

Agnieszka Kowalkowska, PhD.

**Analysis of floral secretory structures in selected
representatives of *Bulbophyllum* Lindl. and *Epipactis* Zinn.
(*Orchidaceae*)**

SUMMARY OF PROFESSIONAL ACCOMPLISHMENTS

University of Gdańsk

Faculty of Biology

Department of Plant Cytology and Embryology

Gdańsk 2018

Table of Contents

| | |
|--|----|
| 1. Name and surname..... | 2 |
| 2. Diplomas, academic degrees..... | 2 |
| 3. Information on previous employment in scientific units..... | 2 |
| 4. Scientific achievement | 2 |
| A. Title of scientific achievement | 2 |
| B. Publications belonging to the scientific achievement..... | 3 |
| C. Discussion of the scientific purpose of the above works and the results achieved, together with a discussion of their possible use | 4 |
| <i>Introduction</i> | 4 |
| <i>Discussion of the scientific purpose and results achieved</i> | 5 |
| <i>Directions and prospects for further research</i> | 9 |
| <i>References</i> | 9 |
| 5. Discussion of other scientific achievements..... | 11 |
| 5.1. Scientific achievements before obtaining a doctoral degree..... | 11 |
| A. Taxonomic research of <i>Orchidaceae</i> | 11 |
| B. Micromorphological analysis in taxonomic studies..... | 12 |
| 5.2. Scientific achievements after obtaining the doctoral degree..... | 12 |
| A. Analysis of secretory tissue in selected species | 12 |
| B. Analysis of anatomic features of gynostemium | 14 |
| C. Cytologic and micromorphological analysis in relation to taxonomic and/or phylogenetic studies in <i>Orchidaceae</i> | 15 |
| 5.3. Other aspects of scientific activity..... | 16 |

1. Name and surname**Agnieszka Kamila Kowalkowska**

2. Diplomas, academic degrees

- ✓ 29.06.2009 – **Ph.D. diploma in biological sciences in the field of biology**,
University of Gdańsk, Faculty of Biology, Diploma no. 3128
Title of PhD dissertation, executed in Department of Plant Taxonomy and Nature Conservation:
„Comparative analysis of floral structures attracting insects in selected species of *Bulbophyllinae* and *Pleurothallidinae* (*Orchidaceae*)”
Supervisor: Dariusz L. Szlachetko, Prof., Ph.D.

 - ✓ 23.06.2003 – **MSc diploma in biological sciences in the field of environmental biology** (with honors), University of Gdańsk, Faculty of Biology, Geography and Oceanology, Diploma no. 5235/B/2003
Title of MSc thesis, executed in Department of Plant Taxonomy and Nature Conservation:
„Taxonomic, geographical and ecological analysis of orchids of Guinea”
Supervisor: Dariusz L. Szlachetko, Prof., Ph.D.
-

3. Information on previous employment in scientific units

- ✓ from 2011-02-01 - assistant professor,
Department of Plant Cytology and Embryology,
 - ✓ from 2010-04-01 to 2011-01-31 - senior scientific and technical specialist,
Department of Plant Cytology and Embryology,
 - ✓ from 2009-10-15 to 2010-03-31 - scientific and technical specialist,
Department of Plant Cytology and Embryology,
 - ✓ from 2008-09-01 to 2009-10-14 - senior technical consultant,
Department of Plant Cytology and Embryology,
 - ✓ from 2007-01-01 to 2008-08-31 - senior technical consultant,
Department of Plant Taxonomy and Nature Conservation,
 - ✓ from 2006-09-01 to 2006-12-31 - senior technician,
Department of Plant Taxonomy and Nature Conservation.
-

4. Scientific achievement* according to Art. 16 Paragraph 2 of the Act of 14 March 2003 r. on academic degrees and academic title, as well as degrees and title in art. (Journal of Laws from 2016, item. 882 with changes in Journal of Laws from 2016, item. 1311.):

A. Title of scientific achievement

Analysis of floral secretory structures in selected representatives of *Bulbophyllum* Lindl. and *Epipactis* Zinn. (*Orchidaceae*)

B. Publications belonging to the scientific achievement

Publications on the genus *Bulbophyllum* Lindl.:

- 1. Kowalkowska AK**, Koziaradzka-Kiszkurno M, Turzyński S (2015) Morphological, histological and ultrastructural features of osmophores and nectary of *Bulbophyllum wendlandianum* (Kraenzl.) Dammer (*B.* section *Cirrhopetalum* Lindl., Bulbophyllinae Schltr., Orchidaceae). *Plant Systematics and Evolution* 301(2): 609-622.

IF = 1.361; IF 5-year = 1.291;

MSHE (Ministry of Science and Higher Education, Poland) = 20 points.

I estimate my contribution for 70%.

- 2. Kowalkowska AK**, Turzyński S, Koziaradzka-Kiszkurno M, Wiśniewska N (2017) Floral structure of two species of *Bulbophyllum* section *Cirrhopetalum* Lindl.: *B. weberi* Ames and *B. cumingii* (Lindl.) Rchb.f. (Bulbophyllinae Schltr., Orchidaceae). *Protoplasma* 254(3): 1431–1449.

IF = 2.457; IF 5-year = 2.658; MSHE = 30 points.

I estimate my contribution for 70%.

- 3. Wiśniewska N, Kowalkowska AK**, Koziaradzka-Kiszkurno M, Krawczyńska AT, Bohdanowicz J (2018) Floral features of two species of *Bulbophyllum* section *Lepidorhiza* Schltr.: *B. levanae* Ames and *B. nymphopolitanum* Kraenzl. (Bulbophyllinae Schltr., Orchidaceae). *Protoplasma* 255(2): 485-499.

IF = 2.457; IF 5-year = 2.658; MSHE = 30 points.

I estimate my contribution for 35%.

