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Original article

Floral nectaries from *Limodorum abortivum* (L) Sw and *Epipactis atropurpurea* Rafin (Orchidaceae): ultrastructural changes in plastids during the secretory process

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Summary — Ultrastructural features of the floral nectaries of 2 orchid species, *Limodorum abortivum* (L) Sw and *Epipactis atropurpurea* Rafin, were compared. In particular, ultrastructural changes were followed in the plastids throughout the process of nectar secretion. There is evidence that plastids play an important role in this process. Before secretion begins, plastids of epidermal and subepidermal cells of the nectaries of both species, accumulated large quantities of starch. However, during the phase of nectar secretion, the plastids were found to contain little if any, starch, suggesting that starch degradation had augmented nectar sugar production. Plastoglobuli were evident during all developmental stages. The tubular reticulum found in plastids of pre-secretory nectaries was still evident during secretion, but was then found to contain an unknown, dense osmiophilic material. The floral nectary of *L abortivum* is sucrose dominant, whereas that of *E atropurpurea* is hexose rich. Therefore, the differences in nectar-carbohydrate composition are apparently not explainable by the similar ultrastructural changes of their floral nectaries. Further investigation is required to determine the biochemical basis for the disparity in nectar sugar composition between these 2 species.

***Epipactis atropurpurea* Rafin / *Limodorum abortivum* (L) Sw / nectary / orchid / plastid / starch**

Abbreviations used in the figures: CW = cell wall; CWp = cell-wall pit; CWi = cell-wall ingrowths; D = dictyosome; ER = endoplasmic reticulum; M = mitochondrion; N = nucleus; NC = nectariferous cells; OD = osmiophilic droplets; P = plastid; PR = plastid reticulum; S = secretion; St = starch; V = vacuole; VB = vascular bundles.

INTRODUCTION

The Orchidaceae is one of the largest and most evolved families in the angiosperms. A large number of genera within this family possess both floral and extra-floral nectaries. Extra-floral nectaries secrete nectar on the outside of buds or inflorescences when the flowers are developing (van der Pijl and Dodson, 1966). Floral nectaries are particularly important due to their adaptative significance related with the ability to attract pollinators. The morphology and location of floral nectaries in the Orchidaceae are variable: a) shallow and cup-like, at the base of the labellum; b) in long spurs, produced either from the fused sepals or from the base of the labellum; c) long, tubular and embedded in the base of the flower alongside the ovary; and d) on the side-lobes or along the central groove of the labellum (see van der Pijl and Dodson, 1966).

Epipactis atropurpurea and *Limodorum abortivum* are 2 examples of this diversity. The labellum of *E atropurpurea* is divided into 2 parts: the outer part (epichile) is tongue-shaped, whereas the proximal part (hypochile) has the shape of a concave gutter. On the other hand, in *L abortivum* each flower has a long, thin and curved nectary spur produced from the internal portion of the labellum. In continuation of our work on these orchids (Pais and Chaves das Neves, 1980; Pais *et al*, 1986; Pais, 1987; Figueiredo and Pais, 1992) we present, in this paper, a study comparing the ultrastructural aspects of the plastids at different stages during the nectar secretion process.

MATERIALS AND METHODS

Plant material

Flowers of *L abortivum* and *E atropurpurea*, of different developmental stages, were collected from plants growing in the field, at Cotovia (Sesimbra, Portugal).

Transmission electron microscopy

Cross-sections of the floral nectaries at different stages of development (pre-secretory, secretory and post-secretory stages), were fixed overnight in 2% glutaraldehyde (GA) in 0.1 M sodium cacodylate buffer, pH 7.2, at 4°C. The material was rinsed thoroughly in the same buffer and postfixed with 2% OsO_4 (aqueous solution) for 2 h. After dehydration in a graded acetone series, the material was embedded in Epon-Araldite according to Mollenhauer (1964). Ultrathin sections were stained with uranyl acetate/lead citrate (Reynolds, 1963), and observed with a Jeol 100C electron microscope at 80 kV.

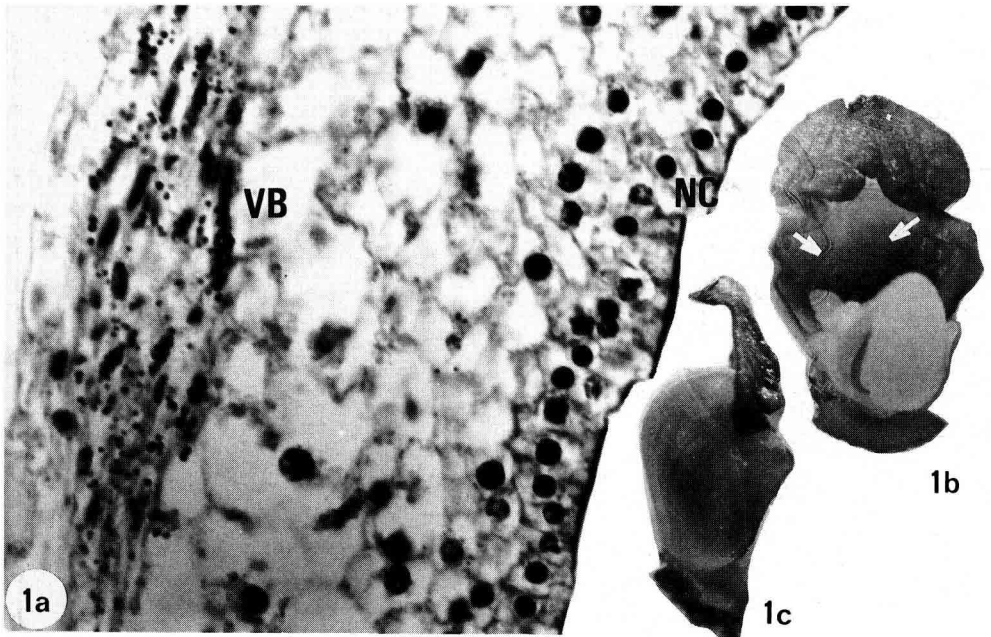
Light microscopy

Semi-thin sections from material fixed within GA/OsO_4 as above were stained with Methylene Blue-Azure A-Safranin (Warmke and Lee, 1976).

