Pollination ecology of *Epipactis helleborine* (L.) Crantz (Orchidaceae, Neottieae) in the south-western Poland

Ekologia zapylania *Epipactis helleborine* (L.) Crantz (Orchidaceae, Neottieae) w południowo-zachodniej Polsce

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ABSTRACT: Pollination ecology of eight populations of *Epipactis helleborine* (L.) Crantz (Orchidaceae, Neottieae) occurring in Lower Silesia in the south-western Poland has been studied. Plants were examined in respect to composition of their nectar and its influence on attracting insects under field conditions. Chemical composition of *Epipactis helleborine* nectar was studied by means of GC/MS SIM. Pollinators and visitors were recorded in natural populations.

KEY WORDS: *Epipactis helleborine*, Orchidaceae, pollinators, vectors, Hymenoptera, Coleoptera, Diptera, nectar composition

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Introduction

There is little detailed information on the pollinating biology of *Epipactis* Zinn (Darwin 1877, Knuth 1909, Proctor & Yeo 1973, Ivri & Dafni 1977, Müller 1988, Ehlers & Olesen 1997), although it is one of the very few orchids occurring commonly both in Europe and in Asia. One species is known to originate form Americas and another one from Africa (Delforge 1994).

In Poland there are 6 species of this genera but only *Epipactis helleborine* (L.) Crantz is not an endangered species. It can be found all around the country in various phytocoenoses, mainly in deciduous and mixed forests. They may be seen on peatbogs, meadows and dunes, as well as highly anthropogenic habitats (Czylok & Rahmonow 1996, Tokarska-Guzik 1996, Jakubska 2003).

The aim of the observations was to define insects which are typical or potential vectors for the pollen of *E. helleborine*, and also an attempt to examine chemical compounds of the nectar.

1. Materials and methods

Eight populations of *Epipactis helleborine* (L.) Crantz growing under varied habitat conditions in the area of Lower Silesia (south-western Poland) were chosen. The studies were carried out at the sites of: Srebrna Góra, Zakrzów, Kotowice, Siechnice, Czernica, Krowiarki Mts., The Stolowe Mts. National Park (Karłów) and Wrocław (Park Zachodni). The size of the populations examined averaged from 69–428 individuals.

The research was conducted during the years 2001–2005, observations were made from 11:00 a.m.–3:00 p.m., from 15 July to 28 August.

The pollinators and visitors insects were captured in the field conditions by authors and identified by specialists.

1.1. Plant materials

The observations were conducted in under field conditions during the peak of flowering period. The herbarium material was deposited in the Department of Biodiversity & Plant Cover Protection, University of Wrocław.

1.2. Chemical analyses

Nectar was collected during the period of plant's maximal secretion, namely between 11 a.m. and 3 p.m. Composition of the nectar was fairly stable amongst the eight populations.

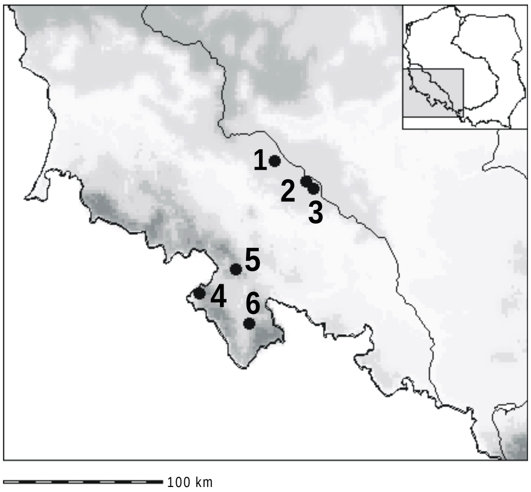

Fig. 1. Sampling sites in Lower Silesia (Ryc. 1. Stanowiska badań na Dolnym Śląsku): 1 – Wrocław (Park Zachodni/Western Park); 2 – Zakrzów, Kotowice, Czernica; 3 – Siechnice; 4 – Srebrna Góra; 5 – Karłów (The Stolowe Mts. National Park); 6 – Krowiarki Mts.