Publication on the genus *Epipactis* Zinn.:

- 4. Kowalkowska AK**, Pawłowicz M, Guzanek P, Krawczyńska AT (2018) Floral nectary and osmophore of *Epipactis helleborine* (L.) Crantz (Orchidaceae). *Protoplasma* DOI: <https://doi.org/10.1007/s00709-018-1274-5>

IF = 2.457; IF 5-year = 2.658; MSHE = 30 points.

I estimate my contribution for 55%.

Scientific achievement, being the basis for applying for post-doctoral degree is presented in 4 monothematic scientific publications published in years 2015-2018 in journals listed in the database Journal Citation Reports. Total IF = **8.732** and total number of MSHE points = **110**. I am the first author in three publications, second - in one, the corresponding author - in all publications.

In the summary, works included in the presented achievement are referred to [pos. 1-4].

C. Discussion of the scientific purpose of the above works and the results achieved, together with a discussion of their possible use

Introduction

In flowers of Orchidaceae, due to pressure from pollinating insects, various structures and pollination mechanisms have developed. The research concerns the secretory structures on flower petals in selected species of *Bulbophyllum* Lindl. - mainly pollinated by *Diptera* (Christensen 1994) and *Epipactis* Zinn. - mainly pollinated by *Diptera* and *Hymenoptera* (Jakubska-Busse and Kadej 2011). In sapromyophilous flowers pollinated by flies, yellow to brown-purple, a scent is the main attractant (van der Pijl and Dodson 1969; Jones and Gray 1976; van der Cingel 1995), which is often similar to decomposing protein compounds, mainly amines, ammonia and indoles (Proctor et al. 1996). The sapromyophilic flowers produce little or no nectar (Jürgens et al. 2006; van der Niet et al. 2011). While flowers pollinated by *Hymenoptera* are characterized by a bright colour, scent pleasant to human and nectar secreted on lip surface (van der Pijl and Dodson 1969).

Fragrances are produced in odor glands (osmophores), which may be morphologically distinguishable or not from other flower tissues (Stern et al. 1987; Vogel 1990). Osmophores can form structures called „antennae”, which are swollen apices of petals (both lateral petals of the inner whorl of the perianth) and/or sepals (petals of the outer perianth: dorsal and both lateral), e.g. in *Bulbophyllum cerambyx* J. J. Sm., *Myoxanthus reymondii* (H. Karst.) Luer, *Restrepia antennifera* Kunth. They can be also localized in prolonged apices of all perianth petals (tepals), especially sepals, e.g. in *Dracula* Luer, *Masdevallia* Ruiz & Pav. or even as glands on the fused sepals (in *Scaphosepalum verrucosum* (Rchb. f.) Pfitzer (= *Scaphosepalum ochthodes* (Rchb. f.) Pfitzer) (van der Cingel 1995). Vogel (1990) described two heterogenic scent centers in *Bulbophyllum ornatissimum*: prolonged osmophores with a smell of cod-liver oil and a trimethylamine odour emitted from lip surface, which was decisive to subject each of the petals of *Bulbophyllum* species to detailed studies. While flowers of *Epipactis* exude strong aromatic compounds, such as eugenol and vanillin, which can be crucial in attracting flies (Jakubska et al. 2005; Jakubska-Busse and Kadej 2011). Due to cytotoxicity, fragrances are released periodically and are not accumulated on the surface of osmophores (Vogel 1990).

The most common short distance attractant is **nectar** (van der Pijl and Dodson 1969), collected in floral or extrafloral nectaries. The term „nectary” refers to the place of production and offering the nectar being visible for pollinators, but it does not imply the same origin and position in flowers (Pacini et al. 2003), because nectaries vary greatly in terms of topography, morphology, anatomy, ultrastructure and secretory processes (Nepi 2017). In the representatives of discussed genera *Bulbophyllum* and *Epipactis*, the superficial nectaries on lips are described (van der Pijl and Dodson 1969; Nilsson 1978; Vogel 1990; Borba and Semir 1998). The research by De Pádua Teixeira et al. (2004) paid my attention to the lip features: the role of nectary was played by the tissue in shallow, longitudinal groove of the lip, and additionally, the papillae on the side lobes displayed the osmophoric function.

Although the ingredients dissolved in nectar can be various constituents: carbohydrates, amino acids, ions, proteins, fragrant compounds (Raguso 2004), enzymes and antioxidants sustaining homeostasis of nectar composition (Carter and Thornburg 2004), and also toxic materials to discourage unwanted consumers (Adler 2001), researchers have become careful in naming the nectar of fluid secreted on lips of *Bulbophyllum*. The labellar secretion was defined as nectar in *B. epiphytum*, *B. glutinosum*, *B. regnellii*, *B. rothschildianum* (De Pádua Teixeira et al. 2004) and in *Bulbophyllum* from section *Didactyle* (Nunes et al. 2014), as lipid-rich secretions in African *Bulbophyllum* (Stpiczyńska et al. 2015), as a protein-rich mucilage in *Bulbophyllum* from section *Racemosae* (Davies and Stpiczyńska 2014; Stpiczyńska and Davies 2016).

Discussion of the scientific purpose and results achieved

The purpose of undertaken research was:

- a) **identification of secretory tissue in flowers** in selected species from the tropical climate: Asian species from the genus *Bulbophyllum* Lindl.: *Bulbophyllum wendlandianum* (Kraenzl.) Dammer [**pos. 1**], *B. cumingii* (Lindl.) Rchb. f. and *B. weberi* Ames [**pos. 2**], *B. levanae* Ames and *B. nymphopolitanum* Kraenzl. [**pos. 3**] and from the temperate climate from the genus *Epipactis* Zinn.: *Epipactis helleborine* (L.) Crantz [**pos. 4**], **characterized in the literature as having superficial nectaries,**
- b) **analysis and description of floral secretory structures** in *Bulbophyllum* species (mainly pollinated by *Diptera*) and *Epipactis* (mainly pollinated by *Diptera* and *Hymenoptera*).

To describe the characteristics of secretory tissue, macro- and micromorphological, histochemical and ultrastructural methods were used.

