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The pollinium of *Loroglossum hircinum* (*Orchidaceae*) between pollination and pollen tube emission

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Key words: *Orchidaceae, Loroglossum hircinum.* – Compound pollen, pollinium, pollen tubes, generative cell, evolution.

Abstract: The structure of the massulae composing the pollinium of *Loroglossum hircinum* was studied before pollination and 12 and 24 hours afterwards. The grains are grouped in tetrads closely packed in massulae. The exine is only present on the outside of the massulae. The intine consists of two layers: a compact layer surrounding the pollen grain and a looser layer surrounding the pollen grain and a looser layer surrounding the tetrad. Twelve hours after pollination, pollen volume and the space between the tetrads increase due to vacuolization. Twenty-four hours after pollination, pollen volume and tetrad spacing are higher due to vacuolization and some grains have emitted pollen tubes. Pollen growth due to vacuole formation, and the absence of common walls between adjacent tetrads lead to crumbling of the massulae. The mature pollen grain does not have apertures: the site of pollen tube emission is determined after pollination. The first grains to germinate are those in the centre of the massula. The vegetative cell nucleus is the first to enter the pollen tube; the generative cell elongates and undergoes the second haploid mitosis shortly after entering the pollen tube.

The pollen of anemophilous plants is dispersed in monads (*Poaceae*), tetrads (*Typhaceae*) or clumps held loosely together with pollenkitt (certain *Compositae, Euphorbiaceae, Oleaceae, Polygonaceae*) (Hesse 1979, Pacini & Franchi 1993, Lisci & al. 1994). The pollen grains of entomophilous plants are released in various kinds of dispersing units: as clumps held together by pollenkitt, viscin threads or elastoviscin (Pacini & Franchi 1993) or as compound pollen (Knox 1984, Knox & McConchie 1986). The most common type of compound pollen found in the orchids is the pollinium (Wolter & Schill 1985, 1986; Zee & Siu 1990). The pollinium is said to be soft when it consists of different subunits known as massulae which can pollinate many flowers (Yeung 1987), whereas in hard pollinia the pollen grains are held together by an external wall of exine. Therefore, the pollinium is deposited as a whole on the stigma of a single flower (Yeung 1987, Zavada 1983). A pollinium may contain 40,000 to 4,000,000 pollen grains (Schill & al. 1992).

The reproductive biology of the orchids is widely unknown, compared to plants of economic interest. Pollen development has been studied in orchids with differ-

ent types of dispersal units (YEUNG 1987). SCHLAG & HESSE (1992) have studied the formation and nature of the generative cell wall and the detachment mode of the generative cell from the intine in *Polystachia pubescens*. Pollination ecology is known to involve seduction with nectar as reward, or deception by means of volatile compounds similar to pollinator pheromones (DAFNI 1987, GERLACH & SCHILL 1991). Pollen germination is peculiar and less well known, involving compound pollen in most cases, with many pollen grains not in contact with the outer surface of the pollinium. The ultrastructure of pollen tube growth in the style has been studied in *Epidendrum* (COCUCCI 1988).

Here we present a study of pollen morphology and germination in *Loroglossum hircinum*, an orchid with soft pollinia. Pollen tubes are emitted within 24 hours after the massula was deposited on the stigma. Pollen germination can only take place after the tetrads have separated due to vacuolization.

Material and methods

Plants of *Loroglossum hircinum* (L.) C. RICH. = *Himantoglossum hircinum* (L.) SPRENGEL growing wild in the evergreen woods around Siena were used for cytological study.

Microscopy. Mature pollinia and pollinia collected 12 and 24 hours after cross pollination were fixed in 4% freshly depolymerized paraformaldehyde in phosphate buffer (pH 7.2), dehydrated in an ethanol series and embedded in LR White (London Resin Co. Ltd.). The sections (2 µm thick) were tested for: a) total insoluble polysaccharides by PAS after aldehyde blockade with dimedone (O'BRIEN & MCCULLY 1981); b) total proteins with bromophenol blue (PEARSE 1968); c) callose with aniline blue (O'BRIEN & MCCULLY 1981); d) RNA, DNA and polycarboxylic acids with toluidine blue O, TBO (O'BRIEN & MCCULLY 1981); e) pectins with alcian blue (PEARSE 1968); f) DNA by fluorescence microscopy with DAPI (COLEMAN & GOFF 1985); g) sporopollenin with auramine O (HESLOP-HARRISON 1977). Stained and unstained sections were observed by interference contrast microscopy.