Nectar was collected from flowers by using capillary and methylene chloride as eluent. Then the methylene chloride extract was dried over anhydrous Na_2SO_4 and evaporated up to volume 0.2 ml followed by analysis GC-FID and GC-MS. This sample of extract was injected into ELWRO N504 gas chromatograph equipped with flame ionization detector - FID.

The instrumental parameters and operational conditions were as follows:

Fused silica capillary column (60 m × 0,25 mm i.d.), a film thickness of 0.25 μm with a temperature program: 50 °C to 300 °C at rate of 3 °C/min, with nitrogen as carrier gas (about 1.2 cm^3/min).

Each analysis was performed in triplicate to assess the reproducibility of the result. In order to define the components present in the sample, extract was analysed by means of Hewlett Packard 5973 GC-MS system.

2. Results

2.1. Insects reactions

Despite the large amount of published works about the biology of the pollinating biology of orchids (Tremblay 1992, Johnson 1997, Luo & Chen 1999, Singer & Sazima 1999, Borba & Semir 2001, Singer & Koehler 2003, Jacquemyn et al. 2005), we still lack detailed data concerning the genera of *Epipactis*. During our research we tried to observe and recognize the entomofauna occurring in these plants.

In the literature data a numerous classifications of visitors are proposed (Obarski 1961, Wojtusiakowa 1969, Grinfeld 1978, Iwan 1988).

We divided the Arthropods observed during the study into 4 groups:

1. pollinators, which are also called typical vectors,
2. visitors (which because of body size might also be a facultative/accidental vector = potential vector),
3. invertebrates visiting orchids, which stay in the relationship with other grups occurring on this plants: Heteroptera, Hymenoptera: Parasitica (Ichneumonidae), Aculeata (Formicidae) and spiders,
4. insects connected with orchid (with vegetative sprouts) because of the fact of feeding with their juices. Aphididae (e.g. *Aphis ilicis* Kaltenbach, 1843) and Homoptera belong to this group.

The most interesting were insects from first two groups: typical vectors and potential vectors. Within first group the most numerous were Hymenoptera (Aculeata: Apidae, Vespidae) and Coleoptera (Coccinellidae). The main part of second group creates Coleoptera (Lagriidae, Cantharidae) and Diptera (Syrphidae).

Fig. 2. a-e. (right)
a) *Vespula* sp. (Hymenoptera) with a pollinium packet on the head. Scale bar = 10 mm (Phot. A. Jakubska);
b) *Myrmica ruginodis* Nylander,1846 (Hymenoptera) after consumption of honey-dew. Scale bar = 10 mm (Phot. A. Jakubska);
c) *Episyrphus balteatus* (Diptera). Scale bar = 10 mm (Phot. A. Jakubska);
d) *Coccinella septempunctata* Linnaeus, 1758 (Coleoptera) on a flower of *Epipactis helleborine*. Scale bar = 7 mm (Phot. A. Jakubska);
e) *Coccinella septempunctata* Linnaeus, 1758 (Coleoptera) with a pollinium packet on the pronotum. Scale bar = 5 mm (Phot. A. Jakubska)

Ryc. 2. a-e. (po prawej)
a) *Vespula* sp. (Hymenoptera) z pakietem pyłkowin na głowie. Skala = 10 mm (Fot. A. Jakubska);
b) *Myrmica ruginodis* Nylander, 1846 (Hymenoptera) w trakcie spożywania nektaru. Skala = 10 mm (Fot. A. Jakubska);
c) *Episyrphus balteatus* (Diptera). Skala = 10 mm (Fot. A. Jakubska);
d) *Coccinella septempunctata* Linnaeus, 1758 (Coleoptera) na kwiecie *Epipactis helleborine.* Skala = 7 mm (Fot. A. Jakubska);
e) *Coccinella septempunctata* Linnaeus, 1758 (Coleoptera) z pakietem pyłkowin na przedpleczu. Skala = 5 mm (Fot. A. Jakubska)

2.2. Chemical analyses

Chemical research revealed presence of over 100 chemical compounds in the nectar of *Epipactis helleborine*. To main attractants contained in the nectar one should include: eugenol, 2,6-dimethoxy-4-(2-propenyl)phenol (methoxyeugenol), ethanol and 4-hydroxy-3-methoxybenzaldehyde (vanillin) (Jakubska et al. 2005).

