The aquatic carnivorous plant Aldrovanda vesiculosa *(Droseraceae) exhibits altered developmental stages in male gametophyte*

Elisabetta Onelli, Mario Beretta, Alessandra Moscatelli, Marco Caccianiga, Michele Pozzi, Nadia Stroppa & Lubomír Adamec

Protoplasma
An International Journal of Animal, Fungal and Plant Cell Biology

ISSN 0033-183X
Volume 258
Number 1

Protoplasma (2021) 258:71-85
DOI 10.1007/s00709-020-01553-6

Springer

Protoplasma (2021) 258:71–85
https://doi.org/10.1007/s00709-020-01553-6

ORIGINAL ARTICLE

The aquatic carnivorous plant *Aldrovanda vesiculosa* (Droseraceae) exhibits altered developmental stages in male gametophyte

Elisabetta Onelli[1] · Mario Beretta[2] · Alessandra Moscatelli[1] · Marco Caccianiga[1,2] · Michele Pozzi[1] · Nadia Stroppa[1] · Lubomír Adamec[3]

Received: 26 May 2020 / Accepted: 31 August 2020 / Published online: 11 September 2020

Abstract

Aldrovanda vesiculosa (Droseraceae) is a rare aquatic carnivorous plant, distributed in Europe, Asia, Africa, and Australia. *Aldrovanda* populations can flower prolifically under favourable conditions, but seed set is very limited. We studied the structure of *Aldrovanda* pollen collected from flowers in different developmental stages (opened and non-opened anthers) from both European and Australian populations to elucidate pollination traits and the basis of poor seed set on the basis of microscopic observation of pollen and anther structure. Microscopic analyses of *Aldrovanda* pollen showed that this plant has pollen arranged in tetrads like other species in the Droseraceae family. In hydrated pollen, cytoplasmic protrusions originate from pores located along the equatorial wall of monads, and can develop into pollen tubes. Interestingly, pollen development from microspores occurs in open anthers, suggesting a delay of the developmental stages. In addition, pollen development displays altered sperm cell formation and precocious pollen germination. Precocious germination may characterize recalcitrant pollen, which naturally do not undergo dehydration before anthesis and remain partially hydrated, particularly in aquatic and wetland plants. These alterations of male gametophyte development could affect fertilization processes, and be the reason for the low reproductive capability of *Aldrovanda* observed both in the field and in cultures. Generally, reduced pollen longevity and very quick germination are considered an adaptation to aquatic or wet environments.

Keywords *Aldrovanda vesiculosa* · Pollen development · Pollen structure · Generative cell · Pollination · Anther structure

Introduction

Aldrovanda vesiculosa L. (Waterwheel plant, Droseraceae) is a rare and endangered aquatic carnivorous plant exhibiting spectacular, rapid movement of its snapping traps. *Aldrovanda* grows on a vast range of territories in Europe, Asia, Africa, and Australia and across various climatic zones—from temperate to tropical and subtropical ones—but its recent natural spread includes only around 50 known sites worldwide (Cross 2012; Adamec 2018). Plants are perennial, rootless, submerged, and free-floating below the water surface, growing in shallow, standing humic waters (Adamec 1995, 2018; Weber 1995; Cross 2012; Fleischmann et al. 2018). Its poorly branched linear shoots have a highly modular structure and are usually ca. 6–20 cm long, including a shoot apex and ca. 14–24 mature leaf whorls. *Aldrovanda* also exhibits distinct and steep morphological, growth, and physiological polarity (Adamec 2000, 2018; Cross 2012). The main feature of this polarity is the permanent, rapid apical shoot growth of 1–1.5 new leaf whorls a day, whereas the basal shoot segments continuously senesce and decay at a similar rate so that shoot length is generally constant ("conveyer belt"–like shoot growth system). New biomass is allocated only to branching and flowering (Adamec 2018). High frequency of branching indicates plant vigour and optimal ecological conditions and is the principal prerequisite for a high relative growth rate. New study on *Aldrovanda vesiculosa* has detailed some aspects of its anatomical structure and novel lifestyle (Atsuzawa et al. 2020).

Handling Editor: Handling Editor: Peter Nick

✉ Lubomír Adamec
lubomir.adamec@ibot.cas.cz

[1] Department of Biosciences, University of Milano, Via Celoria 26, 20133 Milan, Italy

[2] "Città Studi" Botanical Garden, Department of Biosciences, University of Milano, Via Celoria 26, 20133 Milan, Italy

[3] Institute of Botany of the Czech Academy of Sciences, Dukelská 135, CZ-379 82 Třeboň, Czech Republic

All temperate and (sub)tropical *Aldrovanda* populations can flower prolifically under favourable natural conditions and even set fertile seed (e.g. Adamec and Tichý 1997; Adamec 1999; Okada 2008; Cross 2012; Cross et al. 2016). Nevertheless, the plants mainly propagate vegetatively by apical formation of new branches, which may be fairly regular within each successive 5–7 leaf whorl (Adamec 2018). Generally, clonal aquatic plants with frequent branching of the mother shoot exhibit some of the lowest rates of generative reproduction (Herben et al. 2014), which applies well for flowering and seed set of mainly temperate *Aldrovanda* populations. Although several dozens of ecophysiological, biophysical, or genetic studies on *Aldrovanda* have been published so far (for the review, see Cross 2012; Adamec 2018), fewer studies have focused on morphological or ecological aspects of flowering and seed set or germination (e.g. Adamec and Tichý 1997, Adamec 1999; Cross et al. 2015, 2016), and only Sahashi and Ikuse (1973) and Okada (2008) addressed pollen morphology and/or pollination traits.

A single flower bud is initiated at the shoot apex underwater, but near the water surface (Okada 2008; Cross 2012; Adamec 2018). Its erect flower stalk develops on a modified leaf whorl without traps, gradually growing to 12–16 mm length, and at maturity, reaches the water surface. *Aldrovanda* flowers emerge slightly above the water only during anthesis, when they open. The open flower has a subglobose, superior ovary bearing five styles radially, alternating to five slender stamens with yellow anthers. As described by Okada (2008) and Cross (2012), it is important for pollination that some flowers have straight styles (with no contact with anthers), while in others, some styles are bent laterally towards the anthers. The estimated pollen to ovule ratio of 28.5 ± 4.6 indicates a strictly autogamous or self-fertilizing character, which was confirmed both experimentally (Okada 2008) and in closed aquaria without any insects (Adamec 2018). *Aldrovanda* has two different types of flowers which can be distinguished by the stage of floral development: autogamous (opened) and cleistogamous (closed; Adamec and Tichý 1997; Okada 2008; Cross 2012; Cross et al. 2016; Adamec 2018). Under suboptimal ecological conditions, only cleistogamous flowers are formed. All recorded observations confirm that pollination and seed set are only possible in opened flowers, but in temperate populations, both at natural sites and in outdoor cultures, full flower opening is only a few hours (2.5–3), mostly on afternoons when water temperature is highest (Adamec and Tichý 1997; Okada 2008; Cross 2012; Cross et al. 2016; Adamec 2018). High afternoon water temperature ca. > 27 °C is considered a crucial habitat factor, regulating flower opening and thus successful pollination and seed set (Cross 2012). It follows from various studies that the percentage of fertile capsules (floral success of individual flowers) with ripe seeds is extremely variable: from 0 to 53% (*cf.* Adamec and Tichý 1997; Adamec 1999; Okada 2008; Cross et al. 2016). The anthers open immediately after the petals opened and each anther (per stamen) contains 12–22 pollen tetrads (Okada 2008). Sahashi and Ikuse (1973) estimated the individual pollen grain diameter to be ca. 35 × 45 μm and the diameter of a pollen tetrad ca. 66 μm. The pollen grains are three-operculate and have echinate ornamentations (Chanda 1965; Sahashi and Ikuse 1973; Takahashi and Sohma 1982). Neither the longevity of *Aldrovanda* pollen nor pollen germination traits have been observed. It may be hypothesized, however, that it is very short pollen longevity that strongly limits the pollination and thus, the seed set. From 1 to 14 seeds are produced in each capsule (Adamec and Tichý 1997; Okada 2008; Cross 2012; Cross et al. 2016; Adamec 2018).

