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Senescence of unfertilised flowers in *Epiphyllum* hybrids

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Abstract

Epiphyllum hybrids served as a model for the study of reserve remobilisation from unfertilised flowers to the mother stem tissues. Early phases of the tube senescence characterised by wilting were connected with degradation and transfer of reserve substances to the somatic organs of the mother plant. The degradation process began in perianth and stamens and continued through the successive zones (receptacular, pericarpellar and pedicellar) of the flower tube. The phloem-mediated backward substance transport was naturally indicated by the red pigment of the perianth – cactorubin, while integrity of cells and tissues and green colour of the flower tube were still preserved. For the later phases of senescence the loss of permeability and successive breaking of the cell integrity, connected with the colour change of the tube from green to red was evident. The functioning of vascular bundles especially their phloem parts conducting dissolved substances to the sinks in mother stem organs were preserved until late stages of senescence. The recycling and remobilisation of nutrients from all parts of unfertilised ovary and ovules may be considered as a part of the life strategy in the family *Cactaceae* as well as in other taxa evolutionarily adapted to life in extreme environmental conditions.

Additional key words: cactorubin, perianth, substance remobilisation.

Introduction

Programmed cell death (PCD) seems to be an integral part of all developmental processes in plant organs including flowers. The physiological and biochemical data show that especially slowly proceeding forms of PCD such as endosperm degradation (Young and Gallie 1999), aerenchyma formation (He *et al*. 1996) or leaf (Smart 1994) and flower senescence (Rubinstein 2000, O'Donoghue *et al.* 2002) are connected with controlled degradation, remobilisation and reutilisation of cell components. Nutrients from the ageing floral organs (petals) are remobilised and transported to developing ovary in flowers. This type of petal senescence is pollination dependent (Hadfield and Bennett 1997, O´Neill 1997).

The same fate as the petals befall other floral organs which have lost their role in the post-pollination development of the flower, such as sepals, stamens, styles with stigmas, *etc*. Their senescence is connected with the remobilisation of reserve substances from dying parts of flower to the ovary or in the absence of fertilisation to other sinks in the mother plant. Termination and re-utilisation of such ephemeral floral organs is regulated by changes in specific gene expression as it was stated for remobilisation of substances from petals (Woodson 1987, Lawton *et al.* 1990). However, we still do not understand the senescence completely: little is known about the co-ordination of senescence at tissue or organ level (Grbić 2002), relatively small attention has been given to the remobilisation of substances from ovaries and ovules of unfertilised flowers. This process was studied in some details in an endemic shrub *Daphne arbuscula* living in severe life conditions on single locality in Muránska planina mountains in Slovakia (Erdelská 1999). Every year the majority of flowers of this species drop unfertilised. The cell death of individual tissues follows in a regular order. It begins in the nucellus and inner integument, proceeds in the outer integument and ends in the ovary wall. This interesting succession respecting the well preserved function of vascular bundle terminals corresponds with that of programmed recycling

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process of leaves under senescence of deciduous trees (Gan and Amasino 1997). Considering the xerothermic habits and extremely severe life conditions of this species it seems to be possible to consider the recycling of nutrients from ovary and ovules as part of its life strategy (Erdelská and Ovečka 2002).

Some *Epiphyllum* hybrids from the family *Cactaceae* can be used as model objects for the study of reserve remobilisation from unfertilised flowers to the mother stem tissues. The senescence of unfertilised flowers starts uniformly after full anthesis and flower cell deterioration continues rapidly within few days. The perianth of the genus *Epiphyllum*, like some other members of the family *Cactaceae* contains the water-soluble red pigment cactorubin (Hegnauer 1964) from the group of betacyanins (Boke 1980). We found that the pigment cactorubin may serve as natural indicator of substance remobilisation and movement connected with the senescence before flower abscission.

