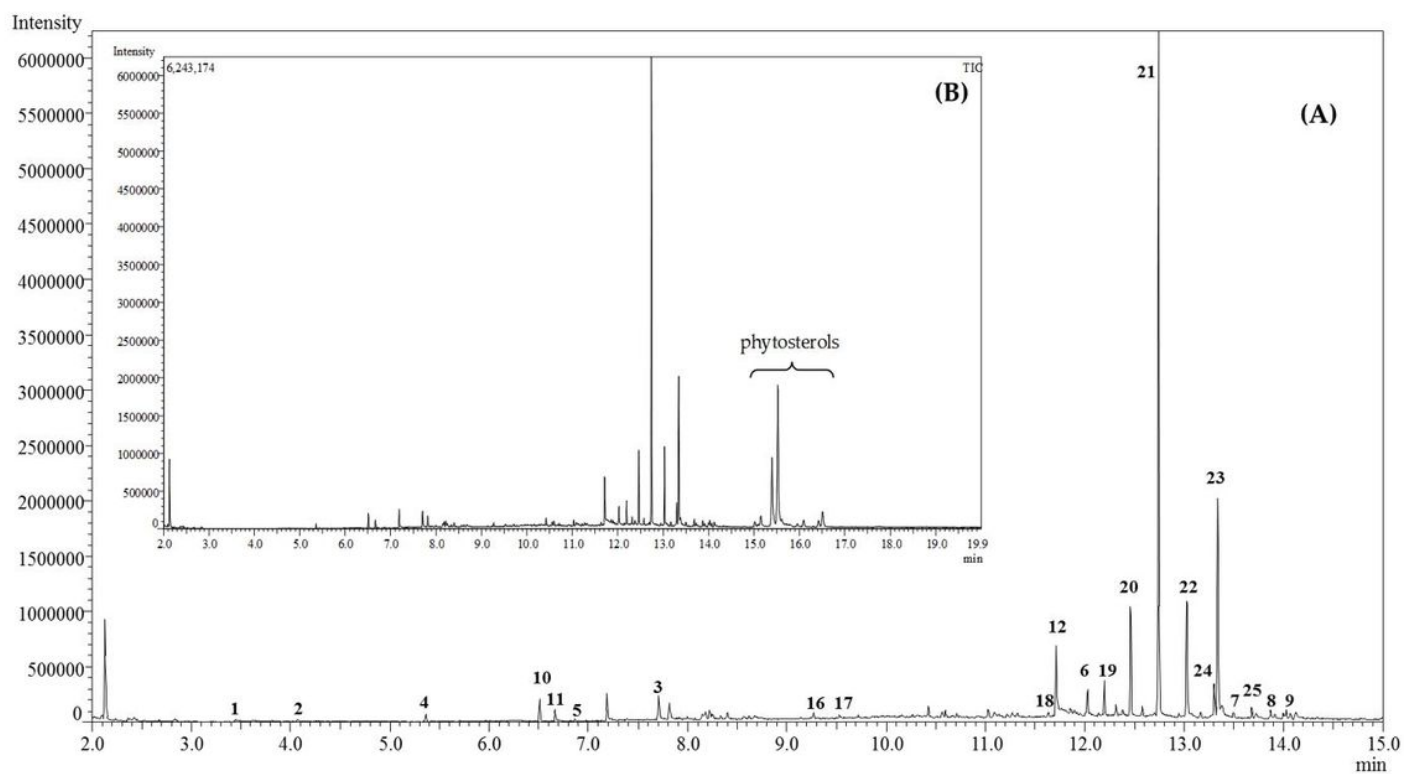


Figure 3

(A) Fragment and B, the full range of GC/MS chromatogram of *Epipactis albensis* flowers extract (dichloromethane solution). The peaks of selected compounds were numbered according to the Table 1.