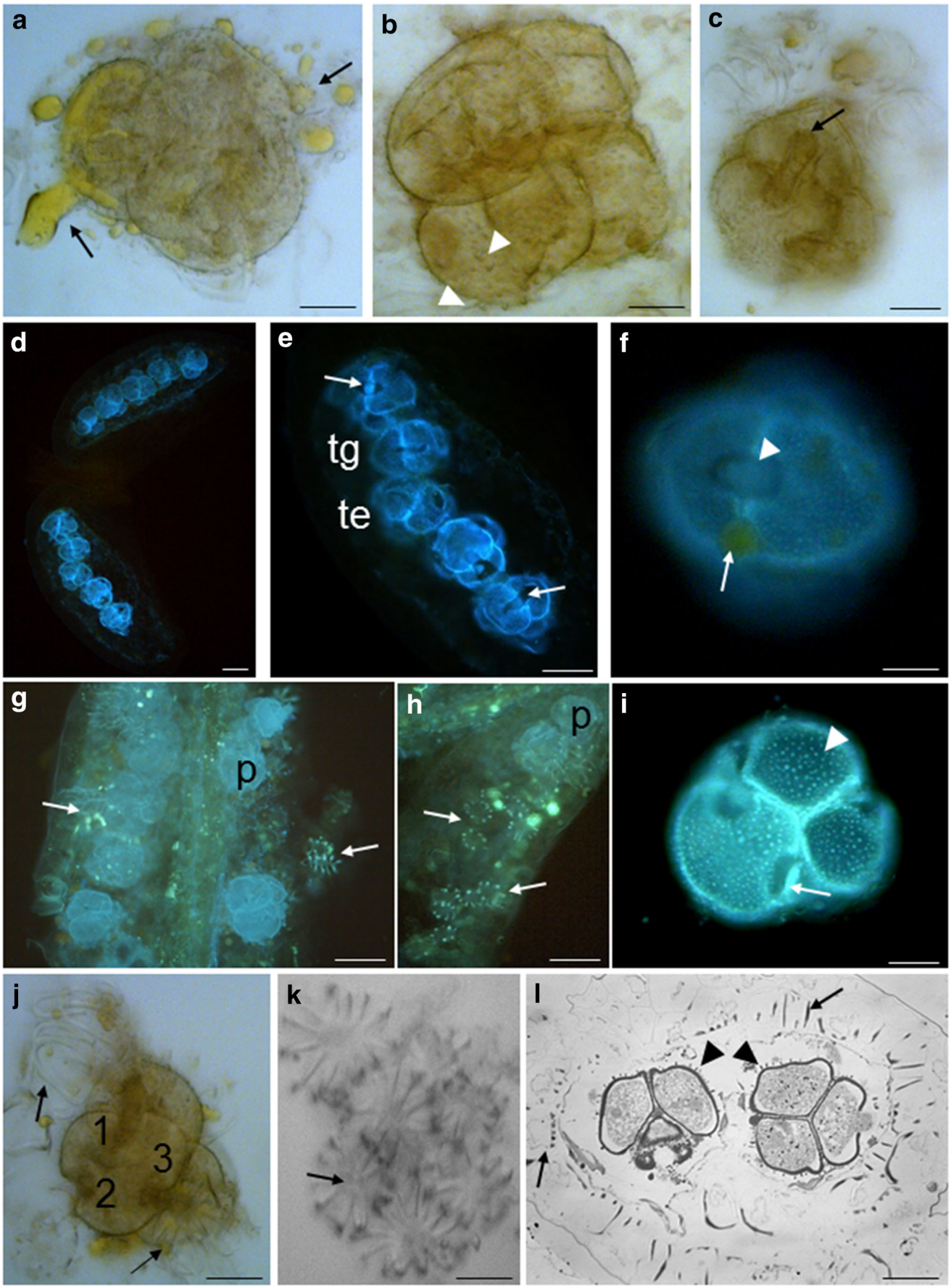
Our aim was to study *Aldrovanda* pollen structure in pollen collected from flowers of different ages and development stages (opened and non-opened anthers) in plants of several world populations, to elucidate the conserved pollination traits of *Aldrovanda* and the reason for its poor seed set. We show that several steps of pollen development that are altered, including pollen grains in which the generative cell/sperm cells (GC/SCs) are not internalized within the vegetative cell (VC), leading to a hypothesis that pollen tubes could not transport SCs to the embryo sac for fertilization. In addition, early pollen germination was detected within anthers, before pollen delivery. These results, together with the short duration of receptivity, could explain the reasons for low seed production and the high level of autogamy observed in *A. vesiculosa*.

Material and methods

Plant origin and sampling

Plants of several *Aldrovanda* populations were used for studying pollen structure. Opened flowers of temperate *Aldrovanda* (originated in SW Hungary; Elansary et al.

Fig. 1 *Aldrovanda vesiculosa* pollen morphology. **a, b, c**: pollen obtained from squashed anthers and observed by light microscopy. Pollen was organized in permanent tetrads of about 65 μm in diameter and arranged in tetrahedral (**a**) or tetragonal (**b**) arrangement. Pollen surface was covered by many drops of pollenkit (**a**; arrows). In dried pollen, the pores were locked by an operculum (**c**; arrows). **d, e, f**: autofluorescence analysis of dried anthers. Few tetrads were presents in the pollen sacs (about 5; **d**, **e**) and both tetrahedral and tetragonal tetrads were present in the same theca (**d**, **e**). The pores of two neighbouring monads appeared aligned along the equator of pollen grains (**e**, **f**; arrows). **g, h, i**: Anthers stained by Aniline Blue. Callose was not present in the tetrads (**g**, **i**). Hydrated pollen showed operculum retraction (**i**; arrow) and distal wall showed intectate and echinate exine (**i**; arrowhead). Callose-stained cell-wall ribs in endothecium cells (**g**, **h**; arrows). **j, k**: squashed anthers observed by LM. The ribs in endothecium cell walls were observed (arrows). Three pores were evident in monads (**j**; numbered as 1, 2, 3). **l**: Anther section showed thickness in endothecium cell wall (arrow) and pollen tetrads with baculae of echinate exine (arrowheads). Tg, tetragonal tetrad; Te, tetrahedral tetrad. Magnification bars: **a**, **b**, **f**: 10 μm; **d**, **e**, **g**, **h**: 50 μm; **c**, **i**, **j**, **k**: 20 μm; and **l**: 30 μm



2010) were collected from a pool in a sand-pit Cep I near Suchdol nad Lužnicí, South Bohemia, Czech Republic, in August 2016 (see Cross et al. 2016). The flowers were dried at ca. 25 °C and kept above silica gel before processing. Flowers of different stages of an *Aldrovanda* population (originated from E Poland; Elansary et al. 2010) were also collected from a fen pool at Karštejn, South Bohemia, Czech Republic (Cross et al. 2016) in July 2017. Tropical *Aldrovanda* plants from Leach Lagoon near Katherine, N.T., N Australia, and subtropical plants from Esperance Bay, W.A., SW Australia (see Elansary et al. 2010), were grown indoors in 3-litre aquaria (Sirová et al. 2003). The litter of robust sedges was used as the main cultivation substrate in these aquaria and the water was considered humic and oligotrophic.

Light and transmission electron microscopy

Flowers from cultivated N and SW Australian and naturalized E Polish populations were collected during June and July 2017, then immediately put in a HEM buffer (0.05-M HEPES, 1-mM $MgCl_2$, 5-mM EDTA, pH 7.4) and kept at 4 °C until processing. Six to 10 flowers were always collected for each flower category (opened or closed). Anthers were squashed and observed in a light microscope Leica DMRB equipped with a MC170HD camera.

To detect callose in the cell walls, dried flowers were stained with 0.1% decolorized Aniline Blue in 100-mM K_2HPO_4/KOH, pH 11, and observed with a Leica DMRB fluorescence microscope equipped with filter set A (Ex BP: 340–380, DM: 400, Em LP 425) and with a MC170HD camera. To observe sections of *A. vesiculosa* pollen, anthers were fixed in 4% formaldehyde in the HEM buffer. Afterwards, they were rinsed with HEM buffer and postfixed in 1% osmium tetroxide. After several rinses by the HEM buffer, samples were dehydrated in ethanol and embedded in LR White resin. Anthers were also fixed in a hypertonic concentration of HEM buffer (with 0.5-M HEPES) to induce plasmolysis. Samples were embedded in the LR White resin as described above.

Semi-fine sections (2 μm) and ultra-thin sections (80 nm) were obtained using a Reichert-Jung microtome. Semi-fine sections were stained by 1% Toluidine Blue and observed with a Leica DMRB light microscope. Ultrathin sections were stained with 3% uranyl acetate and observed with an EFTEM LEO 912AB transmission electron microscope (TEM; Zeiss) working at 100 kV.

Scanning electron microscopy

For SEM analysis, we used open *Aldrovanda* flowers from SW Australian and Hungarian populations and closed flowers from SW and N Australian populations. Pollen grains were acetolyzed according to Erdtman (1960), air dried, sputtered with 25 nm layer of gold in argon plasma (AGAR Automatic Sputter Coater B7341 equipped with a quartz crystal thickness monitor), and then studied using a Leo 1430 scanning electron microscope.

Results

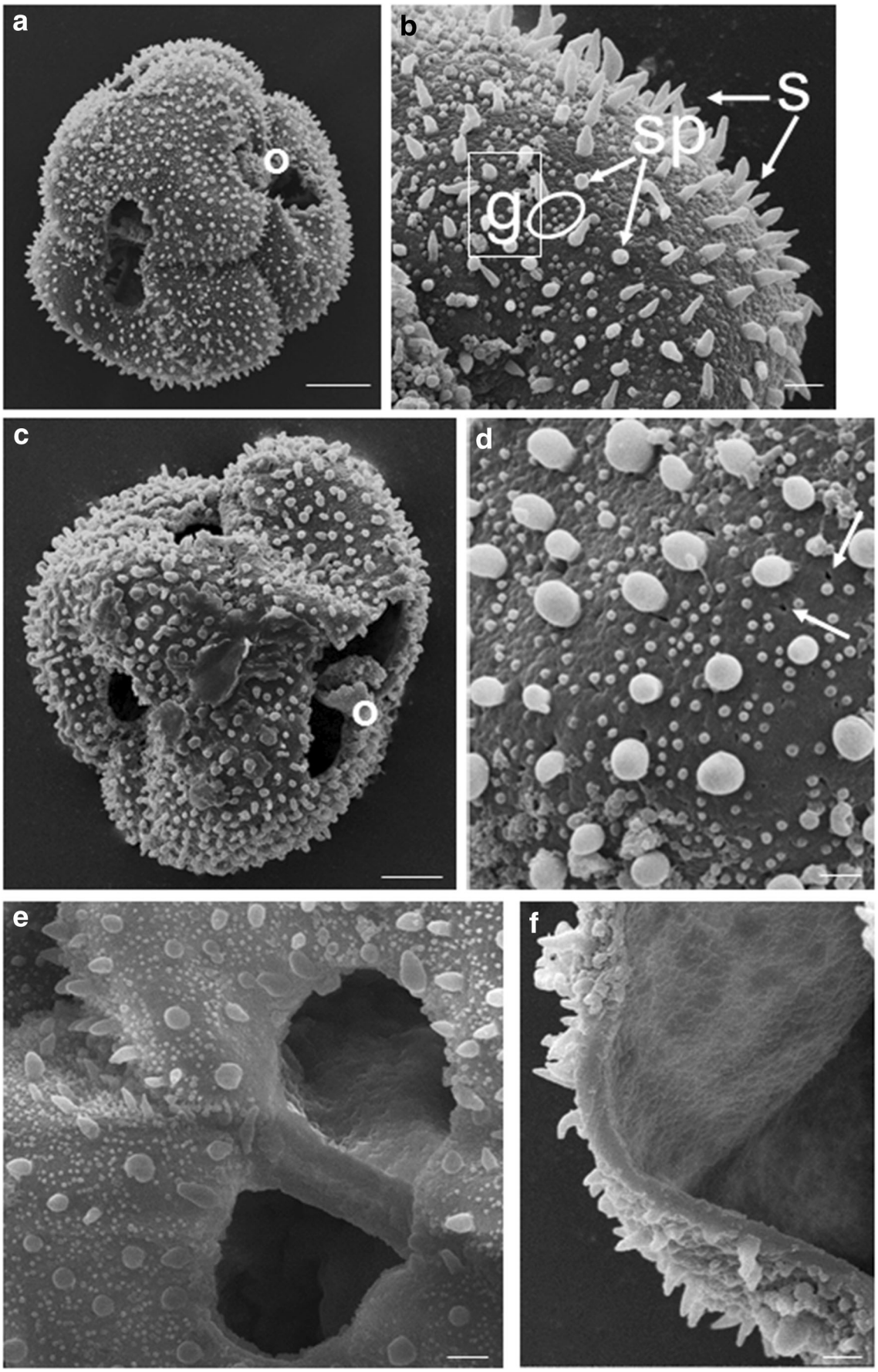
Aldrovanda pollen is organized in permanent tetrads