The presence of many alcohols was identified in the nectar i.e. ethanol, 2,2-diethoxyethanol, methanol, 2-hydroxy-benzenmethanol, 4-hydroxy-benzenmethanol and pentadecanol, heptadecanol, eicosanol as well as benzyl alcohol (Jakubska et al. 2005).

The research of the nectar's composition revealed the presence of substances which might be responsible for the characteristic smell of *E. helleborine*: eicosanoic acid methyl ester, tetracosanoic acid methyl ester, pentadecenoic acid methyl ester, hexadecenoic acid methyl ester and vanillin (Jakubska et al. 2005).

The substances with a potentially narcotic and intoxicating influence were identified e.g. 3-{2-{3-{3-(benzyloxy)propyl}-3-indol, 7,8-didehydro-4,5-epoxy-3,6-d-morphinan and their derivatives. These compounds might be responsible for the characteristic behavior of the visitors known to the literature as „drunken insects" or „sluggish pollinators" (e.g. Ehlers & Olesen 1997).

3. Discussion

Site investigations confirm the suppositions that *Epipactis* lures insects mainly with it's odour. It is considered that morphology of flowers has got a small meaning. *E. helleborine* belongs to species which are morphologically very changeable, both when considering the colour of the flowers and the shape of petals and sepals (Jakubska 2003). The initial results of the observations confirm that attendance of the insects on the light green, white-yellow, light pink and dark pink flowers is similar which suggest at rather small meaning of flowers' colour in the process of luring potential pollinators of this species.

It is possible that eugenol derivatives and vanillin, as well or carboxylic acid esters discovered by us in the nectar of *Epipactis*, have a meaning in the process of luring the insects. One also cannot exclude the synergetic effect of these compounds. These compounds are described in the literature as attractants (Wu & Chu 1990, Harborne 1993, Shelly 2001, Fokialakis et al. 2002).

Separate matter is the presence of ethanol. According to us this compound is formed not only as a result of the metabolism of *Candida* sp., *Aspergilus* sp. and *Cladosporium* sp. as it is suggested by Ehlers & Olesen (1997), but

Table 1. A list of the Arthropods observed on *Epipactis helleborine* (L.) Crantz
Tabela 1. Lista stawonogów obserwowanych na *Epipactis helleborine* (L.) Crantz

Order/Suborder	Family/ Subfamily/ Group	Species	Locality/UTM
ARENEAE	Thomisidae	*Xysticus* sp.	Krowiarki Mts.
	Linyphiidae	*Linyphia* sp.	Krowiarki Mts.
HEMIPTERA/ HOMOPTERA	Aphididae	*Aphis ilicis*[1] Kaltenbach, 1843	Zakrzów (XS37), Kotowice (XS37), Karłów (WR99), Siechnice (XS55), Krowiarki Mts.
COLEOPTERA	Anaspididae	*Anaspis frontalis* (Linnaeus, 1758)	Zakrzów (XS37), Kotowice (XS37), Karłów (WR99), Siechnice (XS55)
	Cantharidae	*Rhagonycha fulva* (Scopoli, 1763)	Zakrzów (XS37), Karłów (WR99)
	Coccinellidae	*Coccinella septempunctata** Linnaeus, 1758	Zakrzów (XS37), Kotowice (XS37), Siechnice (XS55), Karłów (WR99), Srebrna Góra (XS10)
		Propylea quatuordecimpunctata (Linnaeus, 1758)	Zakrzów (XS37), Kotowice (XS37), Siechnice (XS55), Karłów (WR99)
	Nitidulidae	*Meligethes aeneus* (Fabricius, 1775)	Park Zachodni-Wrocław (XS47), Karłów (WR99)
	Lagriidae	*Lagria hirta* (Linnaeus, 1758)	Park Zachodni-Wrocław (XS47)
	Malachiidae	*Malachius bipustulatus* (Linnaeus, 1758)	Karłów (WR99)