In *B. wendlandianum* [**pos. 1**], the nectary was located in the central groove on the inner (adaxial) surface of the lip and consisted of the secretory epidermis and few subepidermal layers. The dense cytoplasm contained abundant mitochondria, expanded endoplasmic reticulum (ER), fully developed dictyosomes, ribosomes, lipid bodies, multivesicular bodies and myelin-like figures. In the vacuole, tannin-like materials were present. A large number of mitochondria is a reflection of high metabolic cell activity, which was previously described in both nectariferous or osmophoric tissues (Pridgeon and Stern 1983; Stpiczyńska et al. 2005b). The presence of numerous ER profiles and dictyosomes is associated with nectar secretion (Figueiredo and Pais 1992; Stpiczyńska et al. 2005a). Moreover, the osmiophilic bodies often occurred (possibly lipoidal) (Pais and Figueiredo 1994; Stpiczyńska 1997; Stpiczyńska et al. 2004). The tannins play a protective role against pathogens, herbivores and UV radiation (Brillouet et al. 2013 and references therein). The entire inner surface of lip displayed secretory features, but histological or ultrastructural differences between papillae on side lobes and labellar groove were not observed, like in Neotropical *Bulbophyllum* (De Pádua Teixeira et al. 2004). The osmophoric activity in *B. wendlandianum* was detected on branched, multicellular appendages of dorsal sepal and petals. The swellings of cuticle proper were revealed in ultrastructural studies of the appendages, which was the first observation of this type on species of the genus *Bulbophyllum*. In addition, the reticulate cuticle was observed, in which the residues of secretory material were noted on the cell

surface in the ends of microchannels, confirming their transport role. The presence of cuticle with microchannels, involved in the transport of fragrance components was described *i.a.* in *Anacamptis pyramidalis* f. *fumeauxiana* (Kowalkowska et al. 2012) or in African *Bulbophyllum* (Stpiczyńska et al. 2015).

In the flowers of *Bulbophyllum cumingii* and *B. weberi* [pos. 2], secretory activity was displayed on dorsal sepals in both species and petals of *B. weberi* (possible osmophores) and on inner (adaxial) surface of lip in both species (putative nectaries). Petals of *B. cumingii* were rather inactive in secretion process. In secretory cells, the dense cytoplasm contained large number of organelles: mitochondria, ER, dictyosomes, lipid bodies, which indicates high metabolic activity in secretory tissues (Pridgeon and Stern 1983; Figueiredo and Pais 1992; Stpiczyńska 1997; Stpiczyńska et al. 2005a). Lips of both species were arcuately curved, with the central longitudinal groove surrounded by two ridges and raised lateral lobes. The secretory tissue, similarly as in *B. wendlandianum* [pos. 1], consisted of one layer of epidermis and few subepidermal layers. Despite the macro- and micromorphological similarities of *B. cumingii* and *B. weberi*, the secretory tissue in the lip groove differed histochemically and ultrastructurally. In *B. weberi*, epidermal cells in cytoplasm contained: lipids, proteins, starch grains, dihydroxyphenols in vacuoles and **pectic acids/mucilage** on lip surface, whereas in *B. cumingii* few lipids, starch grains, no proteins, no dihydroxyphenols and no mucilage was noted. In ultrastructural studies, in *B. weberi* secretory material was observed on lip surface and the vesicles building into plasmalemma, while in the second species, cell wall ingrowths, microchannels and the vesicles were shown. The observations of **ingrowths on inner tangential cell walls of epidermis** in lip of *B. cumingii* and petals of *B. weberi* are the first report on *Bulbophyllum*. The cell wall protuberances may function as transfer cells - highly specialized cells, in which an intensive transport of constituents takes place through the plasmalemma (Gunning and Pate 1974). In both species, the presence of ingrowths and cuticle with microchannels facilitated the nectar exudation and resorption, which was previously described in *Fritillaria* (Stpiczyńska et al. 2012). In the lip of *B. cumingii*, the ingrowths can prove the function of nectary, whereas in petals of the second species, it rather indicates the osmophoric function (without the accumulation of secretions on the surface). The cell wall ingrowths also occurred in *Epipactis atropurpurea* (hexose rich nectar), whereas in *Limodorum abortivum* they were absent (sucrose rich nectar) (Pais and Figueiredo 1994). According to such observations, the sucrose rich nectar could be present on lip surface of *B. weberi* and hexose rich nectar - in *B. cumingii*.

In species of *Bulbophyllum levanae* and *B. nymphopolitanum* [pos. 3], the secretory tissue was detected in lip groove and in the extended apices of sepals and petals (putative osmophores). Moreover, modified stomata with visible associated secretion were observed on lateral petals and lateral sepals of *B. nymphopolitanum*, which might also function as osmophores. The most remarkable feature of the lips epidermis was the occurrence of **the periplasmic space**. Although in *B. levanae*, the space was three-fold larger than in *B. nymphopolitanum*. This feature was perhaps associated with merocrine secretion, in which the cell after secretion remains alive and continues with its secretory activity. Moreover, the synthesized substances could be transported via vesicles (granulocrine

secretion) to the periplasmic spaces, and then on the cell surface (Fahn 1979; Pacini and Nepi 2007; Paiva 2016). This process is common in nectaries or secretory cavities for gums, oils and resins (Paiva 2016). Materials collected in the periplasmic space reduce the space occupied by the protoplast gradually increasing the pressure, and thus, the cytoplasm becomes denser and more compressed (Paiva 2016). In the lip of *B. levanæ*, both periplasmic space, and microchannels coexist, which has not previously been reported in literature. According to Paiva (2016), the secretion through pores or hydrophilic canals occurs in cells that do not develop a subcuticular space.