It has been reported that *Aldrovanda* flowers remain opened only for very short time (about 3 h) and anther dehiscence occurs only after the petals open (Okada 2008; Adamec 2018). Opened flowers were collected at different stages during the short flowering period, since they contained closed anthers (suggesting flowers were collected immediately after opening) or open anthers (suggesting they were collected later; Figs. 1 and 3). Pollen was organized in permanent tetrads of about 65 μm in diameter and arranged in tetrahedral or tetragonal orientations (Fig.1a, b, respectively). This organization of *Aldrovanda* pollen was confirmed also by fluorescence analysis (Fig. 1d–i). The autofluorescence analysis of dried flowers showed that several tetrads were present in the anthers (about 5; Fig. 1d, e). Both tetrahedral and tetragonal tetrads were present in the same theca (Fig. 1e).

The pollen surface was covered by many drops, probably representing pollenkit produced by the tapetum (Fig. 1a, f; arrows). The high amount of this material, the most common adhesive material covering pollen grains (Pacini and Hesse 2005), could explain the difficulty of pollen release from the anthers; in fact, pollen had to be scraped off from the anther envelope. Like other members of the Droseraceae, *Aldrovanda* pollen displayed three equatorial apertures (Figs. 1 j and 3 d), and as also confirmed by SEM analyses (Fig. 2a, e), pores of two neighbouring monads involve the outline of both the distal and proximal sides of pollen grains (Fig. 1c, e, f). In dried pollen, the pores were locked in by an operculum (Fig. 1f, i) and they opened in hydrated pollen by operculum retraction (Fig. 2c).

Anther staining by Aniline Blue showed that callose was not present in tetrads, as expected. On the contrary, callose seemed to be present in the cell wall of anther endothecium cells (Fig. 1g, h). Aniline Blue staining suggested that callose

Fig. 2 SEM micrographs of acetolyzed pollen grains. **a**: tetrad with tetrahedral arrangement of pollen grains with equatorial apertures (o = operculum). **b**: pollen grain showing echinate features on distal face (g = granules, sp = spinules, S = spines). **c**: tetrad with equatorial apertures and retracted opercula (o). **d**: echinate features on distal face and irregular perforations (arrows). **e**: two equatorial apertures of two different pollen grains. **f**: broken pollen grain showing the thickness of the pollen wall. Magnification bars—**a**, **c**: 10 μm; **b**, **f**: 2 μm; and **d**, **e**: 1 μm



was deposited in these wall bars. On the contrary, a solid plate formed in the inner periclinal wall, from which the bars originate, did not contain callose (Fig. 1g, h). In *Aldrovanda*, cell-wall bars were also revealed in the endothecium cells of squashed anthers (Fig. 1j, k) and in anther sections (Figs. 1 l and 3 a, c, g). Autofluorescence and light microscopy (LM) of distal pollen cell walls and tetrad sections revealed that the exine appeared intectate and echinate for the presence of different spinae (Fig. 1b, i, l).

Using SEM analysis, we observed exine ornamentation which revealed that there are no relevant morphological differences between pollen grains of opened and closed flowers (Fig. 2a, c). As already reported for other Droseraceae (Rodondi et al. 2004), there are three main different echinate features on the distal face of each *grain* of the tetrad: spines, spinules, and granules (Fig. 2b). Granules (around 0.1–0.2 μm long, 0.1–0.2 μm wide at the base) are the most numerous and are about three times the sum of spines and spinules, very short, and constant in shape. Spines are around 3 μm long, 1 μm wide at the base and are numerous, with a regular distribution, variable in shape and length, whereas their width remains quite constant. Spinules (around 1 μm long and 1 μm wide at the base) are the least frequent features. The spinules appear like blunt spines and are randomly distributed. Their shapes are similar to granules, but are bigger and sometimes as wide as spines. On the pollen wall, some sporadic and irregular perforations are also present (Fig. 2d). In some broken pollen grains and in some grains without the operculum, it was also possible to measure the exine thickness of 0.7–1.0 μm (Fig. 2e, f).

Inside closed and opened anthers: *Aldrovanda* pollen showed different morphologies

Sections of closed anthers showed tapetum degeneration and cell-wall bands in endothecium cell walls (Fig. 3a). Mature pollen grains, as observed both by LM and TEM, appeared full of amyloplasts (Fig. 3a, b) and no cytoplasmic protrusions were observed at the pollen apertures (Fig. 3a). Observation of pollen obtained by squashed opened anthers and by opened anther sections revealed that in mature pollen, each monad displayed cytoplasmic protrusions originating from each pore (Fig. 3c–g). In *Aldrovanda*, unlike *Dionaea* (Halbritter et al. 2012), the protrusions did not show the persistence of the operculum. LM and TEM analyses showed that only intine covered the cytoplasmic papillae (Figs. 3 e–g and 4 a–c). At the protrusions, the intine appeared thick and was formed with three layers. The innermost region consisted of an inner thin compact layer, a middle layer showing microchannels, and an outer fibril layer (Fig. 4b, layers 1, 2, and 3, respectively). In the area where the exine was present, the fibrillar layer was not observed (Fig. 4a, d) and microchannels disappeared or were reduced considerably in the middle layer (Fig. 4d, arrow).

TEM analyses showed that the exine was organized in a homogenous electron-dense layer from which spinae/spinulae protrude (Fig. 4d–f), as described above, and where fibrillar matrix was observed (Fig. 4f). The presence of spinae/spinulae also characterized the wall between two adjacent monads (Fig. 3 b and 4 e, f). Intine microchannels were also observed in pollen inside closed anthers (Fig. 3b). In relation to the operculum, when the pore is still locked, the inner homogenous layer of sporopollenin was missing and only the thick sculptured cell wall was observed (Fig. 3b).

Aldrovanda pollen displayed different developmental stages in opened anthers

In *Aldrovanda* anthers at anthesis, the vegetative and generative cells (VC and GC) were observed but the GC was not yet internalized within the VC (Fig. 5a, b). In this phase, cytoplasmic protrusions were not observed. TEM revealed that at anthesis, pollen grains showed a vegetative cell with dense cytoplasm, a prominent lobed vegetative nucleus and a stacked rough ER (Fig. 6). In addition, dark lipid drops and small vacuoles were also observed inside the vegetative cell (Figs. 3 e–g; 4 a, d, e; and 6 b). Clusters of vesicles characterized both cytoplasmic protrusions and vegetative cells (Figs. 4 a, d; 5 d; and 6 c). The GC inclusion into the VC appeared delayed with respect to the timing of anthesis. In addition, after the first microspore mitosis, before the GC inclusion, VC did not show a large vacuole but dispersed small vacuoles (Fig. 5 b–d and 6 a, c). In *Aldrovanda*, the GC was also observed inside the VC (Fig. 5a, c), suggesting that GC internalization and subsequent pollen development can occur correctly (Fig. 5c, d). In the same anthers, pollen with either GC included in VC or not included was observed (Fig. 5a–c). The generative cell was surrounded by a thin cell wall and showed some organelles as ER and vesicles and, intriguingly, the GC seldom appeared lobed (Fig. 5d, e).

Moreover, germinated pollen with long pollen tubes were sometimes observed in the old opened anther (Fig. 7). The pollen tubes, which reached a remarkable length (about 300 μm), started by a cytoplasmic protrusion (Fig. 7a) and originated by more monads belonging to the same tetrad (Fig. 7b, c). Inside the anther, some algae with a large nucleus and chloroplasts characterized by single organized thylakoids (Fig. 3g, h) were observed, suggesting the entry of ambient water into the opened flower.