Scanning electron microscopy

The material was fixed with GA/OsO_4 as above, rinsed in water and plunged into liquid propane,

Plate I. Fig 1a. Cross-section of *E atropurpurea* nectary. Notice the nectariferous cells (NC), and the vascular bundles (VB) (x 300). **Fig 1b,c.** Scanning electron micrographs of *E atropurpurea* nectary (arrows) in top **(b)** and longitudinal **(c)** view (x 12). **Fig 2a.** Semi-thin cross-section of the young nectary spur of *L abortivum.* Notice the cells of the nectariferous epidermal layer (NC), and the vascular bundles (VB) appearing in the middle zone of the parenchyma tissue (x 800). **Fig 2b.** Scanning electron micrograph of a cross-section of *L abortivum* spur (x 48).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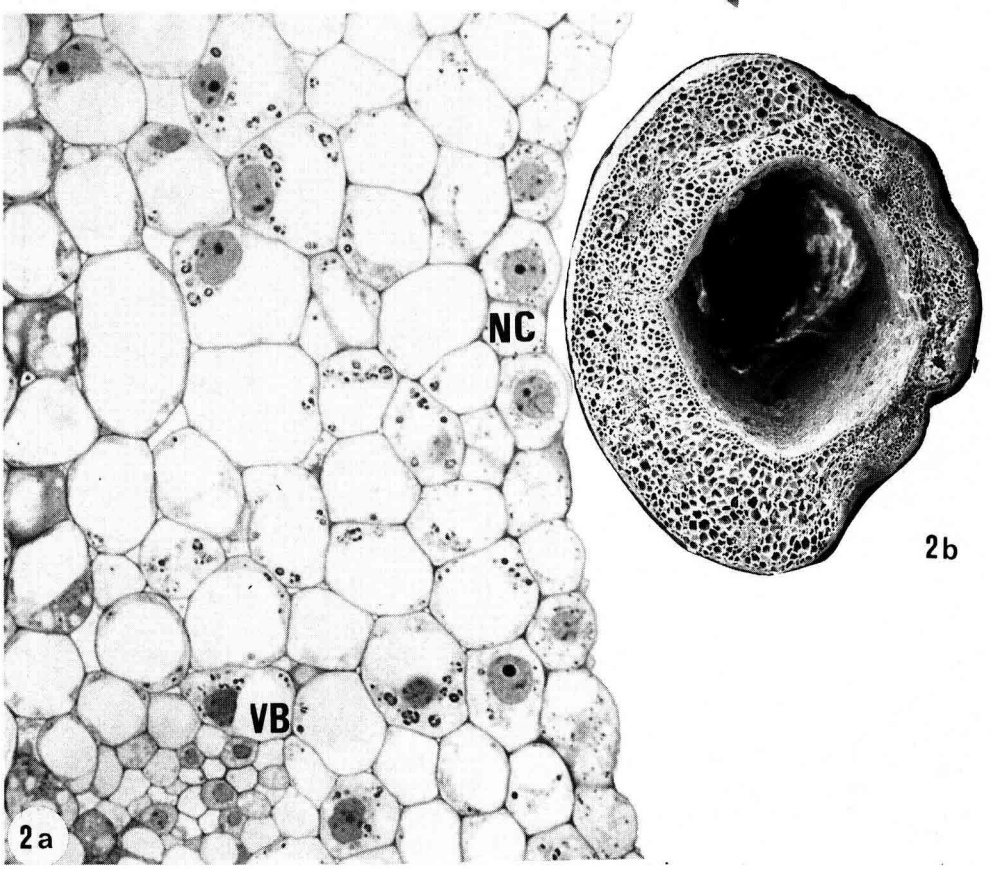
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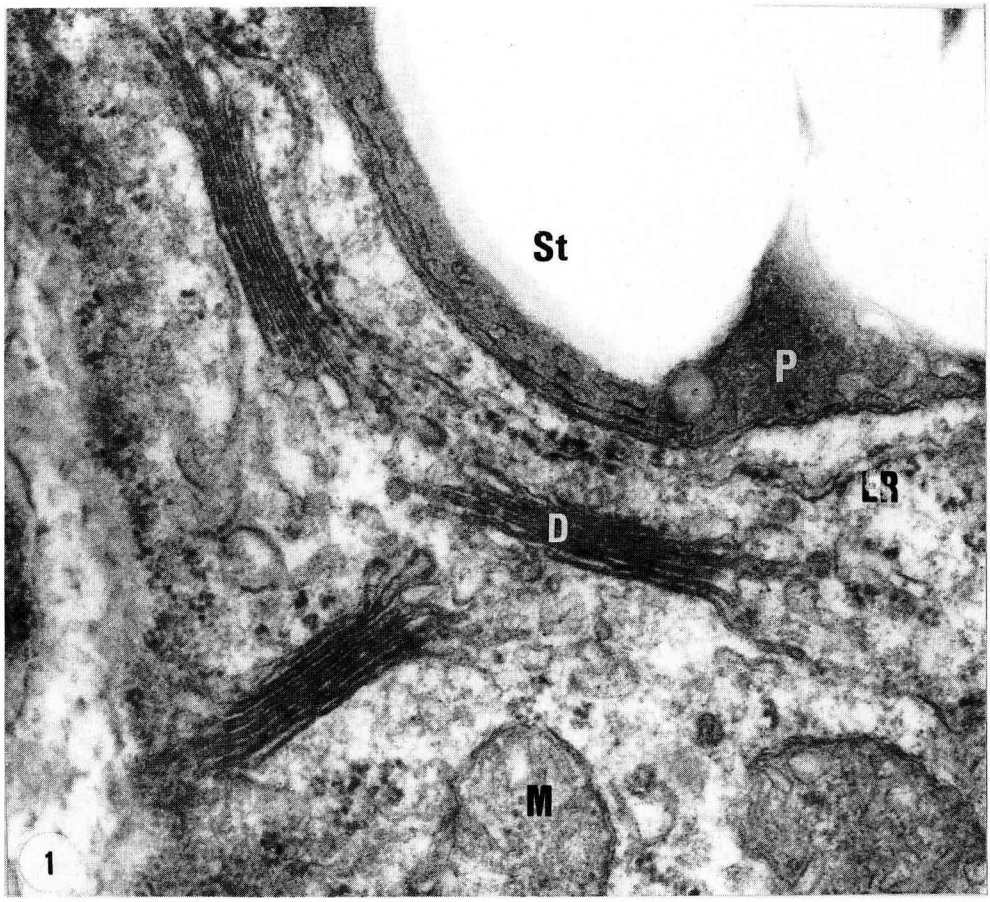
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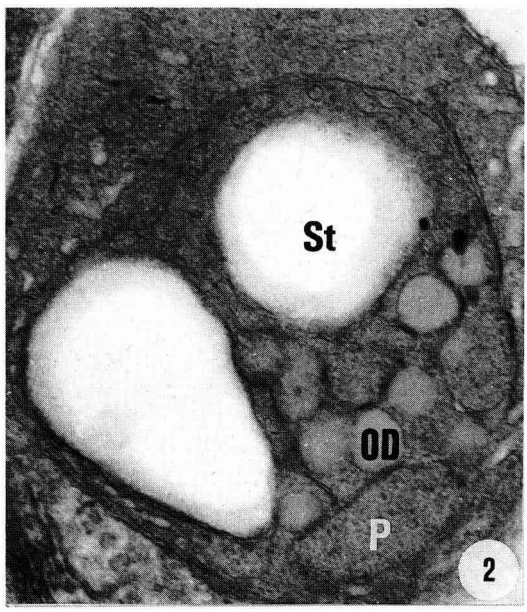
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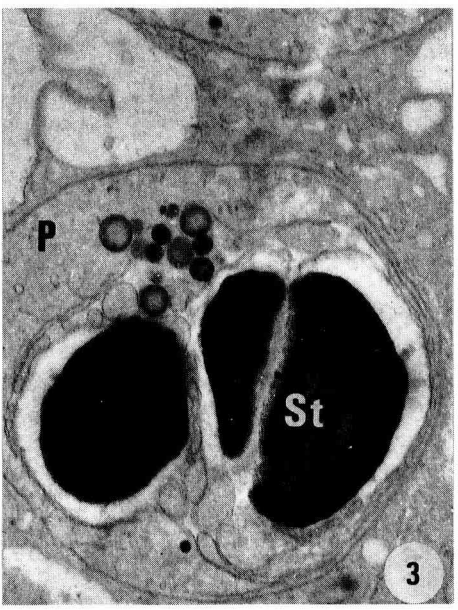
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subcooled in liquid nitrogen. Specimens were freeze-dried at –65°C for 4 d in a Freeze-dryer Polaron E 5300. Dried specimens were sputter-coated with gold in a Polaron E 5350 and observations were made at 15 kV using a Jeol JSM T220 scanning electron microscope.