HYMENOPTERA	Parasitica	Ichneumonidae	*Ichneumon* sp.	Karłów (WR99)
	Aculeata	Formicidae	*Myrmica ruginodis* Nylander, 1846	Zakrzów (XS37), Karłów (WR99)
		Vespidae	*Vespula germanica** (Fabricius, 1793)	Karłów (WR99)
			*Vespula vulgaris** (Linnaeus, 1758)	Karłów (WR99)
			Polistes gallicus (Linnaues, 1767)	Zakrzów (XS37)
			Colletes daviesanus Smith, 1846	Karłów (WR99)
		Apiformes	*Apis mellifera* Linnaeus, 1758	Zakrzów (XS37), Kotowice (XS37), Siechnice (XS55)
			*Psithyrus bohemicus** (Seidl, 1837)	Karłów (WR99)
			Lasioglossum fratellum (Pérez, 1903)	Karłów (WR99)
			Bombus hortorum (Linnaeus, 1761)	Zakrzów (XS37), Kotowice (XS37), Siechnice (XS55), Czernica (XS37)
			*Bombus hypnorum** (Linnaeus, 1758)	Karłów (WR99)
LEPIDOPTERA		Arctiidae/ Syntominae	*Amata phegea* (Linnaeus, 1758)	Zakrzów (XS37), Kotowice (XS37), Siechnice (XS55)
DIPTERA		Syrphidae	*Episyrphus balteatus* (De Geer, 1776)	Zakrzów (XS37), Kotowice (XS37), Siechnice (XS55)
		Mycetophilidae	*Asindulum* sp.	Karłów (WR99)

[1] The species is connected with orchids. However, it is little known about its distribution (SZELEGIEWICZ 1968).
[1] Gatunek związany ze storczykami, jednakże o słabo zbadanym rozmieszczeniu (SZELEGIEWICZ 1968).
* insects, on which pollinia have been found
* owady, na których znajdowano pakiety pyłkowin

it is very probable that the chemical decomposition of some of the compounds present in nectar might be also a source of ethanol and its derivatives (Jakubska et al. 2005).

According to Løjtnant (1974), Müller (1988), Ehlers & Olesen (1997) ethanol is formed as a result of the fermentation process of i.e. e.g. *Candida* sp. These microorganisms get to the lip together with pollinators and visitors. The nectar of *Epipactis* as a very rich source of sugars, e.g. sucrose, glucose and fructose indispensable for this reaction, but according to our observations under natural conditions fermentation process occurs rather relatively rarely, which can result from the presence of compounds identified by us which have strong antimicrobal properties e.g. furfural and syringol, which block the growing process of the yeast (Jakubska et al. 2005).

Chemical compounds revealed during the research, with strong narcotic properties, such as morphine and indol derivatives might be responsible for characteristic reactions of invertebrates visiting the flowers of Broad-leaved Helleborine, known to the literature as "sluggish pollinators effect" (e.g. Ehlers & Olesen 1997).

We have observed insects reactions after visiting the flower.

The effects of the nectar's functioning depend of course on the amount of time spent on the orchid and surely on the size of the insect's body. For the insects with small body size usually a strong slow down of reactions is being observed including the standstill state e.g. *Lagria hirta* (Linnaeus, 1758) and *Coccinella septenpunctata* Linnaeus, 1758. Large insects such as eg. *Bombus* sp., *Vespula* sp. have shown only small disorders in the transection of the flight.

What is interesting, we were not noticed symptoms of aggression in the insects examined when the „narcotic compound" stopped working, which was in contrast to Lawson Tait (Dyakowski 1894).

After analysing available articles concerning the pollinating biology of *Epipactis helleborine*, one can have an impression that only Vespidae (Darwin 1877, Knuth 1909, Proctor & Yeo 1973, Ehlers & Olesen 1997) and Syrphidae (Ivri & Dafni 1977) visit them.

On the research areas, among visitors which are at the same time pollinators *Vespula vulgaris* (Linnaeus, 1758), Fig. 2.a, strongly prevails, *Vespula germanica* (Fabricius, 1793) is found much more rarely.

Within insects most of the observed visitors is *Episyrphus balteatus* (De Geer, 1776), Fig. 2.c, whose presence was confirmed in all examined standings. Site investigations point out that it is the species that feeds with i.e. nectar produced by *Epipactis*. It is difficult to conclude identify if it is a species which pollinates the orchids. It is very possible in a context of literature data, which confirm large importance of Diptera in pollination biology of orchids (Borba & Semir 2001).