The opened anther sections also showed the presence of two sperm cells in the pollen grain, confirming that *Aldrovanda* possesses tricellular pollen (Fig. 8), as do other Droseraceae (Rodondi et al. 2004). Sperm cells revealed a prominent nucleus surrounded by dense cytoplasm containing small vacuoles and a smooth ER (Fig. 8c–e). Interestingly, plasmolysis of cells highlighted that the two SCs were not included inside the VC, since no cytoplasm was observed

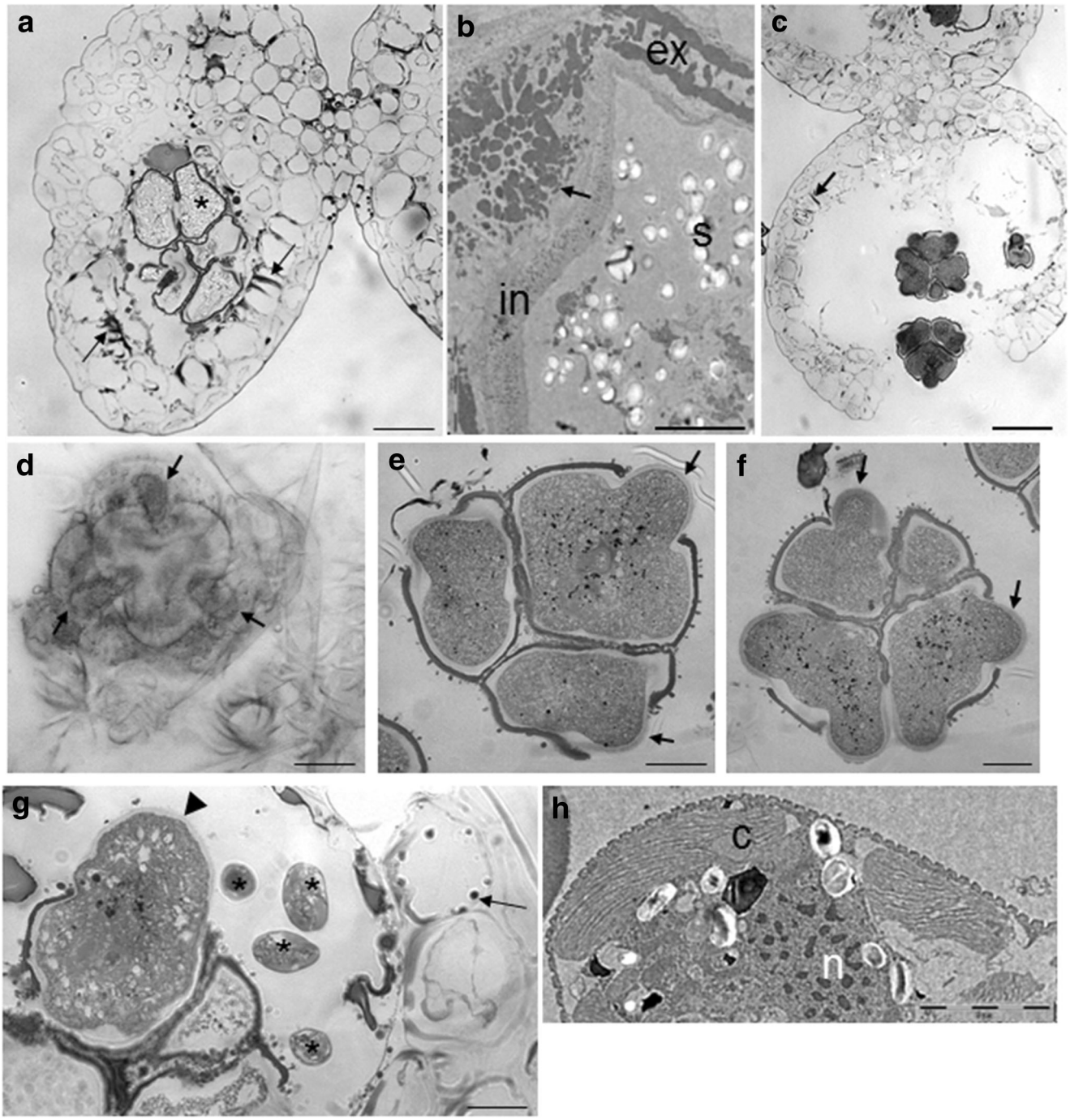
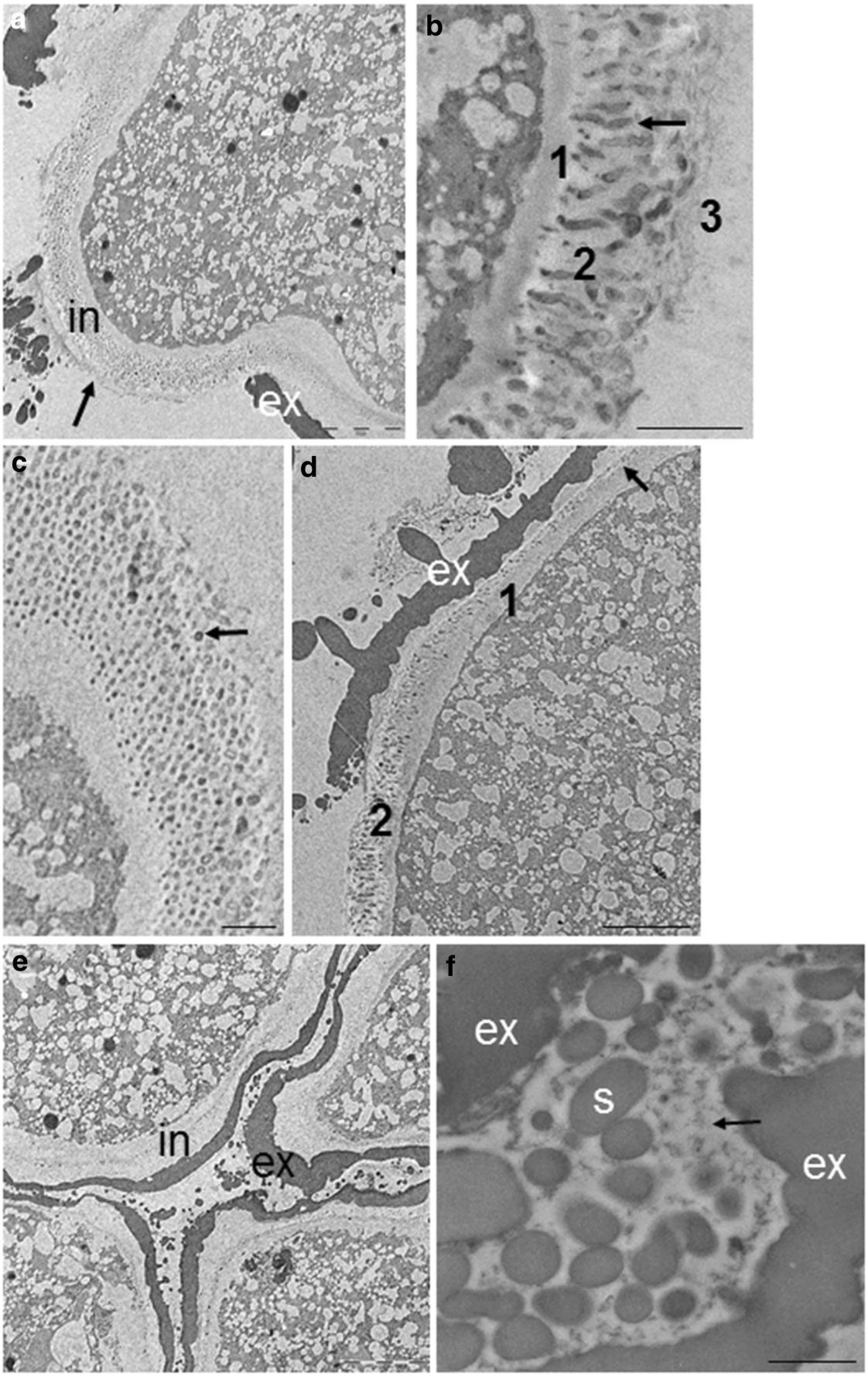


Fig. 3 Pollen morphology in closed and open anthers. **a**, **b**: sections of closed anthers. Tapetum undergoes to degeneration and wall bands in endothecium cell walls were observed (**a**, arrows). Pollen grains appeared filled with amyloplasts (**a**; asterisks) and no cytoplasmic protrusions were observed at pollen apertures (**a**). TEM observations confirmed the presence of numerous small starch granules inside pollen (**b**). Intine (**b**) and exine (**b**) were present. Between two adjacent monads, exine was organized in a homogenous electron-dense layer from which spinae/spinulae protrude (**b**). The operculum showed only the thick sculptured cell wall while the inner homogenous layer of sporopollenine was missing (**b**; arrow). **c-h**: sections of open anthers. Open anthers showed tetrads in which monads display cytoplasmic protrusions that originate from each pore (**c**, **e**, **f**). Protrusions were also observed in squashed anthers (**d**; arrows). In correspondence of protrusion, exine disappeared for the retraction of operculum and only a tick intine was observed (**e**, **f**, **g**; arrows). Starch granules were not present in hydrated pollen and some osmiophilous dark granules were observed as dark spots inside the pollen grains (**e**, **f**, **g**). Inside the pollen sac, some algae were present (**g**; asterisks). These algae were unicellular and displayed a large nucleus and chloroplasts characterized by single thylakoids (**h**); in, intine; ex, exine; s, starch; n, nucleus; c, chloroplast. Magnification bars—**a**: 50 μm; **c**: 40 μm; **d**, **e**, **f**: 10 μm; and **g**, **h**, **b**: 5 μm



◀ **Fig. 4** TEM analyses of pollen cell wall in open anthers. **a-c**: intine structure at the protrusions. Protrusion showed a thick intine organized in three layers: an inner thin compact layer (**b**; 1), a middle layer showing microchannels (**b**, 2; **b** longitudinal/**c** transversal sections; arrows) and an outer fibrillar layer (**b**; 3). **d–f**: cell wall around pollen grains. In the exine, spinae/spinulae protrude from a homogenous electron-dense layer (**d**) also at two adjacent monads (**e**). In this area, microchannels (**d**; 2) disappeared from intine or were reduced considerably (**d**, **e**; arrow) allowing only the persistence of the homogenous layer (**d**; **e**). Fibril matrix, probably derived by tapetum degeneration, was observed in the intercellular space between two adjacent monads (**f**; arrow). The VC cytoplasm showed numerous clusters of vesicles and little vacuoles (**a**, **d**, **e**); in, intine; ex, exine; s, spinae. Magnification bars—**a**, **d**, **e**: 5 μm; **b**, **c**, **f**: 1 μm

around the two SCs and the periplasmic space due to plasmolysis was observed between the cells and the pollen wall (Fig. 8b, c). These data suggest that the GC was not included in the VC, and thus, the mitosis II occurred outside the VC.