Quantitative cytology. Mean microspore surface area and the percentage surface area occupied by tetrads, spaces between tetrads, and pollen tubes at different times, were calculated by a computer image analysis system (Carl Zeiss, Oberkochen, Germany) in sections stained with TBO. Means and standard errors were calculated from ten counts of different non contiguous sections at the same stage. It was not possible to measure microspore volume at different times after pollination because the tetrads were of different geometric forms.

Results

Mature pollinium structure. The pollinarium consists of two club-shaped soft pollinia with 80–100 massulae. The two stipes join at the viscidium (Fig. 1). The massulae consist of closely packed tetrads (Figs. 2, 4). The pollen grains have a polygonal profile, and the tetrads are variable in shape, being mainly tetrahedral towards the outside of the massulae and isobilateral on the inside (Table 1). The exine is without ornamentation and is thicker on the outer side of the outermost tetrads and thinner between the massulae (Fig. 1); it is in the form of plaques (Fig. 4). The intine consists of two layers. The inner layer surrounds the single pollen grain; it is of uniform thickness, stains purplish red with TBO and consists of pectins and carboxylic acids. The outer layer units the pollen grains in tetrads; it is of variable thickness (Fig. 5), stains pink with TBO and consists of pectins only. The outer intine layers of adjacent tetrads are not fused together (Fig. 5) and the

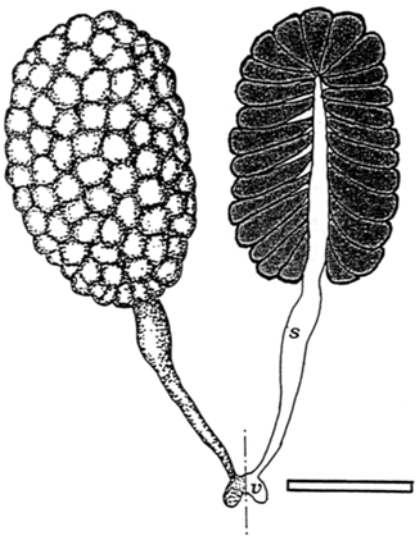


Fig. 1. Diagram of internal and external structure of *Loroglossum hircinum* pollinarium. The massulae, 80–100 in number, are attached to the stripe (*s*); the two pollinia join in the viscidium (*v*) that attaches to the pollinator body. The exine (black line) is thicker on the external surface of the massulae than between the massulae. Bar: 1 mm

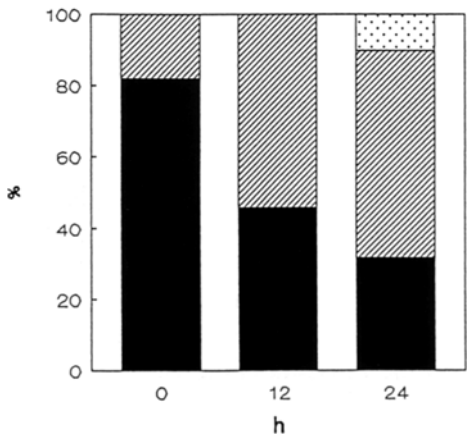


Fig. 2. *Loroglossum hircinum*. Percentage of surface occupied by pollen grains (black area), intercellular spaces (cross hatched) and pollen tubes (dotted), at the time of pollen deposition and 12 and 24 hours afterwards. The pollen tubes appear after 24 hours

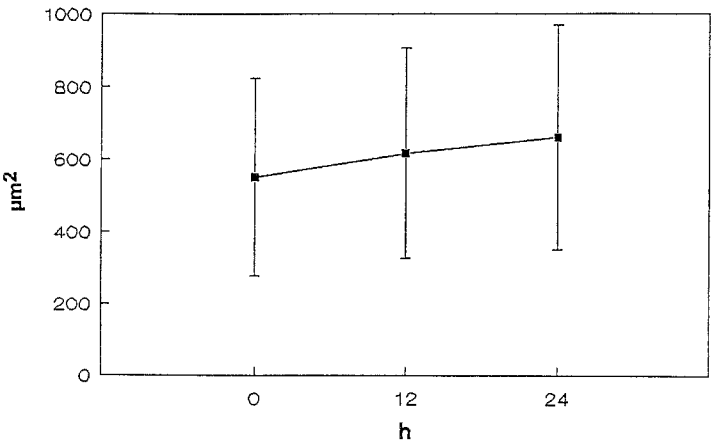


Fig. 3. *Loroglossum hircinum*. Pollen grain surface area (± SD) at the time of pollination and 12 and 24 hours afterwards