RESULTS

Epipactis atropurpurea

The internal portion of the floral nectary of *E atropurpurea* has the shape of a concave gutter (plate I, fig 1b, c). In longitudinal section a nectariferous layer and several layers of parenchyma cells can be seen (plate I, fig 1a).

Both the nectariferous and parenchyma cells of *E atropurpurea* contained, in the pre-secretory stage, numerous amyloplasts filled with starch grains (plate II, fig 1–3). Plastoglobuli and a peripheral reticulum were also evident in these plastids. Dictyosomes and endoplasmic reticulum occurred in the cytoplasrn (plate II, fig 1).

At the secretory stage, a reticulum with osmiophilic content was common in the plastids (plate III, figs 1, 2). At this stage, little or no starch was present inside such plastids. Dictyosomes and ER profiles were abundant both in nectariferous (plate III, fig 1) and parenchyma cells. Cell-wall ingrowths were profuse, but were present only along the external walls of the nectariferous cells (plate III, fig 3).

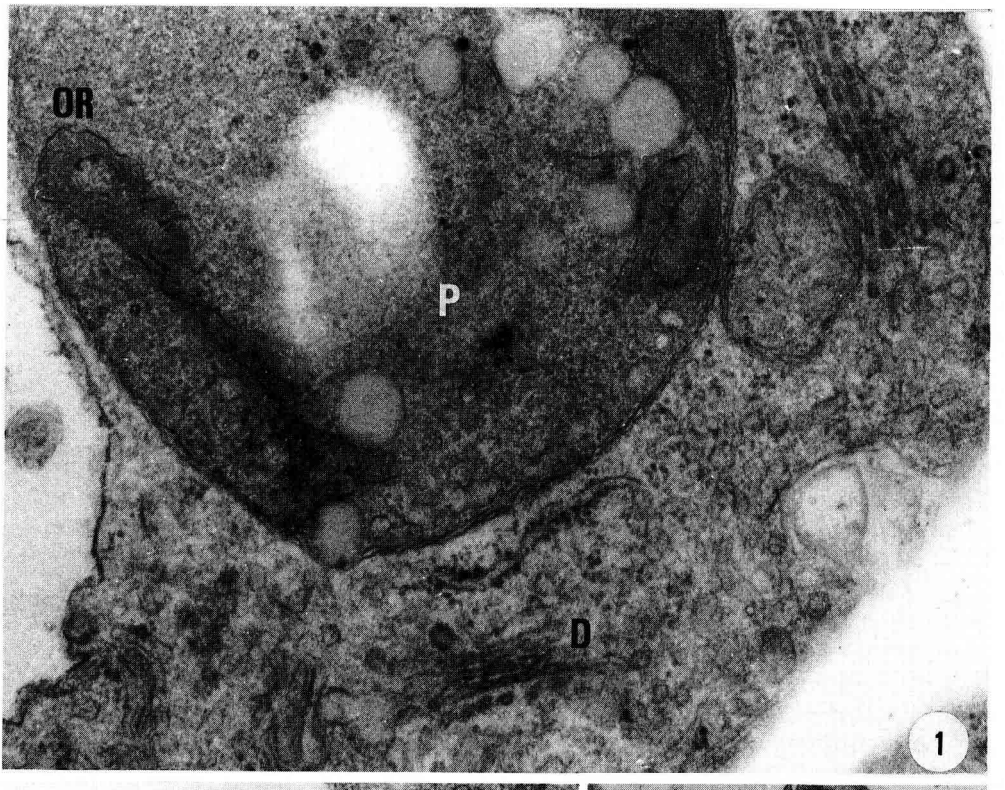
Limodorum abortivum

Like in *E atropurpurea*, when observed in cross-section, the floral nectary from *L abortivum* consists of an internal epidermal cell layer of nectariferous cells surrounding the secretory cavity and various layers of parenchyma cells (plate I, fig 2a,b).

At the pre-secretory stage the nectariferous cells of *L abortivum* showed numerous polymorphic plastids with a dense stroma, an osmiophilic reticulum, some plastoglobuli and starch grains (plate IV, figs 1, 2). During this stage, the parenchyma cells contained numerous amyloplasts with several starch strains filling almost the entire organelle (plate IV, fig 3).

During the secretory stage starch disappeared from the plastids of nectariferous cells and the plastid reticulum appeared filled with osmiophilic contents (plate IV, fig 4; plate V, fig 1). In the cytoplasm of these cells numerous osmiophilic droplets could be seen. Cell-wall pits were also frequently observed between adjacent nectariferous and parenchyma cells (plate V, fig 2). In the nectariferous cells, ER was profuse (plate V, fig 3) with numerous profiles adjacent to plastids (plate V, fig 1). Dictyosomes were also abundant (plate V, fig 3). Later in the secretory stage, the apparent exudation from nectariferous cells of some osmiophilic droplets was observed (plate V, fig 4). The exudation of the nectar components into the secretory cavity occurred by disruption of the cuticle (plate V, fig 5).

Plate II. *E atropurpurea*, pre-secretory stage. **Figs 1–3.** Electron micrographs of sections of nectariferous cells of buds showing numerous plastids (P) with large starch grains (St). Dictyosomes (D) and endoplasmic reticulum profiles (ER) were also frequent (fig 1 x 80 000, fig 2 x 20 000, fig 3 x 8 000).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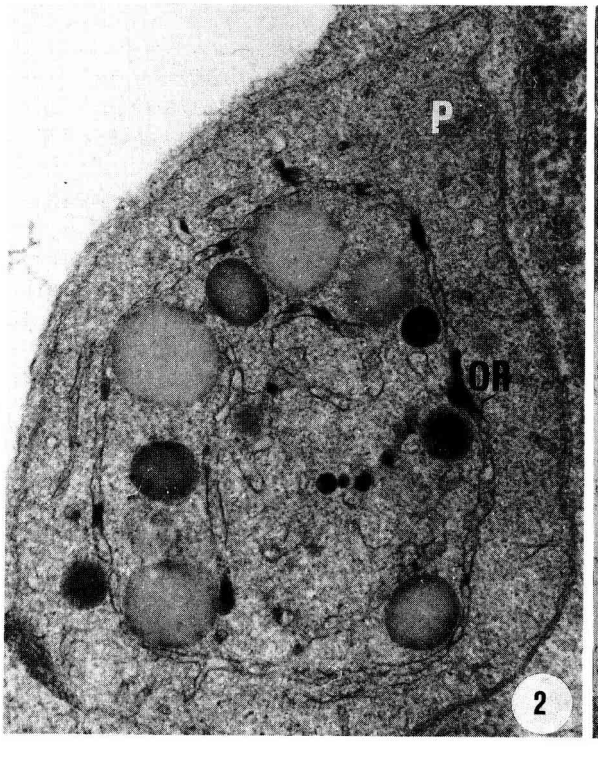
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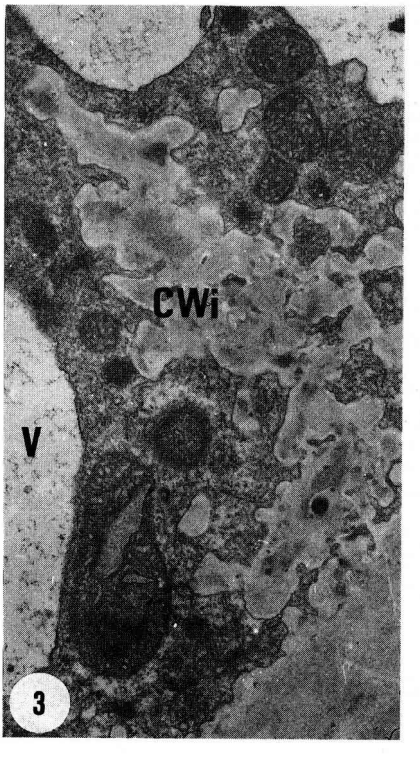
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DISCUSSION