Materials and methods

Epiphyllum plants commonly known today are actually hybrids of epiphytic cacti species native to the jungles of Central and South America, as well as Mexico. We cultivated *Epiphyllum* plants in control room conditions (room temperature, natural photoperiod). Anthesis, wilting of the flowers, as well as cell and tissue ageing were documented through the flowering period photographically in entire organs, in hand-made sections of fresh material and in chemically fixed and wax-embedded material. The fixation was performed using FAA (70 % ethyl alcohol, 40 % formaldehyde, 99 % acetic acid, 18:1:1). Samples after washing were dehydrated in ethyl alcohol and embedded in *Histoplast S* (*Serva*, Heidelberg, Germany). Sections of 7 μm thick were dewaxed and rehydrated prior the staining with PAS (Periodic Acid-Schiff) staining reaction. Resulted staining of starch and polysaccharides was observed using bright-field microscopy. The same staining method gives rise strong cell wall fluorescence under green excitation. For general protein staining sections were stained by 1 % acridin orange and observed under blue excitation radiation. Bright-field and fluorescence microscopy observations were performed with *Olympus BX 51* (Japan) microscope. Starch content and distribution in the fresh hand-made sections were observed after lugol (I+KI) staining.

Dry matter of flowers was determined gravimetrically. Some flowers were removed from the stem at the beginning of the wilting. After weighing they were fastened (fixed) on the thread hanging on the stem of the mother plant for the free desiccation. They were weighed again after two weeks as dried up after additional desiccation in desiccator. The separated control flowers were weighed after the natural flower abscission. Because exogenous application of gibberellins to flowers can induce partial development of fruits without the fertilisation, we used this approach to show changed pattern of tissue ageing in the tube. In selected flowers one drop of 1 % gibberellic acid (GA_3) was applied on the stigma and another one on the upper surface of the flower tube at the time of full anthesis. Treated flowers were observed 7 d after application.

Results and discussion

The structure of the flower at the anthesis: The *Epiphyllum* flower is represented by 4 - 6 cm long green tube bearing male and female generative organs and the rich red perianth 7 - 8 cm long (Fig. 2*a*). The tube is composed of numerous internodia resulting in the presence of numerous scale leaflets on its surface. The flower tube may be divided into three superposed zones (Fig. 1). The short basal *pedicellar zone* or stalk zone connects the tube to the stem of the mother plant. Ovary with numerous ovules is situated in the centre of superposed *pericarpellar zone*. The longest *receptacular zone* bears from inside-to-outside the long style with branched stigma, numerous stamens and more than thirty large but fine or delicate red perianth leaves (Buxbaum 1980).

Separate vascular bundles from each flower member (style, stamens, perianth leaves, scales, ovules) are

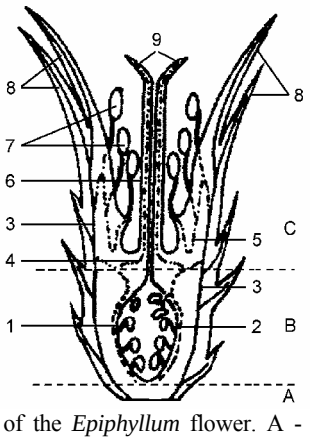

Fig. 1. Scheme of the *Epiphyllum* flower. A - pedicellar zone, B - pericarpellar zone, C - receptacular zone, 1 - ovules in the ovary, 2 - inner vascular bundle, 3 - axial vascular bundle, 4 - styllar vascular bundle, 5 - staminal vascular bundle, 6 - style, 7 - stamens, 8 - perianth, 9 - stigma (from Buxbaum 1980).