Table 1. Types of tetrads in the external and internal part of the pollinium in *Loroglossum hircinum*

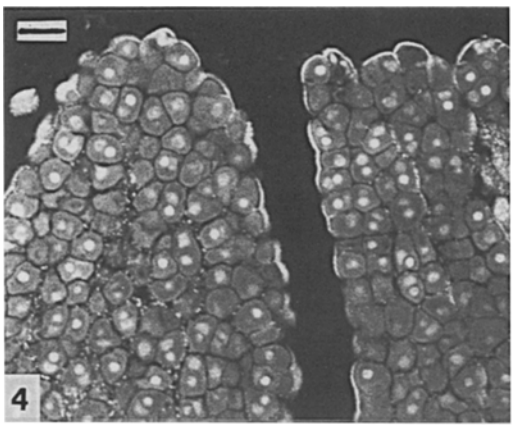
Tetrad type	External tetrads (%)	Internal tetrads (%)
Tetrahedral	73.1	9.8
Isobilateral	26.9	69.5
Decussate		9.8
T-shaped		10.9

tetrads separate when the pollinium is crushed. The generative cell and its nucleus are roundish, whereas the nucleus of the vegetative cell is slightly elongated and paler when stained with DAPI (Fig. 6).

The pollinium 12 hours after pollination. The pollinium detaches easily from the stigma surface during fixing and embedding. Its structure is slightly modified because the space between the tetrads has increased (Figs. 2, 7). The mean surface area of the pollen grains has also increased (Fig. 3) giving them a more rounded appearance. The pollen cytoplasm contains small vacuoles (Fig. 7). The different types of tetrads are easier to distinguish because there is more space between them.

The pollinium 24 hours after pollination. The pollinium detaches less readily from the stigma surface during fixing and embedding. The space between the tetrads has increased (Fig. 2) together with the mean surface area of the pollen grains (Fig. 3). Hence the massulae have increased in volume, are lengthening radially and separating from each other, beginning from the outside. The tetrads also start to separate (Fig. 10). The outer layer of intine has become thinner because the pollen grains have increased in volume (Fig. 3) and become rounder. The percentage of pollen grains germinating is 20–30%, depending on the massula (Fig. 8), but the pollen tubes have not yet penetrated the stigma surface. The first to germinate are