Previous studies on the chemical composition of the floral nectars of *E atropurpurea* and *L abortivum* have indicated clear differences. *L abortivum* has been classified as belonging to a fairly primitive group within the evolutionary sequence of orchids, since its nectar contains mainly sucrose and a low total amino-acid concentration compared to the open *E atropurpurea* nectary (Pais and Chaves das Neves, 1980; Pais *et al,* 1986).

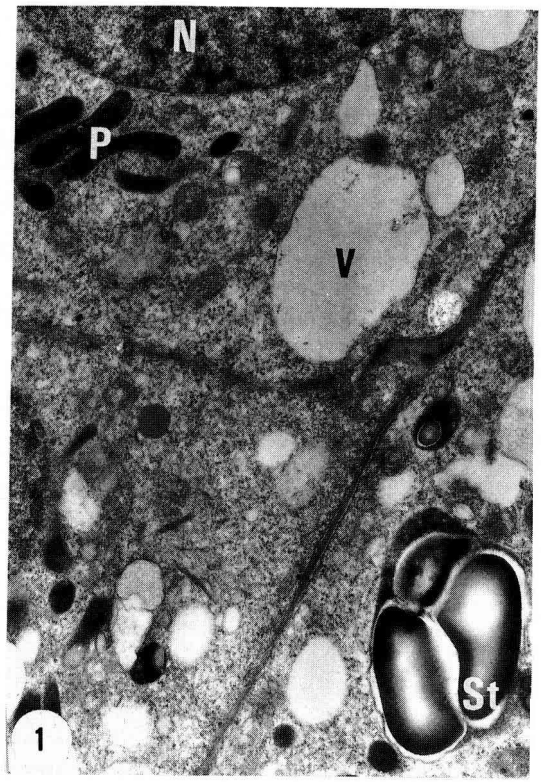
From the ultrastructural point of view the main feature of the nectariferous and parenchyma cells of these orchids were the changes observed in the plastids during the secretion process (Pais, 1987; Figueirido and Pais, 1992). In the presecretory stage, both the nectariferous and parenchyma cells of *E atropurpurea* showed numerous starch grains. This type of plastid could only be seen in the parenchyma cells of *L abortivum*. In the nectariferous cells of *L abortivum*, plastids with an osmiophilic reticulum and small starch grains were found. This type of polymorphic plastids with dense stroma and osmiophilic reticulum are uncommon in nectariferous tissues but have been described in many other secretory structures, especially in those secreting monoterpenes (Fahn, 1988).

The accumulation of nectar both in the nectary of *E atropurpurea* and in that of *L abortivum* occurred simultaneously with starch degradation, which might indicate that the starch accumulated in both parenchyma and nectariferous cells is the source of some of the secreted sugars. Also common to both glands is the presence of a plastid reticulum. Nevertheless, these similar ultrastructural features are not in agreement with the nectar/sugar ratios of both glands: the floral nectar of *L abortivum* is sucrose dominant (Pais *et al,* 1986), whereas that of *E atropurpurea* is hexose rich (Pais and Chaves das Neves, 1980). Therefore, further investigation is required to determine the basis for the differences in the nectar/sugar composition. Probably these differences will reside in the type and amount of enzymes involved in sugar transformations in both glands.

Numerous osmiophilic droplets could be seen in the plastids and cytoplasm of the nectariferous cells in both nectaries. Although the chemical nature of this component is not clear, the appearance of similar osmiophilic droplets has been interpreted as corresponding to the presence of lipids or phenolic material in different types of floral (Peterson *et al,* 1979; Meyberg and Kristen, 1981; Kronestedt *et al,* 1986, Sawidis *et al,* 1989) and extra-floral nectaries (Clair-Maczylajtys and Bory, 1983).

In the outer walls of the nectariferous cells of *E atropurpurea* cell-wall ingrowths could be found, forming a thick labyrinthine layer. These wall ingrowths are common in other outer walls of the secretory cells of nectaries (Durkee *et al,* 1981; Fahn, 1988), nevertheless they were not observed in the *L abortivum* nectary.