Fig. 2. Successive phases of the flower senescence. The flower at the anthesis (*a*), at the beginning of wilting (*b*), in the phase of cell disintegration (*c*), and shortly before the flower abscission (*d*). Wilted perianth and persisting tube of the flower 7 d after the application of GA_3 (*e*). *Arrows* in *a*, *b*, *c*, *d*, and *e* indicate the flower tube. Radial section through the pericarpellar zone at the beginning of substance remobilisation (*f*, *g*). Fresh material (*f*), fresh material stained by I + KI (*g*). Cactorubin-marked reverse flow is located in axial vascular bundles and mucilage cavities (*g*). Cactorubin in the phloem (*arrow*) of the mother stem tissue 5 cm below the attachment of the flower (*h*, fresh material). Degraded tissue of the pericarpellar zone shortly before the abscission of the flower (*i*, fresh material stained by I + KI).

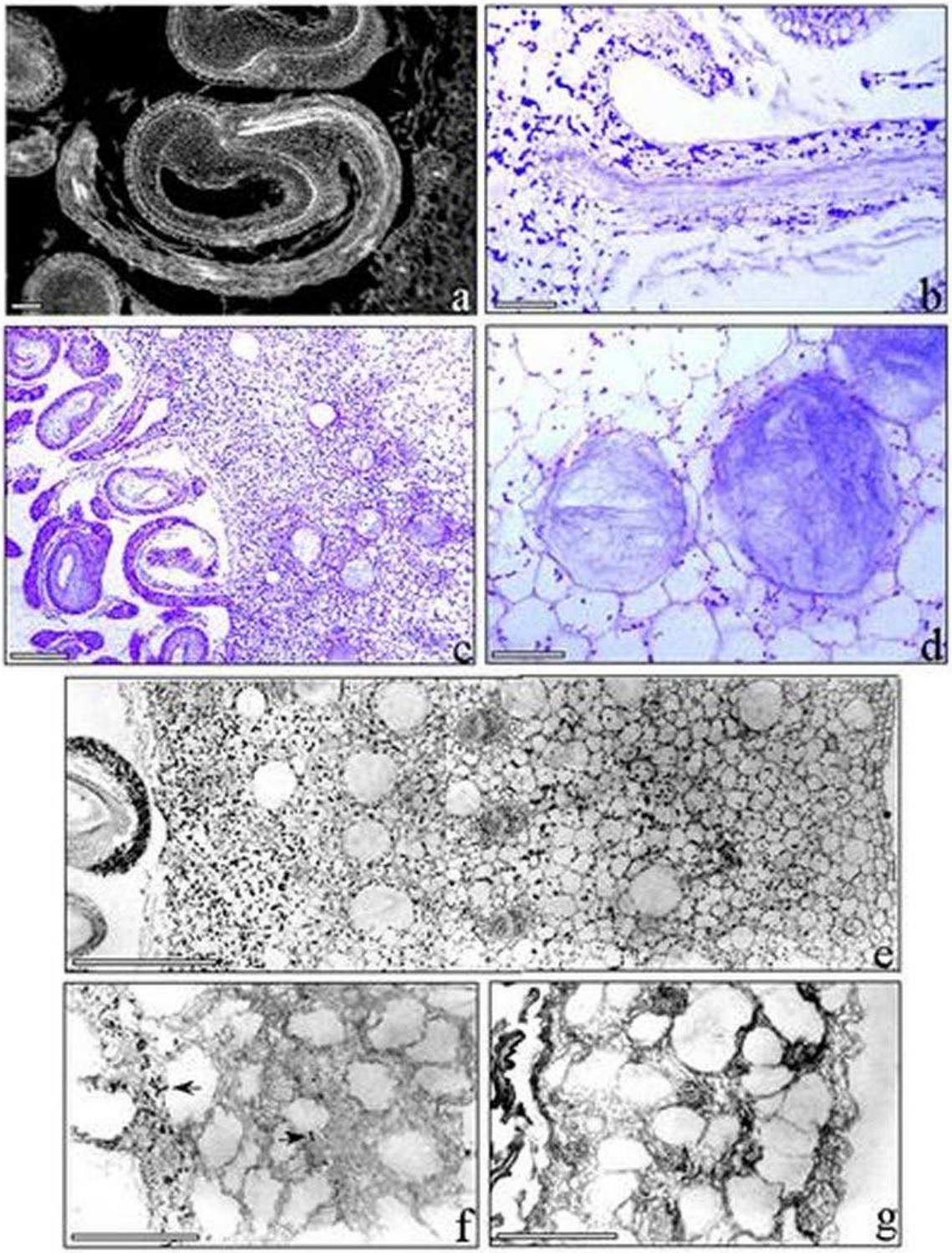

Fig. 3. Ovules and ground parenchyma of the tube at the anthesis. Hemianatropous, bitegmic and crassinucellate ovules with long funiculi (*a*). Connection of funicular bundles with the inner vascular bundles in the tube parenchyma (*b*). Starch reserves in ovules, funiculi and inner tube parenchyma at the anthesis (*c*). Lysigenic cavities are filled with mucilage fibrous content (*d*). Ground parenchyma of the tube at the anthesis and during senescence (*e*, *f*, *g*). Note the width of floral tube (left - ovary with ovules, right - epidermis of the tube), distribution of starch and size of mucilage cavities at the anthesis (*e*), before termination of massive remobilisation (*f*) and after the remobilisation period just before flower abscission (*g*). *Arrows* (*f*) indicate the rest of starch reserves around the inner and axial circle of vascular bundles. Periodic Acid-Schiff staining, bright-field (*b*, *c*, *d*, *e*, *f*, *g*), fluorescence (*a*). *Bar* = 100 μm (*a*, *b*, *d*), 500 μm (*c*, *e*, *f*, *g*).

situated in several circles at different levels of the tube (Figs. 1,4). They gradually unify to the main axial bundles in direction to the flower bottom. The unification is completed in the pedicellar zone of the flower tube (Fig. 1).