Discussion

Aldrovanda vesiculosa displays both cleistogamous and autogamous flowers (Adamec and Tichý 1997; Okada 2008; Adamec 1999; Cross et al. 2016); however, cleistogamous flowers fail to produce seeds and in autogamous flowers, when pollen grains reach the stigma, they are able to form pollen tubes that reach the ovule through a mucilaginous path in the style (Atsuzawa et al. 2020). However, it was observed that in *Aldrovanda*, the number of fertile fruits produced is highly variable and often scarce (Cross 2012; Adamec 2018). In addition, genetic uniformity was observed among different populations of *Aldrovanda*, suggesting that low mutation rates, dominant asexual reproduction, and autogamous reproduction occur (Elansary et al. 2010; Adamec 2018). Data presented in this study were performed on closed and opened flowers, thus on potentially fertile flowers. LM and TEM observations showed altered development of male gametophyte, which could explain the failure of the fertilization process and the rarity of seed set.

Aldrovanda pollen morphology shares some features with other Droseraceae

The morphology of *Aldrovanda* pollen is poorly known (Sahashi and Ikuse 1973; Okada 2008), but it shares some features with other Droseraceae (Kuprianova 1973; Rodondi et al. 2004; Halbritter et al. 2012). In fact, optical and electron microscopy observations of pollen in plants from European and Australian populations showed tetrahedral/tetragonal organization of tetrads, echinate pollen walls, and as reported in species that naturally form permanent tetrads (Blackmore and Crane 1988; Scott et al. 2004; Copenhaver 2005) and produce little or no callose in the intersporal cross-walls of the tetrad. It is not clear which mechanisms allowed monads to remain close to each other. Between monads, some fibrillar materials were observed which could represent pollenkit or tapetum debris. In *Annona*, different species with monad and permanent tetrad pollen were observed. In tetrads of different *Annona* species, several mechanisms for monads adhesion were observed involving both intine and exine bridges or cohesion. In some plants, also callose and cellulose participate in monads adhesion (Copenhaver 2005; Lora et al. 2014). Further analyses could clarify this mechanisms also in Droseraceae.

Instead, callose was present in ribs observed in endothecial cells. In other species, just before anthesis and during the pollen maturation, cell walls of endothecium cells develop characteristic thickened bars which are reported to consist mainly of cellulose and lignin (De Fossard 1969; Whatley 1982; Manning 1996). In *Aldrovanda*, these wall thickenings of the endothecium resemble the palmate ribs described in the Solanaceae (Carrizo García 2002) and have an essential role in anther dehiscence. In fact, in some male sterile *Arabidopsis thaliana* mutants, endothecial wall thickening is affected, resulting in failure of anther opening and pollen release (Dawson et al. 1999; Wilson et al. 2011).

Microscopic observations of *Aldrovanda* pollen also show the presence of cytoplasmic protrusions at pores, a distinctive trait of Droseraceae pollen. In fact, in both *Drosera* and *Dionaea*, cytoplasmic protrusions can emerge from numerous apertures localized in the equatorial area of monads (Rodondi et al. 2004; Halbritter et al. 2012). As already suggested for *Drosera* and *Dionaea* (Halbritter et al. 2012), protrusions in *Aldrovanda* pollen resulted from hydration because in its dry state, pores were occluded by the operculum. Regions of cytoplasmic protrusions showed the presence of microchannels in the intine as well. This microchannelled intine has been reported in pollen of the *Drosera* genus, Poaceae, and other plants as well (Kuprianova 1973; Takahashi 1988; Marquez et al. 1997; Vega-Maray et al. 2003; Eliseu and Dinis 2008). It was suggested that this wall layer plays a role in pollen hydration and activation (Eliseu and Dinis 2008).

A very interesting feature was the presence of tricellular pollen in *Aldrovanda*. In Angiosperms, pollen could be delivered in two different states, depending on the species. In bicellular pollen, the microspore completes only the first asymmetric mitosis prior to pollination giving rise to a large VC and a small GC. Then, after cytokinesis, the GC is internalized into the VC. In tricellular pollen, the internalized GC divides into two sperm cells (SCs) before pollen dispersal giving rise to tricellular pollen (Angold 1968; Pacini and Juniper 1984). Droseraceae is in the latter group, forming tricellular pollen, with two sperm cells within the VC (Rodondi et al. 2004). Similar to other tricellular pollen species, *Drosera* was also described as partially hydrated pollen/

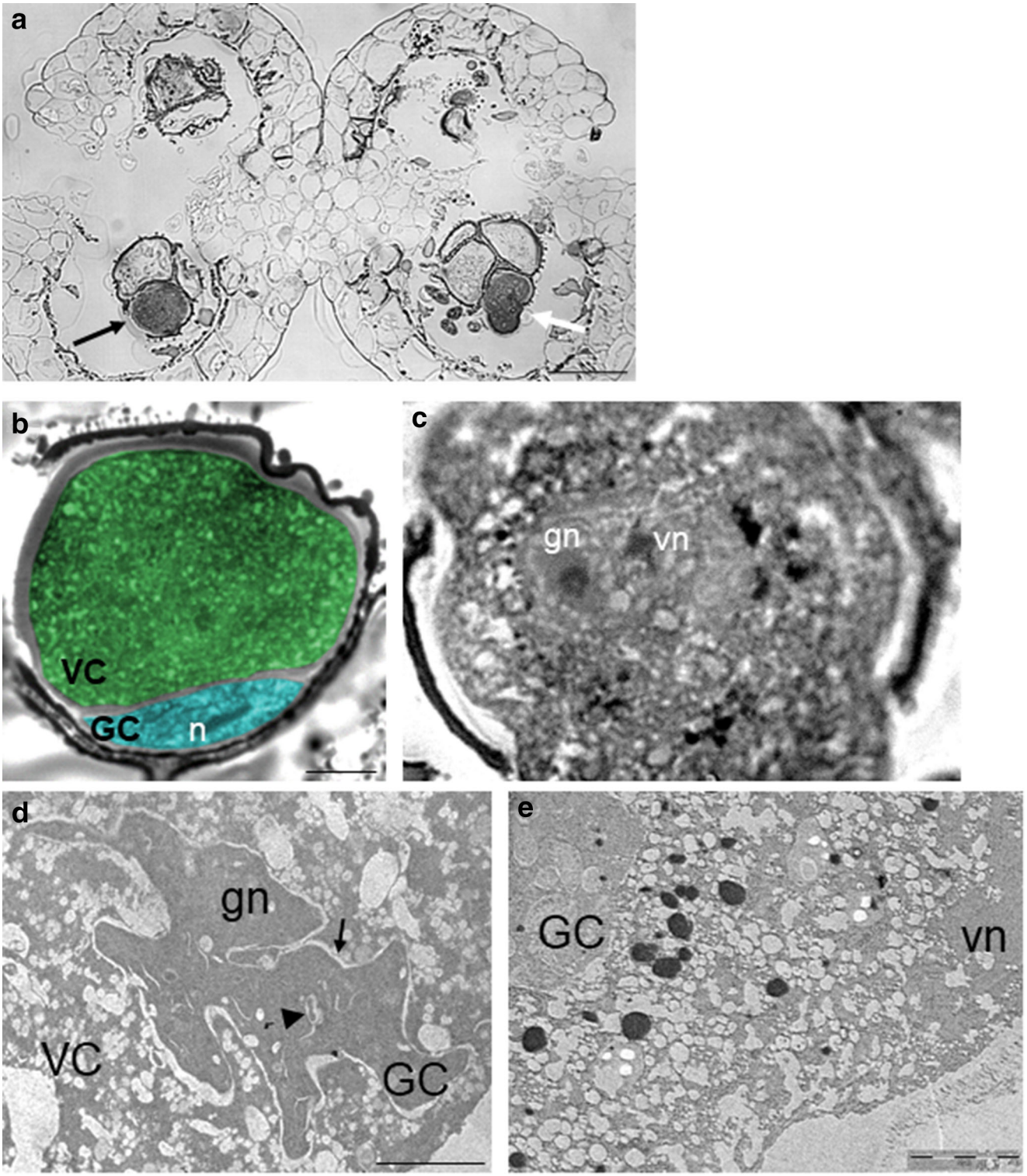
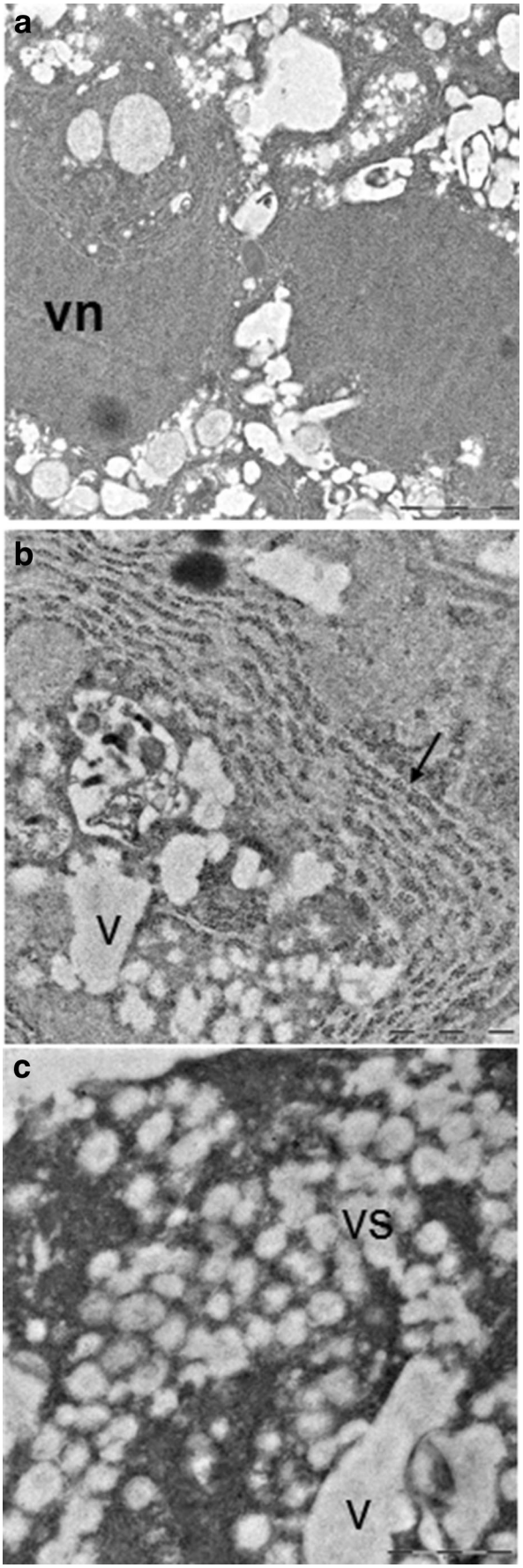