One should stress that in the pollinating biology of the *Epipactis helleborine* the species of great importance are species within Coleoptera included to antophiles (Iwan 1988) e.g.: *Anaspis frontalis* (Linnaeus, 1758)*, Meligethes aeneus* (Fabricius, 1775)*, Malachius bipustulatus* (Linnaeus, 1758) amongst of them, in respect of body size, especially *Rhagonycha fulva* (Scopoli, 1763)*, Coccinella septempunctata* Linnaeus, 1758 and *Propylea quatuordecimpunctata* (Linnaeus, 1758).

Quite an interesting observation was revealing of the presence of the ants on the flowers of *Epipactis* (Fig. 2.b). Their occurrence is thought to be connected with the presence of aphis (green-fly), whose honey-dew is food for ants. We also observed ants drinking nectar, which confirms observations of the former authors (Blüthgen et al. 2003, Davidson et al. 2003, Blüthgen & Fiedler 2004). Nectar of *Epipactis* is a rich source of aminoacids and carbohydrates, which are essential for the development of the animals.

Insects which, in our opinion, together with Hymenoptera can play a vital role in pollination biology of *Epipactis* are ladybirds. *Coccinella* is the common genus which occurs on Lower Silesia. We observed pollinia stuck to the body of these insects after visiting the inflorescence (Fig. 2.e). Coccinellidae are very often found on the flowers of *Epipactis helleborine*, which points at their potentially great importance in the process of exchange and shifting of the pollen. The presence of Coccinellidae should not be connected only with the plant louses which occur often on the vegetative sprouts of the orchids. Ladybirds were present numerously on the plants without plant louses.

Our observations confirm that flowers of *Epipactis* are very often visited by insect. Which suggest tendency to move towards selfpollination observed among some autogamous species within *Epipactis* genus, to us, is the results not from the lack of potential pollinators in the habitat.

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Summary

Eight populations of *E. helleborine* (L.) Crantz originating from an area of Lower Silesia in Poland were examined in respect to composition of their nectar and its influence on attracting insects under field conditions. The research was conducted during the years: 2001–2005. Nectar was collected during the maximum secretion by plants, namely between 11 a.m. and 3 p.m., from 15 July to 28 August. Chemical composition of *E. helleborine* nectar was studied by means of GC/MS SIM. To main attractants contained in the nectar one should include: eugenol, methoxyeugenol, ethanol and vanillin. The substances with potentially narcotic and intoxicating influence were identified, namely derivatives of indole, morphine derivatives and derivatives of phenol. These compounds might be responsible for characteristic behavior of visitors known in the literature as „drunken insects" or „sluggish pollinators" (Jakubska et al. 2005).

Arthropoda representatives observed during the study on *E. helleborine* infloresences have been divided into 4 groups:

1. pollinators, which are also called typical vectors: Hymenoptera (Aculeata: Apidae, Vespidae) and Coleoptera (Coccinellidae);
2. visitors = potential vectors: Coleoptera (Lagriidae, Cantharidae) and Diptera (Syrphidae);
3. invertebrates visiting orchids, but connected with other insects occuring on this species plants: Heteroptera, Hymenoptera: Parasitica (Ichneumonidae), Aculeata (Formicidae) and spiders;
4. insects connected with the orchid, as they feed on sap of its vegetative sprouts (Aphididae, Homoptera).

Among insects pollinating *E. helleborine*, the greatest importance have representatives of Vespidae: *Vespula vulgaris* (Linnaeus, 1758) and *Vespula germanica* (Fabricius, 1793) as well as Coleoptera, e.g.: *Anaspis frontalis* (Linnaeus, 1758), *Meligethes aeneus* (Fabricius, 1775), *Malachius bipustulatus* (Linnaeus, 1758), and regarding their body size, *Rhagonycha fulva* (Scopoli, 1763), *Coccinella septempunctata* Linnaeus, 1758 and *Propylea quatuordecimpunctata* (Linnaeus, 1758) in particular.