The embryological characteristic of *Epiphyllum* corresponds with that of other members of the family *Cactaceae* (Johri *et al.* 1992). The ovules in the ovary are hemianatropous, bitegmic, and crassinucellate (Fig. 3*a*). The micropyle is formed by the inner integument. The central cells of the relatively massive nucellar cap are prolonged longitudinally. The funiculi of the ovules are long and branched. Their vascular bundles are directly connected to small inner vascular bundles of the parenchyma in the pericarpellar zone of the flower tube (Fig. 3*b*). The structure of the placenta is not distinct.

The embryo sacs of *Polygonum* type are relatively large and highly vacuolized. The filiform apparatus of synergids is very distinct, antipodals are ephemeral. The starch reserves are present in great amount in funiculi and outer integuments of the ovules at the time of anthesis (Figs. 3*a*,*b*,*c*). Evidently lower is the amount of starch in other cells of the chalazal region of the ovules, in the inner integument, nucellus and in cells of the embryo sac (Erdelská and Ovečka 2002). Reserve accumulation in the form of starch is evident during the pre-fertilisation developmental phase not only in ovules but also in all tissues of the flower tube (Fig. 3).

The fleshy basic tissue of the tube in all three zones is formed by parenchymatous cells covered with epidermis on the tube surface. In the cross section of the tube the cell size of parenchyma is increasing in the direction from the centre to the surface of the tube, while the starch content at the time of anthesis is decreasing in the same direction (Figs. 3*e*,4*g*). Very prominent is the presence of mucilage cavities in whole parenchymatous part of the tube (Fig. 3*c*,*d*,*e*), increasing in size by lysigenic process during the flower senescence (Figs. 3,4). The presence of mucilage cells is characteristic for the whole family Cactaceae. The mucilage is an acidic polysaccharide and is believed to be involved in sugar storage, some protection functions, and water economy of plants. Transport of ions and water to and from these cells must be assumed (Trachtenberg and Mayer 1981a,b).