The next work concerns the secretory tissue in *Epipactis helleborine* [pos. 4]. The lip consists of two joined parts: the basal part - hypochile and apical part - epichile with knobs. In the cross-section, hypochile was formed by single-layered epidermis, several subepidermal layers and parenchyma, whereas in knobs of epichile, parenchyma contained intercellular spaces and numerous idioblasts. The whole hypochile and knobs of epichile secrete nectar. The scent possibly comes from the aromatic constituents of nectar and is secreted through the epichile tissue and the apices of tepals (osmophores). In the bud stage (about 8 mm long), the pleated surface of hypochile with distended cuticle was revealed, while on the knobs of epichile, small cuticle separation from cell wall was visible. While in *Epipactis palustris* (L.) Crantz (Kowalkowska et al. 2015), the nectary was present on isthmus of the inner surface of hypochile and on the callus of epichile, where the distended cuticle was observed. The observations of distended cuticle, both in *E. helleborine* and *E. palustris* (Kowalkowska et al. 2015), indicated the accumulation of nectar under the cuticle. Then, the nectar can be released outside the cell by the cuticle rupture under the growing pressure to the exterior (Curry et al. 1991). In the cross-sections of buds of *E. helleborine*, a significant secretion was observed on the hypochile, and a small amount on the epichile knobs. Moreover, comparison between pollinated and unpollinated flowers from bud stage until the 16th day of *E. helleborine* anthesis [pos. 4] revealed, that pollinated flowers quickly withered, and in the end of anthesis of unpollinated flowers, **heterogeneous secretion with phenolic fraction** (not observed previously) appeared. It could intensify the scent perception through the potential insect pollinators. Based on these observations, along with the proposed hypothetical scheme of the chemical compounds' influence on the pollinators and visitors insects of *E. helleborine* (Jakubska et al. 2005), the third stage was added: in the last day of anthesis in unpollinated flowers - maximum discharge of nectar from the cells (especially on the knobs) and the intensification of the scent perception through the appearance of phenolic material in secretion. In the 3rd day of anthesis, **the presence of periplasmic space** was noted in flowers of *E. helleborine*, previously noted in *B. levanæ* and *B. nymphopolitanum* [pos. 3]. Cuticular canals (microchannels) occurred in hypochile cells and epichile knobs. Microchannels and periplasmic space co-occurred in the cells of *E. helleborine*, as previously described in *B. levanæ* [pos. 3].

In addition to the above mentioned features of each species, the characteristics of the secretory tissue common to all species have been distinguished. **Plastids with plastoglobuli** were described in the appendages of dorsal sepalum and petals of *B. wendlandianum* [pos. 1], in dorsal sepalum of *B. cumingii*, in petals and lip of *B. weberi*

[**pos. 2**], in lip of *B. levanæ* and *B. nymphopolitanum*, and in petals of *B. levanæ* [**pos. 3**], in lip of *E. helleborine* [**pos. 4**]. Plastids with plastoglobuli were observed both in nectariferous and osmophoric cells (Figueiredo and Pais 1992; Stpiczyńska 1997; Stpiczyńska et al. 2005). In staining on the presence of dihydroxyphenols (FeCl_3), it has been demonstrated that dihydroxyphenolic globules were present in the cytoplasm of all tepals in these species, but not in vacuoles. In contrast, plastids with numerous plastoglobuli were visible in the cytoplasm in ultrastructural studies. Since plastoglobuli were involved in fragrance production and phenolic compounds were known to occur in the floral scent of sapromyophilous species and were thought to attract flies (Jürgens et al. 2006), it was proposed that plastoglobuli are stained with FeCl_3 . The system of associated membranes: the intraplastidal ones, plastid envelope and ER located close to each other allowed for the transport of fragrance components produced in plastoglobuli by ER to plasmalemma or independent movement as lipophilic or osmophilic drops in the cytoplasm (Pridgeon and Stern 1985; Stern et al. 1987; Pais and Figueiredo 1994; Stpiczyńska 1997; Kowalkowska et al. 2012). The secretion was probably transported inside vesicles (originated from ER or dictyosomes), which were fusing with plasmalemma. Based on the observation of irregular plasmalemma and vesicles fusing with plasmalemma or occurring nearby [**pos. 1-5**], it has been proposed the transport of materials on the cell surface in the way of **granulocrine secretion**, which was described *i.a.* in *Gymnadenia conopsea* (Stpiczyńska and Matusiewicz 2001), *Platanthera chlorantha* (Stpiczyńska et al. 2005b), *Anacamptis pyramidalis* (Kowalkowska et al. 2012) and *E. palustris* (Kowalkowska et al. 2015).

Starch grains in plastids, being the source of energy for the production of scent and nectar (Vogel 1990; Nepi 2007; Pacini and Nepi 2007), were observed in the anthesis in *B. cumingii*, *B. weberi* and *B. levanæ* [**pos. 2, 3**]. In flowers of *B. wendlandianum* [**pos. 1**] and *B. nymphopolitanum* [**pos. 3**], starch could be hydrolyzed during anthesis, which requires further research. According to Nepi (2007), the source and quantity of starch in secretory tissue are correlated with the manner of nectar secretion. Species with eccrine secretion contain much starch and those with little or no starch at all may also display granulocrine secretion. In addition, at the bud stage of *E. helleborine* [**pos. 4**], starch utilization was started from epidermal cells. Polimorphic shapes of plastids were observed also in *E. palustris* (Kowalkowska et al. 2015), which is associated with starch reduction (Stpiczyńska et al. 2005a).

Numerous **idioblasts with calcium oxalate crystals in the form of raphides** were observed in tepals of *Bulbophyllum* [**pos. 1-3**], whereas their lower number in both species of *Epipactis* [**pos. 4**]. In dorsal sepalum and pepals of *B. wendlandianum* [**pos. 1**], *B. cumingii* and *B. weberi* [**pos. 2**], development of idioblasts caused an increase in cell volume, which was visible as characteristic groups of elevated cells on the surface of petals. In addition to eliminating the additional calcium from the cytosol (Paiva and Machado 2008), idioblasts reflect the light and direct the attention of insects to the centre of flowers (van der Cingel 1995; Franceschi 2001). In the lips, idioblasts have been predominantly observed under single-layered epidermis of *Bulbophyllum* or in parenchyma of callus/knobs of epichile of *Epipactis*, which also protect the flower against herbivores (Prychid and Rudall 1999).