Figs. 4–9. *Loroglossum hircinum*. – Fig. 4. Detail of two adjacent massulae in mature pollinium (auramine 0). The exine covers only the external part of external tetrads; it is discontinuous, consisting of plaques, one for each part of the tetrad. Bar: 50 µm. – Fig. 5. Parts of adjacent tetrads of mature pollinium (TBO). The pollen grains have two walls, a darker, uniform, inner wall surrounding each grain (arrowheads) and an outer wall of variable thickness uniting the grains of the tetrad (arrows). Only small spaces can be observed between tetrads. Bar: 10 µm. – Fig. 6. Detail of mature pollinium (DAPI). The nucleus of the generative cell is round and fluorescent whereas that of the vegetative cell is oval and less fluorescent. Bar: 50 µm. – Fig. 7. Detail of a pollinium 12 hours after pollination (interference contrast). Small vacuoles (arrowheads) begin to appear in the pollen grains and the spaces between tetrads have increased. Bar: 25 µm. – Fig. 8. Detail of a pollinium 48 hours after pollination (TBO). A pollen grain has recently emitted a pollen tube. Part of the inner intine has been overturned under the impetus of the pollen tube (asterisk). The vegetative nucleus has already entered the tube. Bar: 10 µm. – Fig. 9. Detail of a pollinium 48 hours after pollination (aniline blue). The pollen grains (*) and tubes (arrows) have a thin callose wall. Bar: 25 µm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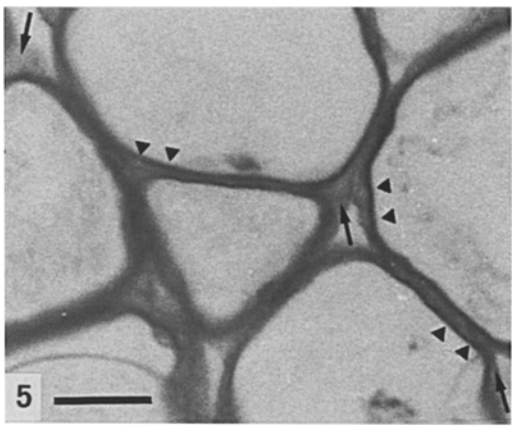
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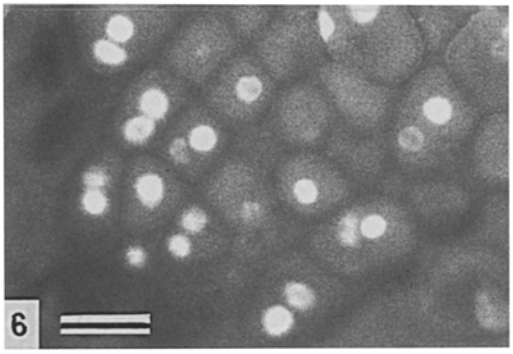
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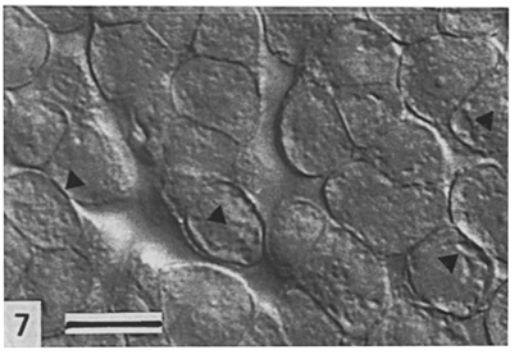
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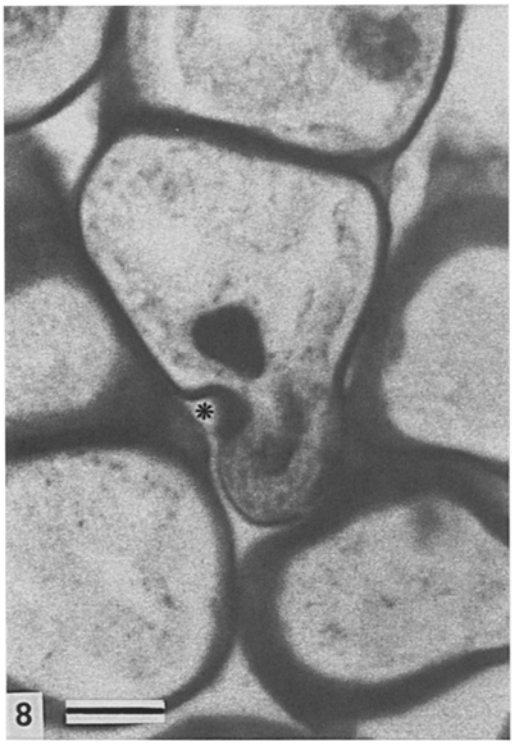
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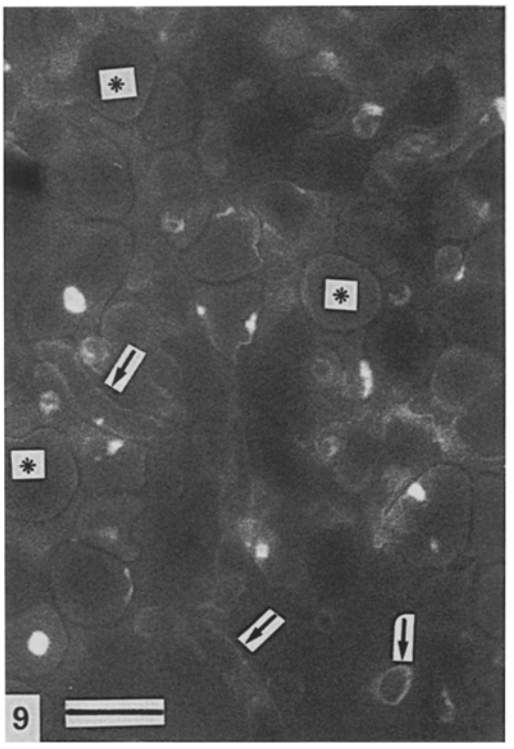
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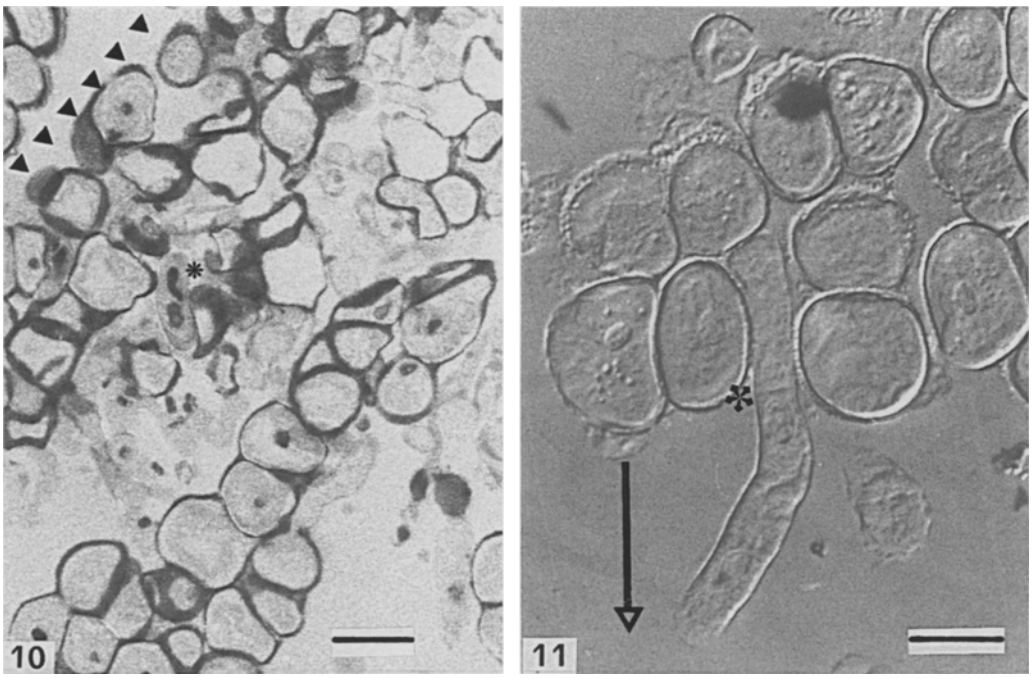