Senescence of unfertilised flowers: *Epiphyllum* is allogamic. In the absence of genetically suitable pollen the unpollinated and unfertilised flowers begin to wilt and degenerate usually on the third day of full anthesis. Wilting of unfertilised flowers is accompanied with remobilisation and transport of substances from perianth, *via* receptacullar zone, pericarpellar zone, and pedicellar zone of the flower tube to the stem part of plants. The red pigment cactorubin became a natural indicator of the remobilisation (Fig. 2). The course of cell and tissue degradation may be followed in some phases:

1) Wilting of the flower began with the change of its orientation from horizontal to the vertical one (Fig. 2*a*,*b*). At this time the flower tube was still green (Fig. 2*b*). Upward-directed flow of cactorubin began from perianth through the main axial bundles of the flower tube to the stem organs *via* phloem (Fig. 2*f*,*g*,*h*). The gradual disappearance of starch from ageing tissues of the green or later lightly pinking flower tube began (Fig. 3*f*). This period of starch loss was connected with dry matter decrease. The starch disappeared at first from outer parts of the tube parenchyma and at the latest from the parenchyma enclosing the vascular bundles close the ovary with ovules (Fig. 3*f*). The lysigenic mucilage cavities in the tube parenchyma enlarged by the successive disintegration of their neighbouring cells, starting with lysis of the cell walls (Fig. 4*a*,*b*,*c*,*d*).

2) Reserve translocation continued by degradation of cell contents within flower tube tissues proceeding in apex-to-base direction. This process was detectable by the colour change from pink-green to final red (Fig 2*c*). During the continuous polysaccharide degradation also the non-polysaccharide substances of flower tube tissues started to be remobilised. The sequential process of saccharide and non-saccharide substance remobilisation in *Epiphyllum* flower tube was detected in parenchyma cells surrounding the axial vascular bundles. Originally starch-filled parenchymatous tissues (Fig. 4*e*,*g*) lost starch and collapsed gradually, simultaneously with the enlargement of mucilage cavities (Figs. 4*f*,*h*). Unlike fast disintegration of surrounding parenchyma cells vascular bundle cell complexes showed higher structural preservation (Fig. 4*f*), what indicates functional participation of the axial vascular bundles in the substance translocation during this period. Axial and peripheral vascular bundles remained regularly arranged in contrast to almost collapsed flower parenchymatous tissue (Fig. 4*i*). At the end of this phase the cactorubin-marked flow penetrated degraded cell contents and stained the remaining tissues of the tube (Fig. 2*c*), and finally also ovary with ovules.

Cell death in ageing flowers was connected with changes in membrane permeability leading to acceleration of ion leakage as it was shown during PCD of daylily petals (Panavas and Rubinstein 1998). Reduced or lost membrane permeability caused the cactorubin spreading in the tissues of flower tube. This process proceeded also in vascular tissue in basipetal direction. However, in the *Epiphyllum* pedicellar zone and contiguous stem parts the red basipetal substance flow still continued through the phloem part of vascular tissues, indicating delay in their senescence (Figs. 4*f*,*i*).

In ageing daylily petals the vascular tissue was the place of increased peroxidase activity (Panavas and Rubinstein 1998). The authors indicated this peroxidase activity as a positive parameter of the functional state of vascular tissue in relation to the flow of reutilised substances. The delay in senescence of tissues close to vascular bundles during the senescence of rice