Fig 5 Generative cell formation in open anthers. **a, b, c**: light microscopy of open anther sections. Vegetative and generative cell were observed (**a**, **b**, **c**: VV green, GC blue) but the GC was not yet internalized into the VC (**a**, black arrow; **b**). Cytoplasmic protrusions were not observed even when the operculum was already retracted (arrow) and only the intine was present at the pore (**a**, arrow; **b**). Dispersed vesicles and small vacuoles were observed in the VC (**b**). In the same anther, pollen with GC including in VC was observed (**a**, white arrow; C). **d**, **e**: GC was internalized into VC. GC appeared lobed and surrounded by a thin cell wall (**d**, arrow). Some organelles such as ER (**d**, arrowhead) and vesicles were observed (**d**, **e**). GC, generative cell; VC, vegetative cell; n, nucleus; gn, generative cell nucleus; vn, vegetative cell nucleus. Magnification bars—**a**, 50 μm; **b**, 10 μm; and **c**, **d**, 5 μm



◀ **Fig. 6** Ultrastructure of the vegetative cell at anthesis. **a**: vegetative cell shows a prominent lobed nucleus and dense cytoplasm. **b**: rough ER was arranged in well-ordered thick stacks. **c**: numerous small vacuoles and cluster of vesicles were also observed. vn, vegetative cell nucleus; v, vacuole; vs, vesicles. Magnification bars—**a**, **b**, **d**: 1 μm; **c**: 2 μm

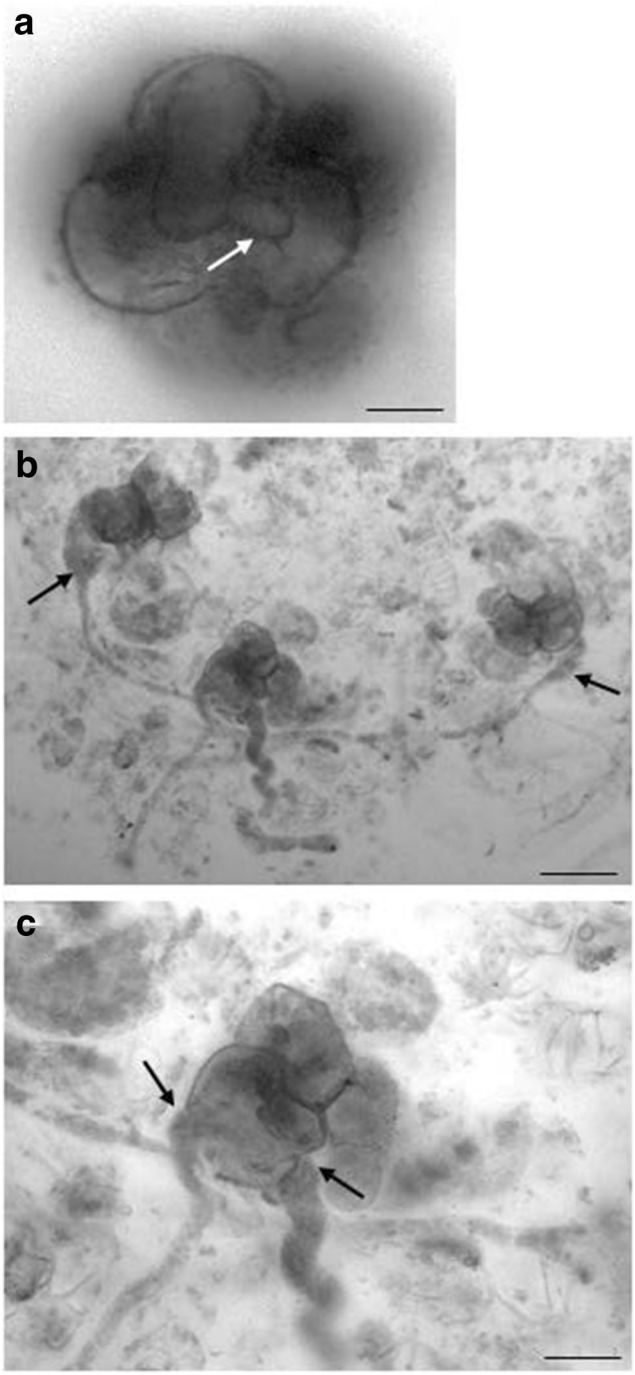


Fig. 7 Germinated pollen tube inside the open anthers. **a**, **b**, **c**: light microscopy of squashed old anthers. Pollen tube germinated by cytoplasmic protrusion (**a**, arrow). Pollen tube reached a remarkable extension (about 300 μm in length; **b**, arrows) and originated simultaneously from more pollen grains belonging to the same tetrad (**c**, arrows). Magnification bars: **a**, 15 μm; **b**, 50 μm; and **c**, 30 μm

recalcitrant plant (Gardner 1975; Franchi et al. 2002). Recalcitrant pollen is dehydration sensitive, and when dispersed, it has reduced longevity and very quick germination (Franchi et al. 2011; Pacini and Dolferus 2019). This feature is also found in different taxa and is considered an adaptation to underwater or wet environments (Brewbaker 1967; Lora et al. 2009; Franchi et al. 2011). In *Aldrovanda*, the short pollen longevity, because of the very short exposure of pollen to air together with a very short flowering time (Adamec and Tichý 1997; Okada 2008; Adamec 2018), could negatively affect fertilization. On the other hand, very rapid pollen germination could be correlated with very short flowering time, facilitating the fertilization process if the time necessary to reach a pollination target is short enough. The density of pollinating targets