Plate III. *E atropurpurea*, secretory stage. **Figs 1–2.** Detail of the plastids (P) of the nectariferous cells of *E atropurpurea* showing osmiophilic reticulum (OR) (fig 1 x 50 000, fig 2 x 40 000). **Fig 3.** Aspect of the cell-wall ingrowths (CWi) frequent in the nectary of *E atropurpurea* (x 12 000).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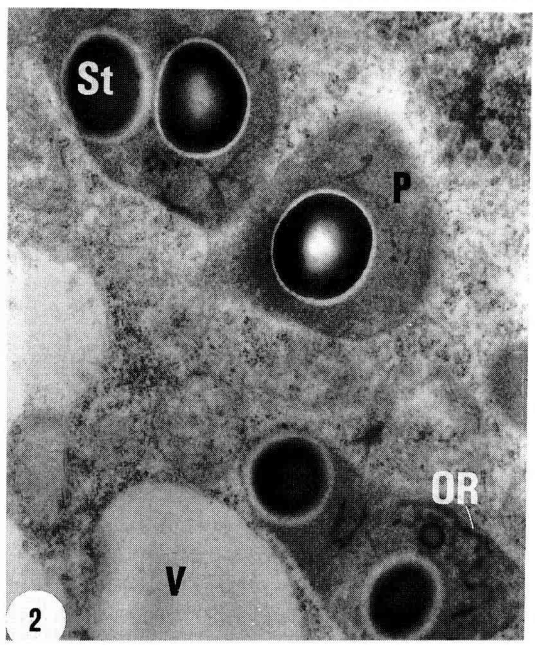
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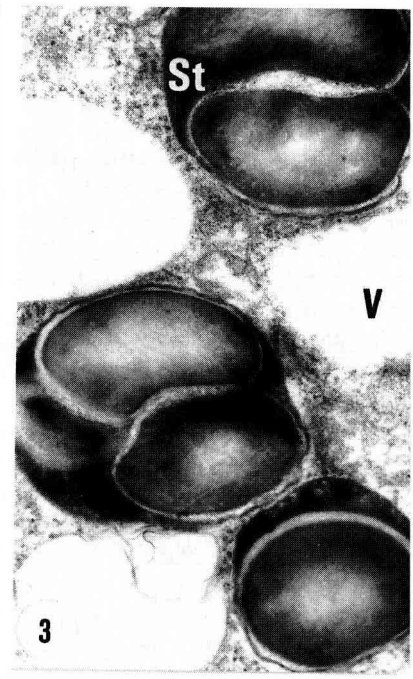
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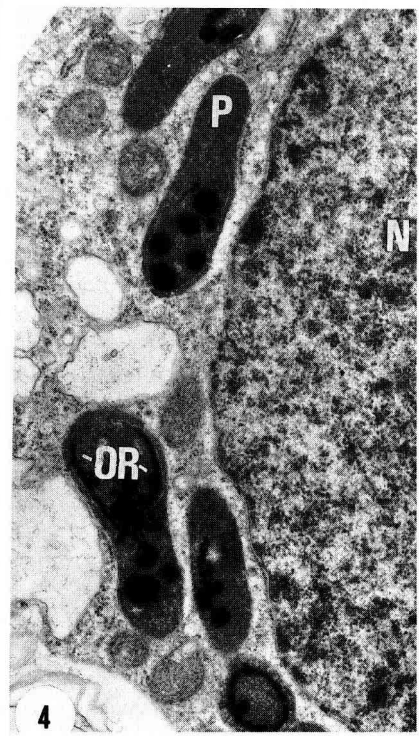
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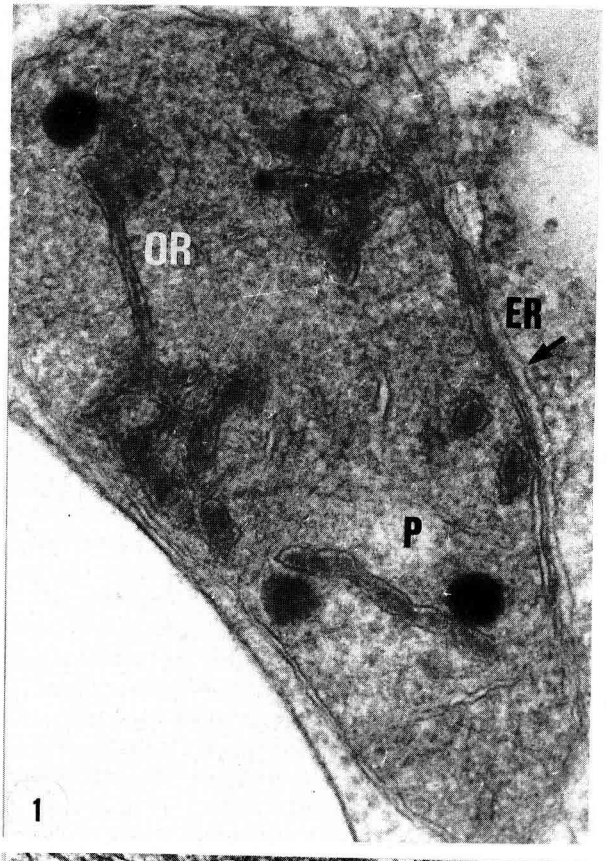
Résumé — Nectaires floraux de *Limodorum arbortivum* (L) Sw et d'*Epipactis atropurpurea* Rafin (Orchidaceae) : modifications de l'ultrastructure des plastides au cours du processus de sécrétion. Nous avons comparé les caractéristiques ultrastructurales de la sécrétion nectarifère de 2 espèces d'orchidées. Des coupes dans les nectaires floraux ont été réalisées à divers stades de développement (avant, pendant et après la sécrétion), fixées avec 2% de glutaraldéhyde, postfixées durant 2 h avec une solution aqueuse de tétroxyde d'osmium (OsO_4) à 2% et étudiées en microscopie électronique à transmission et à balayage. La partie interne du nectaire floral d'*E atropurpurea* a la forme d'une gouttière concave (planche I, fig 1a,b,c). Le nectaire floral de *L abortivum* montre une couche cellulaire épidermique interne de cellules nectarifères parenchymateuses (planche I, fig 2a,b). Avant la sécrétion, les cellules nectarifères et parenchymateuses d'*E atropurpurea* présentent de nombreux amyloplastes remplis de grains d'amidon (planche II, figs 1, 2, 3). Pendant la sécrétion, des structures tubulaires avec un contenu osmiophile se rencontrent fréquemment dans les plastides (planche III, figs 1, 2). À ce stade on trouve peu ou pas d'amidon à l'intérieur de ce type de plastides. La paroi des cellules nectarifères présentent de nombreuses invaginations (plante III, fig 3). Avant la sécrétion, les cellules nectarifères de *L abortivum* présentent de nombreux plastides polymorphes avec des structures tubulaires osmiophiles, des stroma denses, quelques plastoglobules et de petits grains d'amidon (planche IV, figs 1, 2). Au cours de ce stade, les cellules parenchymateuses montrent de nombreux amyloplastes avec plusieurs grains d'amidon remplissant presque entièrement l'organite (planche IV, fig 3). Durant la sécrétion l'amidon disparaît des plastides des cellules nectarifères et les structures tubulaires apparaissent emplies d'un contenu osmiophile (planche IV, fig 4 ; planche V, fig 1). On observe fréquemment des renfoncements dans la paroi cellulaire entre les cellules nectarifères et parenchymateuses voisines (planche V, fig 2). Dans les cellules nectarifères, le reticulum endoplasmique est abondant (planche V, fig 3) avec de nombreux diverticules contigüs aux plastides (planche V, fig 1). Plus tard on peut observer l'exudation du nectar dans la cavité sécrétrice, formée par déchirement de la couche externe de la cuticule (planche V, fig 5). L'ultrastructure des cellules nectarifères et parenchymateuses suggère que les plastides jouent un rôle important dans la sécrétion du nectar. Chez *L abortivum* le nectar accumulé dans l'espace sous-cuticulaire est déversé dans la cavité nectarifère par la rupture de la couche externe de la cuticule. Chez *E atropurpurea* l'exudation du nectar se fait à travers les pores