Streszczenie

Kompleksowe badania ekologii zapylania, obejmujące analizę chemiczną nektaru *Epipactis helleborine* (L.) Crantz, obserwacje zachowania owadów odwiedzających i zapylających, a także identyfikację entomofauny związanej z biologią kruszczyka szerokolistnego prowadzono w 8 populacjach na terenie pd-zach. Polski, tj. w okolicach Srebrnej Góry, Zakrzowa, Kotowic, Siechnic, Czernicy, w rejonie Krowiarek, Parku Narodowego Gór Stołowych (Karłów) oraz na terenie Parku Zachodniego we Wrocławiu. Badania prowadzono w pięciu następujących po sobie sezonach wegetacyjnych w latach 2001–2005, w okresie optimum kwitnienia kruszczyka szerokolistnego, tj. od 15 lipca do 28 sierpnia.

Dostępne, nieliczne dane literaturowe dotyczące biologii zapylania *E. helleborine* wskazywały, iż głównym związkiem odpowiedzialnym za charakterystyczne zachowanie owadów odwiedzających kwiaty tego storczyka jest etanol (Ehlers i Olsen 1997). Efekt działania etanolu obserwowany był w postaci zaburzeń lotu zwanych w literaturze „sluggish pollinators effect" lub „drunken insects".

Przeprowadzone analizy chemiczne wykazały obecność w nektarze kruszczyka szerokolistnego substancji o potencjalnym narkotycznym charakterze, w tym wielopierścieniowych układów aromatycznych, których obecność mogłaby wywoływać efekty przypisywane działaniu etanolu.

Zidentyfikowano m.in. morfinopochodne, indolopochodne, metoksyeugenol oraz wanilinę, a także ok. 100 innych związków chemicznych reprezentujących takie grupy jak: terpeny, alkaloidy, steroidy, kwasy tłuszczowe oraz kwasy karboksylowe.

Przeprowadzone badania wskazują, iż efekt „pijanych owadów" („drunken insects") odwiedzających kwiaty kruszczyka szerokolistnego może być wywołany przez inne związki niż etanol, np. pochodne indolowe, morfinopochodne, czy pochodne fenolowe, których obecność w nektarze wykazały widma GC-MS. Charakterystyczne zachowanie owadów po spożyciu nektaru wydzielanego przez *E. helleborine* może być również efektem synergizmu tych związków (Jakubska i in. 2005).

W trakcie badań stwierdzono, że fundamentalną rolę w procesie wabienia owadów może mieć wydzielany przez kruszczyka szerokolistnego zapach. Do głównych związków odpowiedzialnych za charakterystyczny zapach kruszczyka szerokolistnego należą prawdopodobnie: wanilina, eugenol oraz jego pochodne, a także liczne estry kwasów karboksylowych, a także alkohole, fenole, kwasy organiczne i aldehydy (Jakubska i in. 2005).

Na podstawie przeprowadzonych badań dokonano podziału stawonogów obserwowanych na kwiatostanach *E. helleborine* na 4 grupy:

1. zapylacze (typowe wektory): Hymenoptera (Aculeata: Apidae, Vespidae) oraz Coleoptera (Coccinellidae),
2. owady odwiedzające, które mogą być fakultatywnymi zapylaczami oraz wektorami: Coleoptera (Lagriidae, Cantharidae) a także Diptera (Syrphidae),
3. bezkręgowce odwiedzające *E. helleborine*, ale związane z innymi owadami występującymi na tym gatunku storczyka: Heteroptera, Hymenoptera: Parasitica (Ichneumonidae), Aculeata (Formicidae) oraz pająki,

4. owady związane ze storczykiem, po przez fakt żywienia się jego sokiem pędowym (Aphididae, Homoptera).

Wśród owadów zapylających *E. helleborine* największe znaczenie mają *Vespula vulgaris* (Linnaeus, 1758) oraz *Vespula germanica* (Fabricius, 1793). Fakultatywnym wektorem może być obserwowany na większości badanych stanowisk *Episyrphus balteatus* (Diptera). Warto zauważyć, że duże znaczenie w ekologii zapylania kruszczyka szerokolistnego mają Coleoptera: *Anaspis frontalis* (Linnaeus, 1758), *Meligethes aeneus* (Fabricius, 1775), *Malachius bipustulatus* (Linnaeus, 1758) oraz *Rhagonycha fulva* (Scopoli, 1763), *Coccinella septempunctata* Linnaeus, 1758, a także *Propylea quatuordecimpunctata* (Linnaeus, 1758).


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