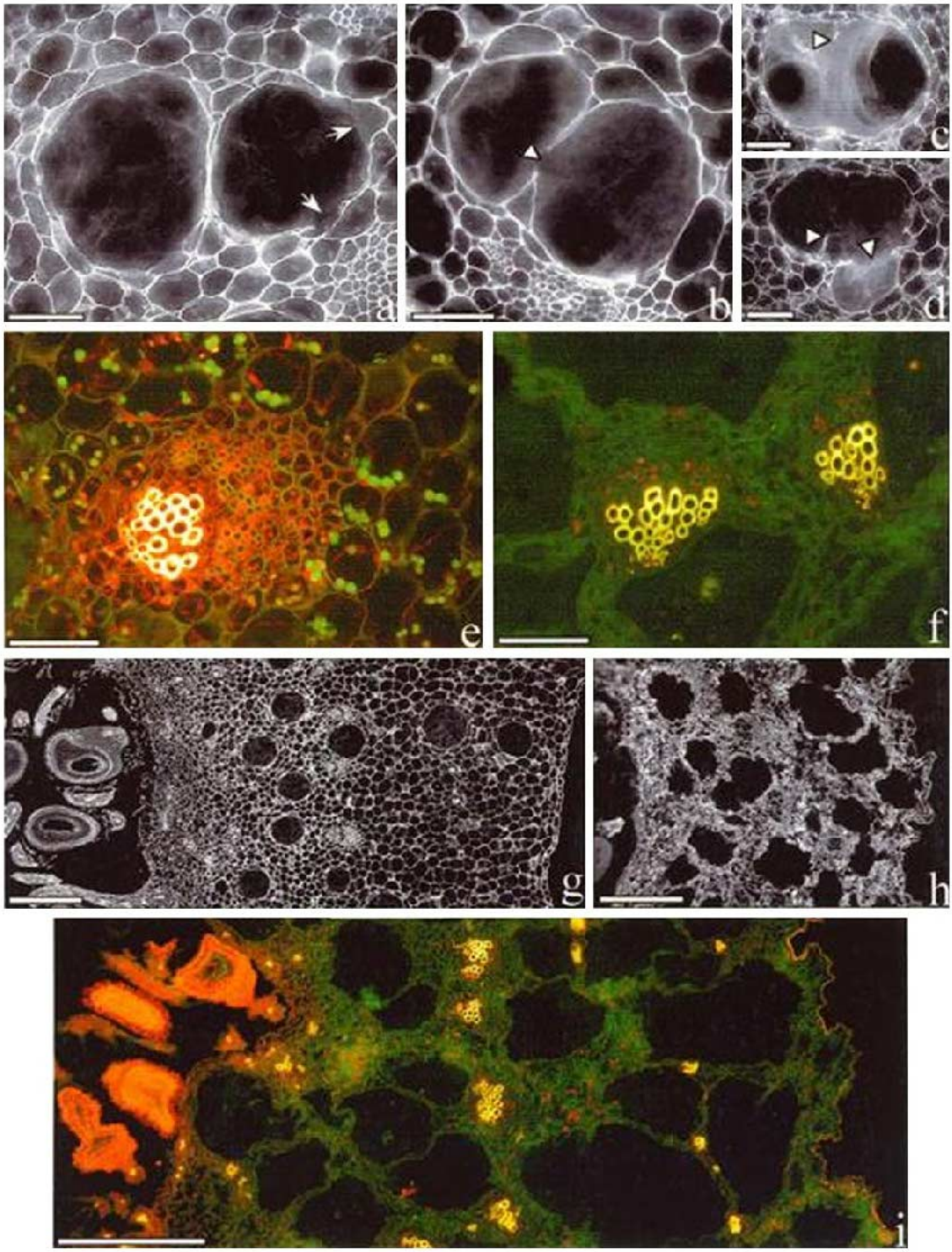

Fig. 4. Lysigenic degradation of ground parenchyma in the tube. The expansion of lysigenic cavities is supported by lysis of individual cell walls at the periphery of the cavity (*a*, *arrows*), and further by lysigenic fusion of cavities (*b*, *c*, *d*, *arrowheads*). Axial vascular bundle cell complex at the anthesis (*e*) and shortly before the end of their function (*f*). Structural changes of the tube tissue before substance remobilisation (*g*) and after the almost completed substance remobilisation (*h*). Arrangement of inner (ovular), central (axial) and outer (peripheral) vascular bundle circles in degrading pericarpellar zone (*i*). Periodic Acid-Schiff fluorescence (*a*, *b*, *c*, *d*, *g*, *h*), acridin orange fluorescence (*e*, *f*, *i*). *Bar* = 100 μm (*a* - *f*), 500 μm (*g* - *i*).