Plate IV. *L abortivum*, pre-secretory stage (figs 1–3); secretory stage (fig 4). **Fig 1.** Electron micrograph of a section of the nectary spur showing nectariferous (left) and parenchyma (bottom right) cells. In the cytoplasm of the nectariferous cells numerous small plastids (P) are evident, while in the parenchyma cells the amyloplasts contained several starch grains (St) filling almost the entire organelle (x 8 000). **Fig 2.** Detail of the plastids (P) of the nectariferous cells showing starch grains (St) and osmiophilic tubular structures (OR) (x 16 000). **Fig 3.** Section of parenchyma cells showing amyloplasts with several starch grains filling the organelles almost entirely (x 12 500). **Fig 4.** Detail of the plastids of the nectariferous cells without starch grains but with osmophilic reticulum (OR) (x 12 000).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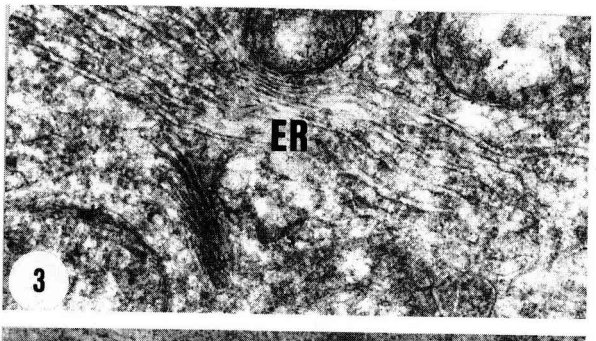
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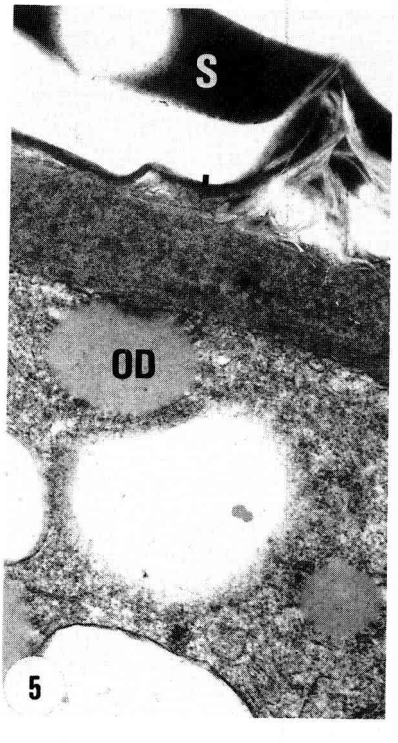
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dation du nectar se fait à travers les pores de la cuticule.

Orchidaceae / *Epipactis atropurpurea* / *Limodorum abortivum* / nectaire / plastide / amidon