Figs. 10, 11. *Loroglossum hircinum*. – Fig. 10. Detail of crumbling massula 48 hours after pollination (TBO). The external tetrads (arrowheads) are less spaced than the central tetrads. Many pollen tubes are visible in the spces between the central tetrads. The vegetative nucleus is visible in one (*) together with the generative cell which is now elongated. Bar: 25 µm. – Fig. 11. Detail of pollinium 24 hours after pollination (interference contrast). In a pollen tube growing towards the stigma (direction of arrow) telophase of the second haploid mitosis is visible (*). Bar: 30 µm

those at the centre of the massula (Fig. 10). The intine is generally thicker where the pollen tube emerges, as if a lid opened out under the pressure of the growing tube (Fig. 8). A thin callosic wall is present in the pollen grains and tubes (Fig. 9). The vegetative nucleus enters the pollen tube first, followed by the generative cell which is no longer round but slightly elongated (Fig. 8). The generative cell elongates considerably and undergoes second haploid mitosis before penetrating the stigma (Fig. 11).

Discussion

Pollinium structure. It is currently held that the intine, but especially the exine, vary in relation to the pollination system (Muller 1979, Burns-Balogh 1983, Zavada 1983, Hesse & Waha 1983), and this is also true for compound pollen (Knox & McConchie 1986, Fitzgerald & al. 1993). Since orchids have different types of pollen dispersing units, exine and intine have different patterns. *Epidendrum scutella* and many other orchids have three types of exine: that surrounding each pollen grain, that uniting the tetrad and that surrounding the massula (Cocucci & Jensen 1969). *Peristylus spiranthes* likewise has three types of exine but between the microspores of the tetrads it is discontinuous (Zee & Siu 1990). The

intine is sometimes bi-layered: that separating the pollen grains of a tetrad has persistent cytomictic channels, as a result the generative cells are immersed in the same cytoplasm. In *Loroglossum*, the exine is only present on the outside of the outermost tetrads of each massula, and it is thickest on the outermost tetrads of the pollinium; moreover, it is without ornamentation. The absence of the exine in most pollen grains may be due to the fact that the pollen dispersing unit in this case is the massula which does not adhere directly to the pollinator's body but is carried in the pollinarium. In Fig. 4 the massulae are hydrated by the fixative, but when they are dry, there should be no space between the exine of an external tetrad and that of the adjacent one, in order to prevent water loss at anthesis. It should be called in mind that in orchids the pollen is not completely exposed to the air but sheltered inside the anther until the pollinator arrives. The purpose of this arrangement may be to prevent pollen dehydration. In *Oncidium cheirophorum* the pollen is offered to the pollinator for as long as 30 days (Clifford & Owens 1988).