coleoptiles was interpreted in the context of remobilisation process (Inada *et al.* 1998). The functional state of vascular tissue has been characterised as long persisting in the senescence (Matile and Winkenbach 1971, Bieleski 1995), and translocation of most of the soluble sugars and amino acids from degrading daylily petals by phloem transport has been documented (Bieleski 1995). Substance remobilisation is a highly controlled process and particular class of genes encoding enzymes participating in the remobilisation from the flower was identified (Bleecker and Paterson 1997, O´Neill 1997, Do and Huang 1997, Panavas *et al.* 1999, Fernandez *et al.* 2000, Sugawara *et al.* 2002, Wagstaff *et al.* 2002, O'Donoghue *et al.* 2002, González-Carranza *et al.* 2002, Grbić 2002).

3) In the last phase of senescence the cell contents become totally exhausted (Fig. 2*d*,*i*). The width of the flower tube was considerably reduced in comparison to green flower tube (Figs. 3*e*,*g*, 4*g*,*h*). The abscission of the whole flower followed after total dehydration of cell wall remnants in all tissues (Fig. 2*d*), including ovary with ovules (Fig. 2*i*).

The flower and petal senescence in *Epiphyllum* exerted wilting as one of defined ways of petal termination in contrast to abscission of turgid petals (van Doorn 2001). In addition to macro- and microscopical documentations the substance transfer and remobilisation was confirmed also by changes in the flower mass by comparing the dry mass of remobilised flowers after their natural abscission with the dry mass of flowers artificially removed at the beginning of wilting. Dry mass of artificially detached flowers (0.405 ± 0.06 g) was almost doubled in comparison to flowers with natural senescence (0.235 ± 0.036 g). The results clearly showed that 42 % of dry mass was re-utilised.

Fertilisation makes difference between unfertilised flowers and flowers continuing in development in the mean of pattern of substance remobilisation. Unfertilised flowers are reutilised completely by mother plant, while dying organs like perianth become to be source for a newly established sink in fertilised flower – developing fruit with seeds. Parthenocarpy can mimic the fertilisation in respect of source-to-sink ratio. It is well known that setting and partial development of fruits can be induced by exogenous application of gibberellins to flowers (Gustafson 1960, George *et al*. 1984, Gillaspy *et al.* 1993, Swain and Olszewski 1996). After GA_3 application on the stigma and the upper surface of the flower tube at the time of full anthesis we observed substantially changed trend of substance remobilisation from the flower organs. The wilting and successive substance remobilisation were not affected in the perianth, stamens, style and finally also in unfertilised ovules. However, the tube tissues remained green and without any sign of wilting or degradation for about three months (Fig. 2*e*). The fate of tissues in prospective fruit was changed only by application of GA_3, which could indicate a basic nature of the regulation. In addition, physiological integrity of the tissues in the tube after GA_3 application prevented cactorubin spreading in the basal part of the tube, even it was transported to the stem vascular tissue. This observation further supports the conclusion that just a loss of membrane functions is responsible for the cactorubin leakage in late stages of tube senescence.

Conclusions: Nutrient substance investment to flowers is typical for sexual reproduction of Angiosperms. In the full absence of fertilisation and seed development a part of the material invested is remobilised and translocated backwards to some sinks in somatic organs of the mother plant through the process of controlled senescence. The substance translocation from unfertilised *Epiphyllum* flowers to the stem organs *via* phloem was distinctly identified by cactorubin, the red pigment of petals. The disappearance of starch from ageing tissues and dry matter decrease of entire flowers was characteristic for this process. The reserve remobilisation from unfertilised flowers may be considered as a part of the life strategy of plant species adapted to hard life conditions and/or restricted pollination, and a contribution to the tendency of continual reutilisation of all substances involved in the nutrient circulation in the nature.

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