As the most important scientific achievements included in the series of publications presented in the habilitation process, I consider:

Description for *Bulbophyllum* species for the first time:

- swellings of cuticle on the appendages of dorsal sepalum and petals of *B. wendlandianum*,
- cell wall ingrowths in lip of *B. cumingii* and petals of *B. weberi*,
- periplasmic spacer in lips of *B. levanae* and *B. nymphopolitanum*,
- coexistence of periplasmic spaces and microchannels in lip epidermis of *B. levanae*.

Description for *Epipactis* species for the first time:

- heterogenous secretion with additional phenolic fraction in the last stage of anthesis of unpollinated flowers in *E. helleborine*,
- coexistence of periplasmic spaces and microchannels in *E. helleborine*, like in *B. levanae*.

Directions and prospects for further research

Various structures in the secretory tissue noted for the first time encourage for further flower studies of *Bulbophyllum* and *Epipactis*. These studies are a contribution to understanding the pollination biology of these species and planning their further species protection. Studies on the sapromiophilic species of *Bulbophyllum* and on autogamy in *Epipactis* are continued under two doctoral dissertations, in which I am the auxiliary supervisor (Natalia Wiśniewska, MSc, Patrycja Guzanek MSc).

References

- Adler LS (2001) The ecological significance of toxic nectar. *Oikos* 91:409-420
- Borba EL, Semir J (1998) Wind-assisted fly pollination in three *Bulbophyllum* (*Orchidaceae*) species occurring in the Brazilian campos rupestres. *Lindleyana* 13: 203-218
- Brillouet J-M, Romieu C, Schoefs B, Solymosi K, Cheynier V, Fulcrand H, Verdeil J-L, Conéjéro G (2013) The tannosome is an organelle forming condensed tannins in the chlorophyllous organs of *Tracheophyta*. *Ann Bot.* 112(6): 1003-1014.
- Carter C, Thornburg RW (2004) Is the nectar redox cycle a floral defense against microbial attack? *Trends Plant Sci* 9: 320-324
- Christensen DE (1994) Fly pollination in the *Orchidaceae*. W: J Arditti (red.) *Orchid biology: Reviews and Perspectives VI*: 415-454. New York: John Wiley and Sons
- Curry KJ, McDowell LM, Judd WS, Stern WL (1991) Osmophores, floral features, and systematics of *Stanhopea* (*Orchidaceae*). *Am J Bot* 78: 610-623
- Davies KL, Stpiczyńska M (2014) Labellar anatomy and secretion in *Bulbophyllum* Thouars (*Orchidaceae: Bulbophyllinae*) sect. *Racemosae* Benth. & Hook. F. *Ann Bot* 114(5): 889-911
- De Pádua Teixeira S, Borba EL, Semir J (2004) Lip anatomy and its implications for the pollination mechanisms of *Bulbophyllum* species (*Orchidaceae*). *Ann Bot* 93: 499-505
- Fahn A (1979) Ultrastructure of nectaries in relation to nectar secretion. *Am J Bot.* 66: 977
- Figueiredo ACS, Pais MS (1992) Ultrastructural aspects of the nectary spur of *Limodorum abortivum* (L.) Sw. (*Orchidaceae*). *Ann Bot* 70: 325-331
- Franceschi VR (2001) Calcium oxalate in plants. *Trends Plant Sci* 6: 331-331

- Gunning BES, Pate JS (1974) Transfer cells. In: Robards AW (ed) Dynamic aspects of plant ultrastructure. McGraw-Hill, London, pp. 441-480
- Jakubská A, Prządło D, Steininger M, Anioł-Kwiatkowska J, Kadej M (2005) Why does pollinators became „sluggish”? Nectar chemical constituents from *Epipactis helleborine* (L.) Crantz (*Orchidaceae*). *Appl Ecol Environ Res* 3(2): 29-38
- Jakubská-Busse A, Kadej M (2011) The pollination of *Epipactis* Zinn., 1757 (*Orchidaceae*) species in Central Europe - the significance of chemical attractants, floral morphology and concomitant insects. *Acta Soc Bot Pol* 80: 49-57
- Jones DL, Gray B (1976) The pollination of *Bulbophyllum longiflorum* Thouars. *Amer Orchid Soc Bull* 45: 15-17
- Jürgens A, Dötterl S, Meve U (2006) The chemical nature of fetid floral odours in stapeliads (*Apocynaceae-Asclepiadoideae-Ceropegieae*). *New Phytol* 172: 452-468
- Kowalkowska AK, Margońska HB, Kozieradzka-Kiszkurno M, Bohdanowicz J (2012) Studies on the ultrastructure of a three spurred *fumeauxiana* form of *Anacamptis pyramidalis*. *Plant Syst Evol* 298: 1025-1035
- Kowalkowska AK, Kostelecka J, Bohdanowicz J, Kapusta M, Rojek J (2015) Studies on floral nectary, tepals' structure and gynostemium morphology of *Epipactis palustris* (L.) Crantz (*Orchidaceae*). *Protoplasma* 252(1): 321-333.
- Nepi M (2007) Nectary structure and ultrastructure. In: Nicolson SW, Nepi M, Pacini E (eds) Nectaries and nectar. Springer, Rotterdam, pp 129-166.
- Nilsson LA (1978) Pollination ecology of *Epipactis palustris* (*Orchidaceae*). *Bot Notiser* 131:355-368
- Nunes ELP, Smidt EC, Stützel T, Coan AI (2014) What do floral anatomy and micromorphology tell us about Neotropical *Bulbophyllum* section *Didactyle* (*Orchidaceae: Bulbophyllinae*)? *Bot J Linn Soc* 175: 438-452
- Pacini E, Nepi M, Vesprini J (2003) Nectar biodiversity: a short review. *Plant Syst Evol* 238: 7-21
- Pacini E, Nepi M (2007) Nectar production and presentation. In: Nicolson SW, Nepi M, Pacini E (eds) Nectaries and nectar. Springer, Rotterdam, pp 167-214
- Pais MS, Figueiredo ACS (1994) Floral nectaries from *Limodorum abortivum* (L.) Sw. and *Epipactis atropurpurea* Rafin (*Orchidaceae*): ultrastructural changes in plastids during the secretory process. *Apidologie* 25: 615-626
- Paiva EAS (2016) How do secretory products cross the plant cell wall to be released? A new hypothesis involving cyclic mechanical actions of the protoplast. *Ann Bot* 117: 533-540
- Paiva EAS, Machado SR (2008) The floral nectary of *Hymenaea stigonocarpa* (*Fabaceae, Caesalpinioideae*): structural aspects during floral development. *Ann Bot* 101(1):125-133
- Pridgeon AM, Stern WL (1983) Ultrastructure of osmophores in *Restrepia* (*Orchidaceae*). *Am J Bot* 70: 1233-1243
- Pridgeon AM, Stern WL (1985) Osmophores of *Scaphosepalum* (*Orchidaceae*). *Bot Gaz* 146: 115-123
- Proctor M, Yeo P, Lack A (1996) The natural history of pollination. Harper Collins Publishers, London
- Prychid CJ, Rudall PJ (1999) Calcium oxalate crystals in monocotyledons: a review of their structure and systematics. *Ann Bot* 84: 725-739
- Raguso RA (2004) Flowers as sensory billboards: progress towards an integrated understanding of floral advertisement. *Curr Opin Plant Biol* 7: 434-440
- Stern WL, Curry KJ, Pridgeon AM (1987) Osmophores of *Stanhopea* (*Orchidaceae*). *Am J Bot* 74: 1323-1331
- Stpiczyńska M (1997) The structure of nectary of *Platanthera bifolia* L. (*Orchidaceae*). *Acta Soc Bot Pol* 62: 5-9