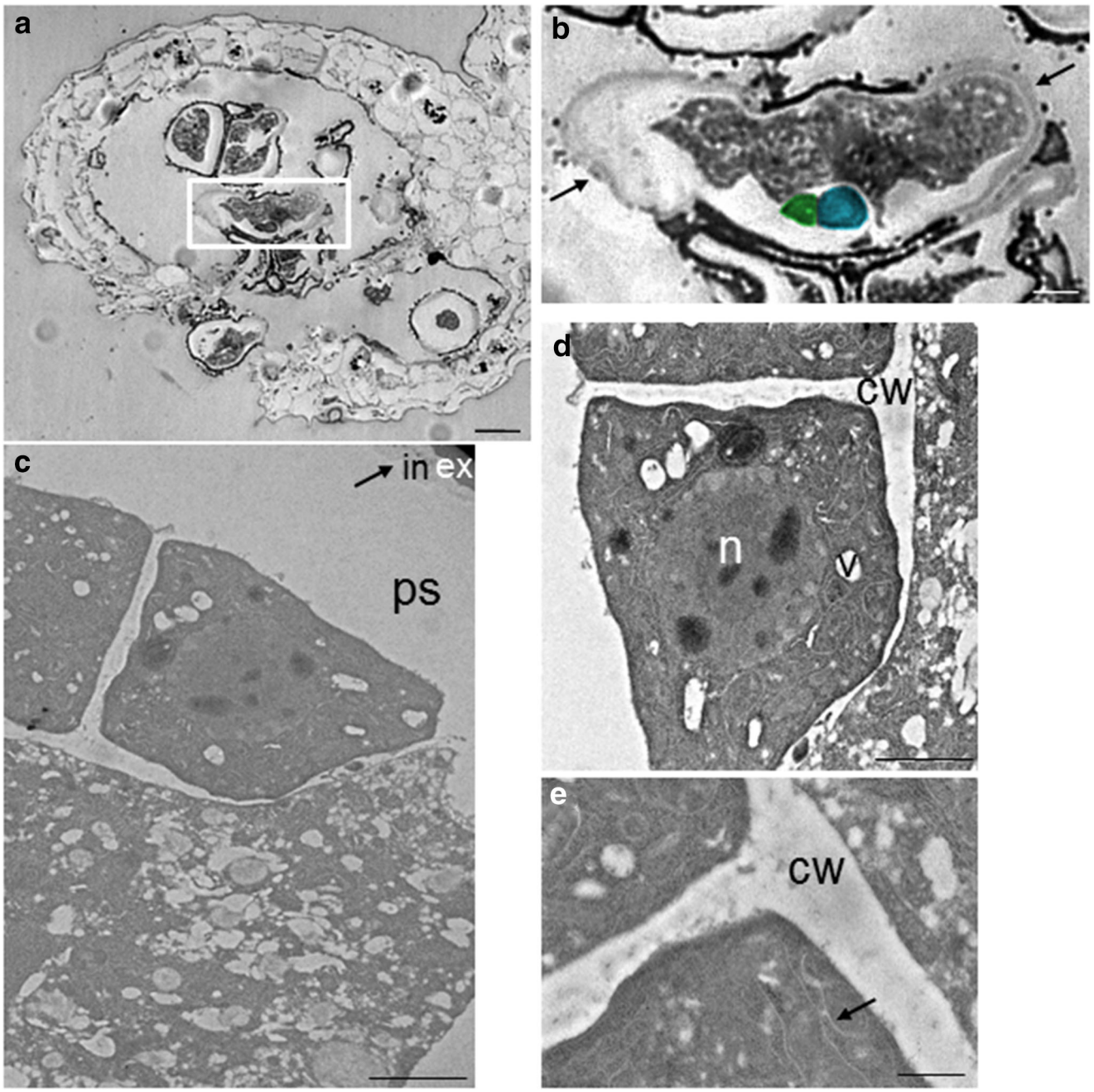


Fig. 8 *Aldrovanda vesiculosa* pollen was tricellular. **a**, **b**: light microscopy of open plasmolysed anthers. Pollen showed cytoplasmic protrusions but not pollen tubes (**b**; arrows). Magnification of pollen in the box of panel **a** showed that sperm cells (**b**; blue and green cells) were not included inside the vegetative cell. **c**, **d**, **e**: TEM analyses of sperm cells in plasmolysed pollen. Plasmolysis creates a periplasmic space between sperm cells and pollen cell wall and no cytoplasm was observed around sperms (**c**; arrow). Sperm cells were surrounded by a thin cell wall and showed a prominent nucleus with dense cytoplasm containing little vacuoles and smooth ER (**d**, **e**; arrow); ps, periplasmic space; in, intine; ex, exine; cw, cell wall; n, nucleus; v, little vacuole. Magnification bars: **a**, 25 μm; **b**, 10 μm; **c**, 3 μm; **d**, 2 μm; and **e**, 0,5 μm

in the area and the number of receptive flowers/inflorescences per plant also determine the success of pollination (Pacini and Dolferus 2019).

However, pollen longevity after dispersal strongly depends on environmental conditions and high temperature could affect hydrated/recalcitrant pollen (Pacini and Dolferus 2019). High water temperature during flowering is crucial for the floral success in *Aldrovanda* (Cross 2012; Adamec 2018); the recent climate change could thus be an important factor for reproductive success of *Aldrovanda*, at least in some parts of its distribution range. Because metabolite homeostasis is important for cell protection during pollen dehydration, further studies could also better characterize this process in *Aldrovanda*.

Aldrovanda showed an altered pattern of pollen development

In order to better understand the origin of the low reproductive success of *A. vesiculosa*, we investigated the development of male gametophyte. Pollen grains usually complete their development before anthesis and pollen presentation (Pacini and Dolferus 2019). In *Arabidopsis thaliana*, a plant with tricellular pollen, the first mitotic division of microspores starts during the tapetum degeneration, whereas the second division forming the two sperms occurs when endothecium lignification is completed and anthesis has not yet occurred (Nagpal et al. 2005; Cecchetti et al. 2008). Auxin and jasmonic acid are involved in the coordination of pollen maturation and anther dehiscence (Nagpal et al. 2005; Cecchetti et al. 2008) and several genes were identified to control pollen/anther ripening (Gómez et al. 2015).

Interestingly, in *Aldrovanda*, these later pollen maturation steps occurred after anther dehiscence. In fact, in open anthers, pollen displayed different developmental stages, from the end of mitosis I, when GC and VC formed, to germinated pollen. This suggests a delay of pollen maturation or premature anther opening. However, the presence of pollen showing GC inclusion in VC or pollen without CG inclusion in the same anther together with the presence of a differentiated endothecium layer with lignified wall thickening in closed anthers suggested that anther dehiscence occurred as expected. Moreover, because the anther dehiscence occurred immediately after the flower opening and in the opened flower, both open and closed anthers were collected; a delay in pollen developmental pattern was hypothesized.

Through microscopic analysis, we found evidence that *Aldrovanda* pollen also showed both normal and altered developmental pattern. Generally, it was reported for other plant species that during pollen development, water was stored in large vacuole and starch was accumulated, while in mature pollen, large vacuole disappeared and numerous vesicles or little vacuoles contained soluble carbohydrates derived by hydrolysis of starch in order to protect pollen from osmotic stress (Pacini et al. 2011; Firon et al. 2012; Carrizo García et al. 2017; Pacini and Dolferus 2019). In *Aldrovanda* VC, starch hydrolysis and large vacuole disappearance seemed to occur early, before the GC inclusion, suggesting a modification of water/carbohydrate pattern which could affect water homeostasis and then pollen viability.

Another modification of *Aldrovanda* pollen development related to sperm formation: In most pollen grains, the GC was included in the VC and sometimes the GC appeared lobed. As reported for the pollen grains of other species (e.g. see Cresti et al. 1979; Heslop-Harrison et al. 1988), during pollen maturation and pollen tube formation, the GC undergoes progressive changes in shape and GC microtubules play a role in cell reshaping (cf. Heslop-Harrison et al. 1988). However, the presence of two sperm cells outside the VC in several pollen grains suggested that GC was not included in the VC, and thus, the mitosis II occurs outside the VC. In this case, if pollen germinates, two sperms cannot be transmitted to the female gametophyte through the pollen tube, which could be one mechanism of fertilization failure. Overall, these data revealed a delay of pollen maturation and severe modifications of developmental patterns so that when pollen is exposed to the environment, it can be immature and not ready for the fertilization process. In this case, the availability of functional pollen is further restricted in flowers with a very short opening time as for *Aldrovanda* (Adamec and Tichý 1997; Okada 2008; Adamec 1999, 2018).

Aldrovanda pollen development in open anthers stopped during the phase of pollen tube formation and growth. It is possible that the presence of very sticky pollenkit trapped mature pollen into the anthers preventing them to reach the stigma and thus avoiding or reducing fertilization. Nevertheless, germination of pollen inside the anthers is a feature of cleistogamous species (Lord 1979; Pacini and Franchi 1982); however, numerous autogamous species also show this pollen behaviour (Pacini and Franchi 1982; Kaur et al. 2005; Sahai et al. 2016). The precocious germination may characterize recalcitrant pollen, which naturally do not undergo dehydration before anthesis and remain partially hydrated, particularly in plants living in aquatic or wet habitats (Pacini and Franchi 1982), as for the aquatic plant *Aldrovanda*. The entry of ambient water into the opened flower, indicated by the presence of algae in the loculi, might affect the hydration status of pollen and induce germination. However, precocious pollen germination inside the anthers was also observed in various *A. thaliana* mutants (Johnson and McCormick 2001; Xie et al. 2010; Wang et al. 2012), suggesting that pollen dormancy is not controlled exclusively by the dehydration level but also by genetic mechanisms. As reported in *A. thaliana*, the presence of gametes is not necessary for pollen tube growth (Glöckle et al. 2018). Similarly, it is possible to assume that also in *Aldrovanda*, pollen tube growth is independent of the correct development of the male gametophyte (Glöckle et al. 2018).

Conclusions

In *Aldrovanda*, as observed for recalcitrant pollen, transport on a compatible stigma is a prerequisite for successful fertilization, or alternatively, autogamy occurs. The reason for the high level of autogamy observed in *Aldrovanda* could be the precocious pollen germination inside the anthers. In addition, the low level of seed production observed could also be ascribed to an alteration of pollen development because in some cases, the presence of sperm cells outside the VC was observed as a consequence of lack of GC internalization. For this reason, although the *Aldrovanda* pollen tube grows, it could often be unable to deliver sperm to the embryo sac for double fertilization. Knowledge of molecular mechanisms regulating the GC internalization could help us understand the alteration in *Aldrovanda* pollen development. Unfortunately, no mutants have yet been described with this defect. The assessment of the frequency of such an alteration within and among different populations growing in different geographic areas and under different environmental conditions could shed light on the reproductive behaviour of *Aldrovanda* throughout its wide distribution range.

Acknowledgements Sincere thanks are due to Mr. Curtis Lubbe (Institute of Botany CAS, Třeboň, Czech Rep.) for English correction. Thanks are also due to two anonymous reviewers for correcting the manuscript and valuable comments.