The different type, number and form of the tetrads on the outside and inside of the massula are related to the manner in which the tetrads are packed inside the pollinium, helping to avoid water loss. The pollen grains of the outer tetrads are certainly a disadvantage in this regard with respect to the inner ones, until the pollinator arrives.

Most of the pollen grains have only a uniform intine wall; in *Loroglossum*, instead, it consists of two distinct layers with different functions. The inner layer surrounds the individual grains and distends when the pollen increases in volume due to vacuole formation. With the methods of observation used in this study no openings were apparent, which suggests that they are not predetermined. Since the grains are packed together, the site of pollen tube emission is probably determined after the tetrads become spaced from each other. The outer layer of the intine unites the grains of the tetrad. Figure 5 suggests that the external intine is not common to adjacent tetrads. This is confirmed by the fact that the tetrads come apart with gentle pressure as well as after pollination, when the pollen volume increases due to vacuolization. If the tetrads had a common intine, it would be difficult for them to separate from each other. The function of the outer intine layer is also to allow rearrangement of the grains of a tetrad and distancing of the tetrads, as a consequence of the increase in pollen grain volume. Since there are no common walls, unlike in others compound pollen (Knox & McConchie 1986), the tetrads of *Loroglossum* stay together by virtue of the way they are packed.

Vacuole formation and change in pollen grain form. *Loroglossum hircinum* pollen germinates a long time after pollination. Although some grains have germinated after 24 hours, the pollen tubes have not yet penetrated the stigma. This is evident from the fact that some pollinia detach from the stigma during fixing and embedding. The late emission of pollen tubes is probably due to the fact that space must first be created in which they can grow. Whenever pollen germinates, vacuolization occurs (Heslop-Harrison 1987) but in *Loroglossum hircinum* and perhaps in all *Orchidaceae* with pollen in massulae, this is the first and only vacuolization that occurs during male gametophyte development (Fig. 12). In all the other species studied so far, one or two vacuolizations, followed by formation of new cytoplasm (Pacini 1994), occur between microspore release and opening of the anther (Fig. 12). Orchid pollen is very small compared to pollen of other families