- Stpiczyńska M, Matusiewicz J (2001) Anatomy and ultrastructure of spur nectary of *Gymnadenia conopsea* (L.) *Orchidaceae*. *Acta Soc Bot Pol* 70: 267-272
- Stpiczyńska M, Davies KL, Gregg A (2004) Nectary structure and nectar secretion in *Maxillaria coccinea* (Jacq.) L.O. Williams ex Hodge (*Orchidaceae*). *Ann Bot* 93: 87-95
- Stpiczyńska M, Davies KL, Gregg A (2005a) Comparative account of nectary structure in *Hexisea imbricata* (Lindl.) Rchb.f. (*Orchidaceae*). *Ann Bot* 95: 749-756
- Stpiczyńska M, Milanesi C, Faleri C, Cresti M (2005b) Ultrastructure of the nectary spur of *Platanthera chlorantha* (Custer) Rchb. (*Orchidaceae*) during successive stages of nectar secretion. *Acta Biol Crac* 47: 111-119
- Stpiczyńska M, Nepi M, Zych M (2012) Secretion and composition of nectar and the structure of perigonal nectaries in *Fritillaria meleagris*. *Plant Syst Evol* 298: 997-1013
- Stpiczyńska M, Davies KL, Kamińska M (2015) Diverse labellar secretions in African *Bulbophyllum* (*Orchidaceae: Bulbophyllinae*) sections *Ptiloglossum*, *Oreonastes* and *Megaclinium*. *Bot J Linn Soc* 179(2): 266-287
- Stpiczyńska M, Davies KL (2016) Evidence for the dual role of floral secretory cells in *Bulbophyllum*. *Acta Biol Cracov Ser Bot* 58: 57-69
- van der Cingel NA (ed) (1995) An atlas of Orchid Pollination. Balkema, Rotterdam
- van der Niet T, Hansen DM, Johnson SD (2011) Carrion mimicry in a South African orchid: flowers attract a narrow subset of the fly assemblage on animal carcasses. *Ann Bot* 107:981-992
- van der Pijl L, Dodson CH (1969) Orchid flowers: their pollination and evolution. University of Miami Press, Coral Gables
- Vogel S (1990) The role of scent glands in pollination: on the structure and function of osmophores. Amerind, New Delhi

5. Discussion of other scientific achievements

In addition to four publications that make up the scientific achievement discussed above, my scientific achievement consists of 17 publications with a total **IF = 19.936 (MSHE = 282 points)**. In six of the publications I am the first author, in seven - corresponding author. Below is a brief overview of these works.

5.1. Scientific achievements before obtaining a doctoral degree

The area of my scientific interests included the following research topics:

A. Taxonomic research of *Orchidaceae*

During the master's thesis, I classified African species working with herbarium sheets from the Muséum National d'Histoire Naturelle from Paris and making specialized drawings, the main effect of which is a monograph [pos. 1, 2], for which I received, together with the promoter, the 1st degree Rector's Team Award. Other works concern taxonomic revision [pos. 3], describing new species [pos. 4] or forms [pos. 5] for science.

1. Szlachetko DL, **Kowalkowska AK** (2007) Inventaire préliminaire de la flore d'orchidées de Guinée, Afrique occidentale. *Richardiana* 7(4): 174-184.

2. Szlachetko DL, **Kowalkowska AK** (2007) Contributions to the Orchid Flora of Guinea. Polish Botanical Studies 25: 1-259.
3. Margońska HB, **Kowalkowska AK** (2008) Taxonomic revision of *Dienia* (*Malaxidinae*, *Orchidaceae*). Annales Botanici Fennici 45: 97-104.
4. Szlachetko DL, **Kowalkowska A** (2008) Two new species of *Disperis* (*Orchidaceae*, *Orchidoideae*) from Central West Africa. Polish Botanical Journal 53(1): 1-3.
5. Margońska HB, **Kowalkowska AK** (2008) Une nouvelle forme de *Anacamptis pyramidalis* (*Orchidaceae*). Richardiana 8(1): 1-5.

B. Micromorphological analysis in taxonomic studies

The aim of research on selected species of *Malaxidinae* [pos. 6] was to describe the features of micromorphological lips in six groups, separated on the basis of taxonomic works. The work describes the features of epidermal cells: striation of the cuticle, the presence of raphides and remains of secretions on the cell surface.