Funding This research was partly supported by the Czech Long-term research development project No. RVO 67985939 (for LA).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Adamec L (1995) Ecological requirements and the European distribution of the aquatic carnivorous plant *Aldrovanda vesiculosa* L. Folia Geobot Phytotax 30:53–61

Adamec L (1999) Further notes on flowering and seed set of *Aldrovanda vesiculosa*. Flytrap News (Sydney) 12(4):9–12

Adamec L (2000) Rootless aquatic plant *Aldrovanda vesiculosa*: physiological polarity, mineral nutrition, and importance of carnivory. Biol Plant 43:113–119

Adamec L (2018) Biological flora of Central Europe: *Aldrovanda vesiculosa* L. Perspect Plant Ecol Evol Syst 35:8–21

Adamec L, Tichý M (1997) Flowering of *Aldrovanda vesiculosa* in outdoor culture in the Czech Republic and isozyme variability of its European populations. Carniv Plant Newsl 26:99–103

Angold RE (1968) The formation of the generative cell in the pollen grain of *Endymion non-scriptus* (L). J Cell Sci 3:573–578

Atsuzawa K, Kanaizumi D, Ajisaka M, Kamada T, Sakamoto K, Matsushima H, Kaneko K (2020) Fine structure of *Aldrovanda vesiculosa* L.: the peculiar lifestyle of an aquatic carnivorous plant elucidated by electron microscopy using cryo-techniques. Microscopy (in press):dfaa019. https://doi.org/10.1093/jmicro/dfaa019

Blackmore S, Crane PR (1988) The systematic implications of pollen and spore ontogeny. In: Humphries CJ (ed) Ontogeny and systematics. Columbia University Press, New York, USA, pp 83–115

Brewbaker JL (1967) The distribution and phylogenetic significance of binucleate and trinucleate pollen grains in the angiosperms. Am J Bot 9:1069–1083

Carrizo García C (2002) An approach to the diversity of endothecial thickenings in Solanaceae. Flora 197:214–223

Carrizo García C, Nepi M, Pacini E (2017) It is a matter of timing: asynchrony during pollen development and its consequences on pollen performance in angiosperms—a review. Protoplasma 254: 57–73

Cecchetti V, Altamura MM, Falasca G, Costantino P, Cardarelli M (2008) Auxin regulates *Arabidopsis* anther dehiscence, pollen maturation and filament elongation. Plant Cell 20:1760–1774

Chanda S (1965) The pollen morphology of Droseraceae with special reference to taxonomy. Pollen Spores 7:509–528

Copenhaver GP (2005) A compendium of plant species producing pollen tetrads. J N Carol Acad Sci 121:17–35

Cresti M, Ciampolini F, Pacini E, Sarfatti G, Donini B (1979) Ultrastructural features of *Prunus avium* L. pollen tube in vivo. Caryologia 32:433–440

Cross A (2012) *Aldrovanda*. The waterwheel plant. Redfern Natural History Productions, Poole

Cross AT, Skates LM, Adamec L, Hammond CM, Sheridan PM, Dixon KW (2015) Population dynamics of the endangered aquatic carnivorous macrophyte *Aldrovanda vesiculosa* at a naturalised site in North America. Freshw Biol 60:1772–1783

Cross AT, Adamec L, Turner SR, Dixon KW, Merritt DJ (2016) Seed reproductive biology of the rare aquatic carnivorous plant *Aldrovanda vesiculosa* (Droseraceae). Bot J Linn Soc 180:515–529

Dawson J, Sozen E, Vizir I, Van Waeyenberge S, Wilson ZA, Mulligan BJ (1999) Characterization and genetic mapping of a mutation (ms35) which prevents anther dehiscence in *Arabidopsis thaliana* by affecting secondary wall thickening in the endothecium. New Phytol 144:213–222

De Fossard RA (1969) Development and histochemistry of the endothecium in the anthers of in vitro grown *Chenopodium rubrum* L. Bot Gaz 130:10–22

Elansary HOM, Adamec L, Štorchová H (2010) Uniformity of organellar DNA in *Aldrovanda vesiculosa*, an endangered aquatic carnivorous species, distributed across four continents. Aquat Bot 92:214–220

Eliseu SA, Dinis AM (2008) Ultrastructure and cytochemistry of *Eucalyptus globulus* (Myrtaceae) pollen grain. Grana 47:39–51

Erdtman G (1960) The acetolysis method, a revised description. Sven Bot Tidskr 54:561–564

Firon N, Nepi M, Pacini E (2012) Water status and associated processes mark critical stages in pollen development and functioning. Ann Bot 109:1201–1213

Fleischmann A, Cross AT, Gibson R, Gonella PM, Dixon KW (2018) Systematics and evolution of Droseraceae. In: Ellison AM, Adamec L (eds) Carnivorous plants: physiology, ecology, and evolution. Oxford University Press, Oxford, pp 45–57

Franchi GG, Nepi M, Dafni A, Pacini E (2002) Partially hydrated pollen: taxonomic distribution, ecological and evolutionary significance. Plant Syst Evol 234:211–227

Franchi GG, Piotto B, Nepi M, Baskin CC, Baskin JM, Pacini E (2011) Pollen and seed desiccation tolerance in relation to degree of developmental arrest, dispersal, and survival. J Exp Bot 62:5267–5281

Gardner RO (1975) A survey of the distribution of binucleate and trinucleate pollen in the New Zealand flora. New Zealand J Bot 13:361–366

Glöckle B, Urban WJ, Nagahara S, Andersen ED, Higashiyama T, Grini PE, Schnittger A (2018) Pollen differentiation as well as pollen tube

guidance and discharge are independent of the presence of gametes. Development 145:e152645

Gómez JF, Talle B, Wilson ZA (2015) Anther and pollen development: a conserved developmental pathway. J Integr Plant Biol 57:876–891

Halbritter H, Hesse M, Weber M (2012) The unique design of pollen tetrads in *Dionaea* and *Drosera*. Grana 51:148–157

Herben T, Šerá B, Klimešová J (2014) Clonal growth and sexual reproduction: tradeoffs and environmental constraints. Oikos 124:469–476

Heslop-Harrison J, Heslop-Harrison Y, Cresti M, Tiezzi A, Moscatelli A (1988) Cytoskeletal elements, cell shaping and movement in the angiosperm pollen tube. J Cell Sci 91:49–60

Johnson SA, McCormick S (2001) Pollen germinates precociously in the anthers of raring-to-go, an *Arabidopsis* gametophytic mutant. Plant Physiol 126:685–695

Kaur S, Nayyar H, Bhanwra RK, Kumar S (2005) Precocious germination of pollen grains in anthers of soybean (*Glycine max* (L.) Merr.). Soybean Genet Newsl 32:1–10

Kuprianova LA (1973) Pollen morphology within the genus *Drosera*. Grana 13:103–107

Lora J, Herrero M, Hormaza JI (2009) The coexistence of bicellular and tricellular pollen in *Annona cherimola* (Annonaceae): implications for pollen evolution. Am J Bot 96:802–808

Lora J, Herrero M, Hormaza JI (2014) Microspore development in *Annona* (Annonaceae): differences between monad and tetrad pollen. Am J Bot 101:1508–1518

Lord E (1979) The development of cleistogamous and chasmogamous flowers in *Lamium amplexicaule* (Labiatae); an example of heteroblastic inflorescence development. Bot Gaz 140:39–50

Manning JC (1996) Diversity of endothecial patterns in the angiosperms. In: D'Arcy WG, Keating RC (eds) The anther, form, function and phylogeny. Cambridge University Press, Cambridge, pp 136–158

Marquez J, Seoane-Camba JA, Suarez-Cervera M (1997) The role of the intine and cytoplasm in the activation and germination processes of Poaceae pollen grains. Grana 36:328–342

Nagpal P, Ellis CM, Weber H, Ploense SE, Barkawi LS, Guilfoyle TJ, Hagen G, Alonso JM, Cohen JD, Farmer EE, Ecker JR, Reed JW (2005) Auxin response factors ARF6 and ARF8 promote jasmonic acid production and flower maturation. Development 132:4107–4118

Okada H (2008) Pollination system of *Aldrovanda vesiculosa* (Droseraceae), a critically endangered aquatic plant in Japan. Makinoa New Ser (Kochi) 7:93–100

Pacini E, Dolferus R (2019) Pollen developmental arrest: maintaining pollen fertility in a world with a changing climate. Front Plant Sci 10:e679

Pacini E, Franchi GG (1982) Germination of pollen inside anthers in some non-cleistogamous species. Caryologia 35:205–215

Pacini E, Hesse M (2005) Pollenkitt – its composition, forms and functions. Flora 200:399–415

Pacini E, Juniper B (1984) The ultrastructure of pollen grain development in *Lycopersicum peruvianum*. Caryologia 37:21–50

Pacini E, Jacquard C, Clément C (2011) Pollen vacuoles and their significance. Planta 234:217–227

Rodondi G, Beretta M, Andreis C (2004) The genus *Drosera* L. in northern Italy: pollen morphology as a taxonomic tool. Plant Biosyst 138: 157–164

Sahai K, Rawat KK, Gupta D (2016) A note on precocious pollen germination in *Woodfordia fruticosa* (L.) Kurz. Trop Plant Res 3:606–610

Sahashi N, Ikuse M (1973) Pollen morphology of *Aldrovanda vesiculosa*. J Jap Bot 48:374–379

Scott RJ, Spielman M, Dickinson HG (2004) Stamen structure and function. Plant Cell 16(Suppl 1):S46–S60

Sirová D, Adamec L, Vrba J (2003) Enzymatic activities in traps of four aquatic species of the carnivorous genus *Utricularia*. New Phytol 159:669–675

Takahashi H (1988) Ontogenetic development of pollen tetrads of *Drosera capensis* L. Bot Gaz 149:275–282

Takahashi H, Sohma K (1982) Pollen morphology of the Droseraceae and its related taxa. Sci Rep Res Inst Tohoku Univ (Biol) 38:81–156

Vega-Maray A, Fernández-González D, Valencia-Barrera R, Seoane-Camba J, Suárez-Cervera M (2003) Ultrastructural modifications in the apertural intine of *Parietaria judaica* L. (Urticaceae) pollen during the early stages of hydration. Grana 42:220–226

Wang Y, Chu YJ, Xue HW (2012) Inositol polyphosphate 5-phosphatase-controlled Ins(1,4,5)P3/Ca2+ is crucial for maintaining pollen dormancy and regulating early germination of pollen. Development 139:2221–2233

Weber HE (1995) *Aldrovanda*. In: Hegi G (ed) Illustrierte Flora von Mitteleuropa, Band IV, Teil 2A, 3. Auflage. Blackwell Wissenschafts, Berlin, pp 34–37

Whatley JM (1982) Fine structure of the endothecium and developing xylem in *Phaseolus vulgaris*. New Phytol 91:561–570

Wilson ZA, Song J, Taylor B, Yang C (2011) The final split: the regulation of anther dehiscence. J Exp Bot 62:1633–1649

Xie B, Wang X, Hong Z (2010) Precocious pollen germination in *Arabidopsis* plants with altered callose deposition during microsporogenesis. Planta 231:809–823