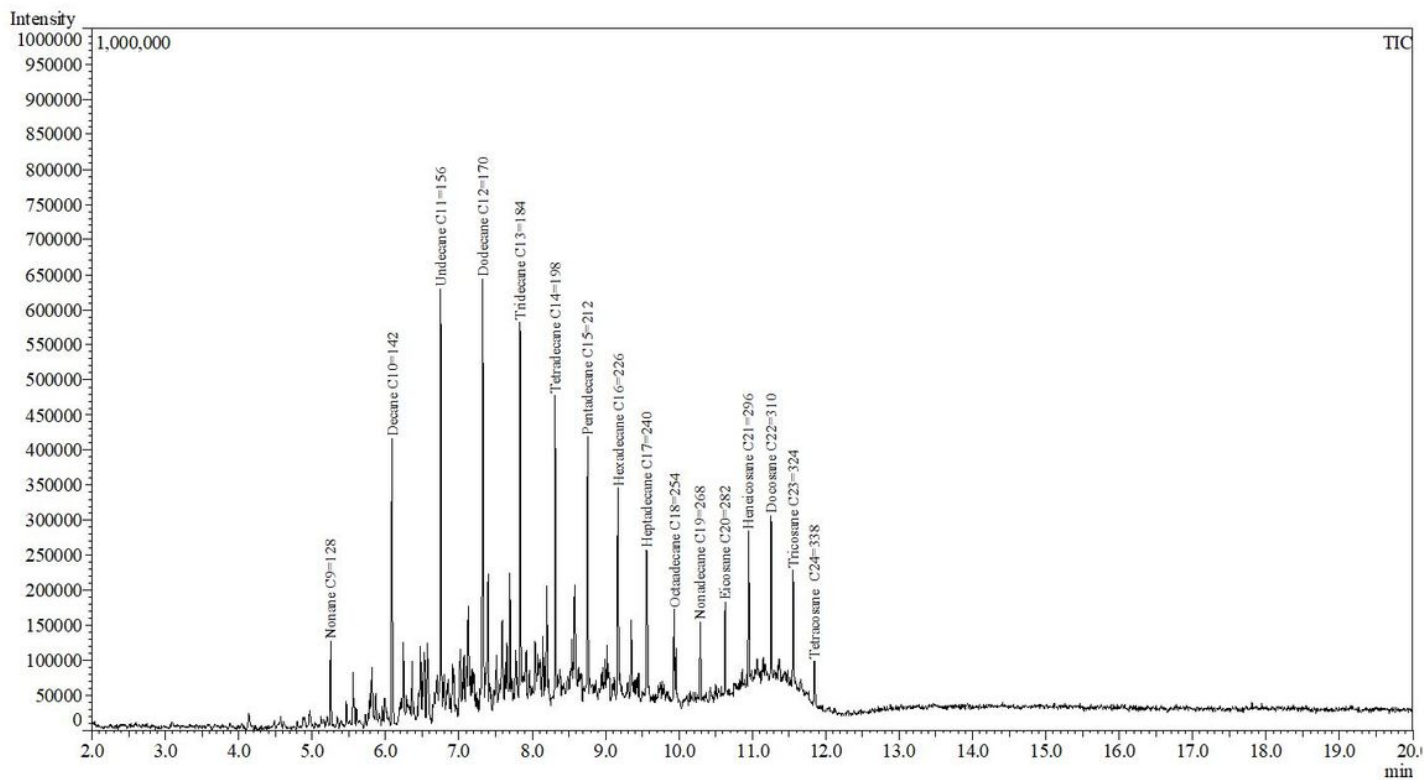


Figure 4

GC-MS chromatogram of sample containing C9-C24 hydrocarbons.

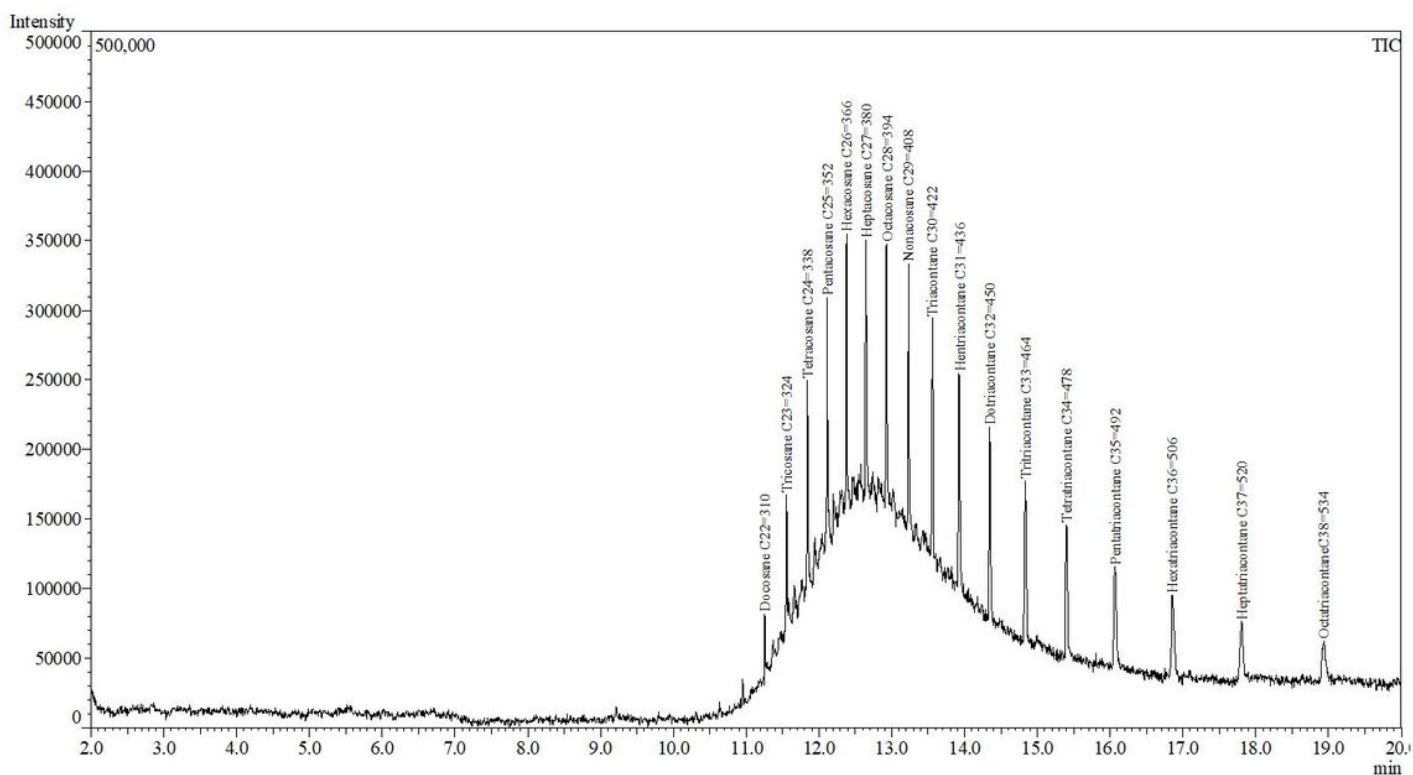


Figure 5

GC-MS chromatogram of sample containing C22-C38 hydrocarbons.