6. **Kowalkowska AK**, Margońska HB (2009) Diversity of labellar micromorphological structures in selected species of *Malaxidinae* (*Orchidales*). Acta Societatis Botanicorum Poloniae 78(2): 141-150.

5.2. Scientific achievements after obtaining the doctoral degree

The area of my scientific interests covers the following research topics:

A. Analysis of secretory tissue in selected species

I started the research of floral structures of *Orchidaceae* attracting insects as part of my PhD thesis under the supervision of prof. dr. hab. Dariusz Szlachetko at the Department of Plant Taxonomy and Nature Conservation at the Faculty of Biology of the University of Gdańsk. The enormous diversity of floral structures of the representatives of *Bulbophyllinae* and *Pleurothallidinae* on the micromorphological level was a contribution to extending the research of these structures with histochemical and ultrastructural methods that would describe the nature of secretory tissue. As an employee of the Department of Plant Cytology and Embryology, in 2010 I obtained funding for the NCN project: "Floral secretory structures of the genus *Cirrhopetalum* Lindl. *sensu latissimo*" (now *Cirrhopetalum* is considered a section in the genus *Bulbophyllum*). Experiences gained thanks to cooperation with prof. dr. hab. Jerzy Bohdanowicz and dr hab. Małgorzata Kozieradzka-Kiszkurno allowed me to work out my own research workshop: to get acquainted with anatomical and ultrastructural techniques, as well as to improve the technique of making and analyzing microscopic preparations.

[Pos. 7, 8] include examination of the lip and spur in the new form of *fumeauxiana* *Anacamptis pyramidalis*. In typical *Anacamptis* flowers, the spur grows at the lip base, which is the petal of the inner whorl of the perianth. In the *fumeauxiana* form, additional spurs developed on the lateral sepals - the petals of the outer whorl. Spurs growing on petals of two

whorls have not been described before. This type of mutation has been confirmed in molecular studies. A comparison of spur of the lip and lateral sepals revealed the same anatomical features: a single-layer internal and external epidermis, a round cell shape of the outer epidermis, a thick undulated cuticle. At the base of both spurs, a small multicellular appendix was present, strongly stained in reaction for the protein detection. Residues of secretions were visible on both surfaces of the lip spur, which probably functions as an osmophore, whereas in the spur of lateral sepals - on the inner surface. Research on the *fumeauxiana* form has been extended with ultrastructural studies [pos. 8] and secretory activity was demonstrated on the lip surface, swellings, apices of lateral sepals, both surfaces of spurs: the lip and lateral sepals. Characteristic features of osmophoric cells were noted: dense cytoplasm with numerous profiles of ER, mitochondria, plastids with plastoglobuli and tubular structures, large cell nucleus, lipid drops and vesicles fusing with plasmalemma. These studies were the basis for the conclusion that the spur functions as an osmophore. In addition, the similarity of flower morphology and anatomy, flowering time and the same group of pollinating insects: *A. pyramidalis*, *A. pyramidalis* f. *fumeauxiana* and *Gymnadenia conopsea* suggest a possible mechanism based on food deception - an imitation of the presence of nectar in *Gymnadenia* spurs.

[Pos. 9] includes examination of the lip, tepals' structure and gynostemium morphology of *Epipactis palustris*. This publication, only for formal reasons, is not a part of the scientific achievement. The lip consists of two movably joined parts: the basal part (hypochile) with central broad isthmus and apical part (epichile) with callus. The whole surface of lip callus and abaxial side of isthmus have the features of secretory cells. The exudation at first appears on callus, later on isthmus, what could be a strategy to prolong the emission of volatile substances and nectar, and this means to prolong luring pollinators. This hypothesis was supported by the research in transmission electron microscopy (TEM). The plastids noted in callus were without starch, whereas the isthmus' cells contained partly hydrolyzed starch. Some plastids in callus had polymorphic shapes, which was related to a starch reduction. During the depletion of starch in callus cells, the number of plastoglobuli within the plastids increased, and also lipid bodies appeared in the cytoplasm. Whereas, in isthmus cells, proplastids with phytoferritin were noted. The endoplasmic reticulum in contact with plasmalemma and the vesicles fusing with plasmalemma in secretory cells of callus and isthmus show a way of granulocrine secretion. The cross-sections of sepals revealed the presence of numerous stomata with large substomatal cavities on the strongly undulated external surface. The pollen grains adhering to the rostellum-viscidium prove previous ecological observations that the rostellum-viscidium is not a barrier preventing self-pollination.

The next publication [pos. 10] concerned the examination of flowers of the neoheterotrophic orchid *Epipogium aphyllum* without chlorophyll. The conducted analysis proved that nectar is secreted on the upper surface of papillae placed in groups on multicellular elevations, as well as inside the spur, mainly at its apex. Secretion on the papillae was present through the whole anthesis, whereas the cells in spur were active periodically. Nectar secretion was not dependent on the colour of *E. aphyllum* shoots.

Micromorphological and ultrastructural studies of *Viola odorata* [pos. 11] showed the presence of nectaries - the appendages of anthers directed to the spur. Their surface was covered with papillae. Nectar was secreted by modified stomata, concentrated mainly on the apex of nectary.

In the publication [pos. 12], 4 species within the *Dactylorhiza incarnata/maculata* complex were selected for the study. Secretory activity in the epidermis at the entrance to the spur (probable osmophores) and internal epidermis of spurs (probably trace nectar) in *Dactylorhiza incarnata*, *D. maculata* subsp. *fuchsii* and *D. majalis* subsp. *majalis*. The secretion was absent on papillae of *D. maculata* subsp. *maculata*, although the presence of poorly developed microchannels, vesicles in the periplasmic space and plastoglobuli in plastids may be responsible for the production of a small amount of scent or nectar. Results of the research showed that the examined taxa of *Dactylorhiza* were well separated by their fragrance profiles, which may be important in the isolation of flowers against pollinating insects. Vanillin precursors, mainly observed in *D. incarnata* and *D. majalis* subsp. *majalis* are considered important in attracting potential pollinators.