Zusammenfassung — Florale Nektarien bei *Limodorum abortivum* (L) Sw und *Epipactis atropurpurea* Rafin (Orchidaceae): Ultrastrukturelle Veränderungen in den Plastiden während des Sekretionsprozesses. Wir führten eine vergleichende Untersuchung über die Ultrastruktur und die chemische Zusammensetzung der Nektarsekretion in Blüten bei zwei Orchideenarten, *Limodorum abortivum* und *Epipactis atropurpurea* durch. Querschnitte der Nektarien wurden in verschiedenen Entwicklungsstadien (vor, während und nach der Sekretion) für die Transmission- und Rasterelektronenmikroskopie in 2% Glutaraldehyde fixiert und 2 Stunden mit 2% wässeriger Osmiumtetroxidlösung nachfixiert. Der innere Teil der Nektarien von *E atropurpurea* hat die Form einer konkaven Rinne (Tafel I, Abb 1a,b,c). Die Nektarien von *L abortivum* weisen eine innere epidermale Zellschicht aus nektarerzeugenden Drüsenzellen auf, die mit verschiedenen Lagen von Parenchymzellen das Sekretreservoir umgibt (Tafel I, Abb 2a,b). Sowohl die Drüsen- als auch die Parenchymzellen von *E atropurpurea* zeigten im Stadium vor der Sekretion viele mit Stärkekörnern gefüllte Amyloplasten (Tafel II, Abb 1, 2, 3). Im sekretorischen Stadium traten vermehrt tubuläre Strukturen mit osmiophilem Inhalt in den Plastiden auf (Tafel III, Abb 1, 2). In diesem Stadium enthielten die Plastiden nur noch wenig oder keine Stärke mehr. Die Zellwand der Drüsenzellen wiesen zahlreiche Einstülpungen auf (Tafel III, Abb 3). Vor Beginn der Sekretion enthielten die nektarerzeugenden Zellen von *L abortivum* zahlreiche polymorphe Plastiden mit tubulären osmiophilen Strukturen, dichtem Stroma, einigen Plastoglobuli und kleinen Stärkekörnern (Tafel IV, Abb 1, 2). Gleichzeitig wiesen die Parenchymzellen zahlreiche Amyloplasten mit einigen Stärkekörnern auf, die fast die ganze Organelle ausfüllen (Tafel IV, Abb 3). Während der Sekretion verschwand die Stärke aus den Plastiden der Drüsenzellen und die mit osmiophilen Inhalt gefüllten tubulären Strukturen traten auf (Tafel IV, Abb 4; Tafel V, Abb 1). Häufig wurden Vertiefungen der Zellwand zwischen aneinander grenzenden Drüsen- und Parenchymzellen beobachtet (Tafel V, Abb 2). In den nektarerzeugenden Zellen war das Endoplasmatische Reticulum sehr gut entwickelt (Tafel V, Abb 3), zahlreiche Ausläufer grenzten an die Plastiden. Später konnte die Ausscheidung einiger Nektarprodukte in der Zelle beobachtet werden (Tafel V, Abb 4). Die Ausscheidung der Nektarkomponenten in das Sekretreservoir

Plate V. *L abortivum*, secretory stage (figs 1–4); post-secretory stage (fig 5). **Fig 1.** Detail of a plastid of nectariferous cells showing tubular osmiophilic structures and plastoglobuli in the stroma. Notice an ER profile closely associated with the plastic envelope (↓) (x 60 000). **Figs 2–5.** Sections of nectariferous cells of *L abortivum* spur showing some ER profiles and osmiophilic droplets (OD) in the cytoplasm. **Fig 2.** Notice the cell-wall pit (CWp). **Fig 3.** Conspicuous endoplasmic reticulum (ER) of nectariferous cell. **Fig 4.** Plastids (P) without starch. Note the apparent release of osmiophilic material to the cell wall (right). **Fig 5.** Some secreted material (S), released into the nectary spur through some disruption zones (↓), appears over the cuticule (fig 2 x 20 000, fig 3 x 28 000, fig 4 x 28 000, fig 5 x 26 000).

erfolgte durch Aufreißen der äußeren Kutikulaschicht (Tafel V, Abb 5). Die Ultrastruktur der Drüsen- und Parenchymzellen läßt vermuten, daß die Plastiden eine wichtige Rolle bei der Nektarsekretion spielen. Bei *L abortivum* sammelt sich der Nektar in einem subkutikularen Raum und wird durch Aufreißen der oberen Kutikulaschicht in das Nektarreservoir abgegeben. In *E atropurpurea* erfolgt die Nektarausscheidung durch Poren in der Kutikula.

Orchidaceae / *Epipactis atropurpurea* / *Limodorum abortivum* / Nektarium / Plastide / Stärke

REFERENCES

Clair-Maczylajtys D, Bory G (1983) Les nectaires extrafloraux pédicellés chez l'*Ailanthus glandulosa*. *Can J Bot* 61, 683-691

Durkee L, Gaal D, Reisner W (1981) The floral and extrafloral nectaries of *Passiflora*. I. The floral nectary. *Am J Bot* 68, 453-462

Fahn A (1988) Secretory tissues in vascular plants. *New Phytol* 108, 229-257

Figueiredo AC, Pais MS (1992) Ultrastructural aspects of the nectary spur of *Limodorum abortivum* (L). *Ann Bot* 70, 325-331

Meyberg M, Kristen U (1981) The nectaries of *Aptenia cordifolia*, ultrastructure, translocation of ^{14}C-labelled sugars, and a possible pathway of secretion. *Z Pflanzenphysiol* 104, 139-147

Mollenhauer HH (1964) Plastic embedding mixtures for use in electron microscopy. *Stain Technol* 39, 111-115

Pais MS, Chaves das Neves H (1980) Sugar content of the nectar exudate of *Epipactis atropurpurea*. *Apidologie* 11, 39-45

Pais MS, Chaves das Neves H, Vasconcelos AMP (1986) Amino-acid and sugar content of the nectar exudate of *Limodorum abortivum* and *Epipactis atropurpurea*. *Apidologie* 17, 125-136

Pais MS (1987) Ultrastructure des nectaires floraux d'*Epipactis atropurpurea* et production du nectar. *Ann Sci Bot* 8, 17-28

Peterson RL, Scott MG, Miller SL (1979) Some aspects of carpel structure in *Caltha palustris*. *Am J Bot* 66, 334-342

van der Pijl L, Dodson CH (1966) *Orchid Flowers. Their Pollination and Evolution*. University of Miami Press, Miami, FL, USA

Reynolds ES (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J Cell Biol* 17, 208-212

Sawidis T, Emeftheriou EP, Tsekos I (1989) The floral nectaries of *Hibiscus rosasinensis*. III. A morphometric and ultrastructural approach. *Nord J Bot* 9, 63-71

Warmke HE, Lee SLJ (1976) Improved staining procedures for semithin epoxy sections of plant tissues. *Stain Technol* 51, 179-185