7. **Kowalkowska AK**, Margońska HB, Kozieradzka-Kiszkurno M (2010) Comparative anatomy of the lip spur and additional lateral sepal spurs in a three-spurred form (f. *fumeauxiana*) of *Anacamptis pyramidalis*. *Acta Biologica Cracoviensia Series Botanica* 52(1): 13-18.
8. **Kowalkowska AK**, Margońska HB, Kozieradzka-Kiszkurno M, Bohdanowicz J (2012) Studies on the ultrastructure of a three-spurred *fumeauxiana* form of *Anacamptis pyramidalis*. *Plant Systematics and Evolution* 298: 1025-1035.
9. **Kowalkowska AK**, Kostelecka J, Bohdanowicz J, Kapusta M, Rojek J (2015) Studies on floral nectary, tepals' structure and gynostemium morphology of *Epipactis palustris* (L.) Crantz (*Orchidaceae*). *Protoplasma* 252(1): 321-333.
10. Świączkowska E, **Kowalkowska AK** (2015) Floral nectary anatomy and ultrastructure in mycoheterotrophic plant – *Epipogium aphyllum* Sw. (*Orchidaceae*). *The Scientific World Journal* 2015: 1-11.
11. Wiśniewska N, Bohdanowicz J, **Kowalkowska AK** (2015). Micromorphology and ultrastructure of the floral nectaries of *Viola odorata* L. (*Violaceae*). *Modern Phytomorphology* 7: 59-66.
12. Naczka AM, **Kowalkowska AK**, Wiśniewska N, Haliński ŁP, Kapusta M, Czerwicka M (2018) Floral anatomy, ultrastructure and chemical analysis in *Dactylorhiza incarnata/maculata* complex (*Orchidaceae*). *Botanical Journal of the Linnean Society* 187(3): 512–536, DOI: <https://doi.org/10.1093/botlinnean/boy027>

B. Analysis of anatomic features of gynostemium

Published works concern the use of anatomical features of gynostemium in studies of autogamy in *Dendrobium biflorum* [pos. 13] and *Epipogium aphyllum* [pos. 14]. For the first time, unique forms of autogamy were noted in the studies of autogamy of *D. biflorum* [pos. 13]: pollen grains germinated directly from the anther locules, passing by the stigma or falling down or sliding down to the stigmatic surface. Pollen tubes were visible in the stylar canal. This form of autogamy can be a pollination method on the Society Islands (and even throughout French Polynesia), when there are no pollinating insects or high in the mountains, where there are difficult environmental conditions. In *E. aphyllum* [pos. 14], the most important observation of mine was the germination of pollen grains grouped in tetrads after two days of hand pollination of the flower. In the third and fourth day, there was a mass growth of pollen tubes along the stylar canal and at the entrance to the ovary.

- 13. Kowalkowska AK, Margońska HB (2012)** Notes on the self-pollination of *Dendrobium biflorum*. *Acta Societatis Botanicorum Poloniae* 81(3): 223-228.
- 14. Krawczyk E, Rojek J, Kowalkowska AK, Kapusta M, Znanińska J, Minasiwicz J (2016)** Evidence for mixed sexual and asexual reproduction in the rare European mycoheterotrophic orchid *Epipogium aphyllum*, *Orchidaceae* (ghost orchid). *Annals of Botany* 118(1): 159-172.

C. Cytologic and micromorphological analysis in relation to taxonomic and/or phylogenetic studies in *Orchidaceae*

[Pos. 15] includes a chapter in a monograph, in which I included a description of preparation of formulations for scanning microscopy. [Pos. 16] contains a description of morphological and/or micromorphological features of the lip and gynostemium of *Vargasiella venezuelana* and *Warrea costaricensis* relevant in taxonomic and phylogenetic studies. [Pos. 17] includes the research of *Paphiopedilum canhii* karyotype and macro- and micromorphological studies of gynostemium, lips and leaf surface in selected representatives of subgenera and *Paphiopedilum* sections to demonstrate that *P. canhii* forms an independent phylogenetic line.

- 15. Kowalkowska AK (2013)** Scanning Electron Microscopy (SEM), in: Taxonomic redefinition of subtribe *Malaxidinae* (*Orchidales*, *Malaxidae*). Margońska HB, Kowalkowska AK, Górniak M, Rutkowski P. Koeltz Publishing House, 691pp., rozdział w monografii.
- 16. Szlachetko DL, Górniak M, Kolanowska M, Mytnik-Ejsmont J, Kowalkowska AK, Koliński T (2014)** Taxonomic position and phylogeny of the genus *Vargasiella* (*Orchidaceae*, *Vandoideae*) based on molecular and morphological evidence. *PLoS ONE* 9(6): e98472. DOI: 10.1371/journal.pone.0098472.
- 17. Górniak M, Szlachetko DL, Kowalkowska AK, Bohdanowicz J, Canh CX. (2014)** Taxonomic placement of *Paphiopedilum canhii* (*Cypripedioideae*; *Orchidaceae*) based

on cytological, molecular and micromorphological evidence. *Molecular Phylogenetics and Evolution* 70: 429-441.

5.3. Other aspects of scientific activity

I presented the results of my research at national and international conferences in the form of 8 oral presentations and 16 posters. I participated in a total of 12 projects, for which it was obtained funding from the funds of the Ministry of Science and Higher Education and later the National Science Center (3 projects) and funds from the University of Gdańsk (9 projects). In 4 projects, I was a supervisor, including 1 of the NCN project. In 4 projects, I was an executor, including 1 from the Ministry of Science and Higher Education and 1 supervisory grant. In 4 projects, I was the content supervisor (auxiliary supervisor function in two PhDs). Twice I received the Team Award of the Rector of the University of Gdańsk for the monograph and series of scientific publications. In the years 2005-2013, five times I spent on short-term research internships in research centers in Great Britain and Austria.

A detailed list of my scientific, didactic and popularizing achievements can be found in Appendix 3.

Agnieszka Kowalkowska