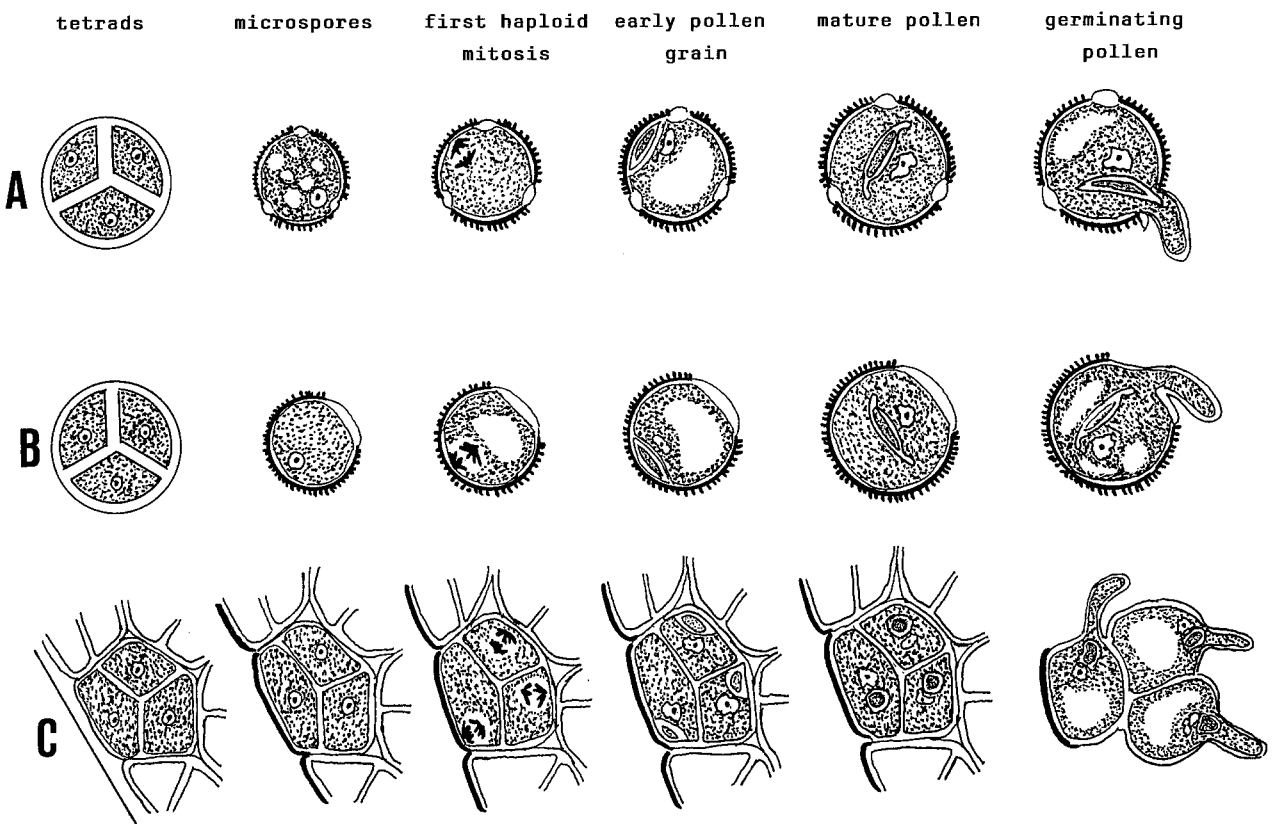

Fig. 12. Comparative examples of vacuole formation during male gametophyte development. Vacuoles always form after germination; during pollen development, there may be two vacuolizations as in many dicots (*A*), one vacuolization as in many monocots (*B*) or none as in the *Orchidaceae* (*C*). The pollen grains of monocots and dicots increase in volume as a consequence of vacuolization; in orchids, the volume increase in extremely limited

(PANDOLFI & al. 1993); orchid pollen in massulae and pollinia is polyhedral rather than spherical or ovoid as usual. The small volume is due to the lack of vacuoles (PANDOLFI & al. 1993). A similar situation is present in the genus *Acacia* in which the pollen dispersing unit is the polyad (FITZGERALD & al. 1993).

Once germination has begun, most of the cytoplasm migrates into the pollen tube with the generative cell, the vacuole grows and it finally becomes extracellular due to formation of the callose plug.

Germination. In angiosperms, the time elapsing between pollen deposition on the stigma and emission of the pollen tube varies from species to species: it is c. one minute in *Secale cereale* (HESLOP-HARRISON 1979), c. 3 minutes in *Cucurbita pepo* (NEPI & PACINI 1993), c. 60 minutes in *Helleborus foetidus* (HESLOP-HARRISON & al. 1986) and c. 3 hours and 30 minutes in *Lycopersicum peruvianum* (PACINI & SARFATTI 1978). Although many pollen grains reach the stigma at the same time in *Loroglossum hircinum*, they do not germinate synchronously, those in the centre of the massula germinating before those on the periphery. This may be because the tetrads at the centre of the massula are the first to be isolated and to have space for

the tubes to grow. The grains of the outermost tetrads would have space for the tubes to grow; but the exine without apertures prevents pollen tubes from growing on the outside.

A similar example of graded germination in a species with pollen dispersed in monads surrounded by pollenkitt, is that of *Cucurbita pepo*, in which there are 411 ± 52 ovules per ovary. A pollinator brings an average of 224 pollen grains every time it visits a flower, but because many grains do not adhere to the stigma, an average of 88 visits are necessary (Nepi & Pacini 1993). In this case germination takes place in a few minutes and a wave of pollen tube emissions follows each insect visit. When there are few ovules per ovary, it is of on the other hand of great importance for the grains to emit a pollen tube immediately to have a chance for fertilization (Ottaviano & Mulcahy 1989). With many ovules per ovary, as in the orchids, those in certain positions may be fertilized first (Den Nijs & Miotay 1991, Le Deunff & al. 1993).

The generative cell and the second haploid mitosis. At maturity, the pollen grains of the *Orchidaceae* are binucleate and have a spherical generative cell in contrary to the spindle-shaped one of all other angiosperm pollen grains (Pandolfi & al. 1993). These authors suggest that a spherical generative cell requires less energy than a spindle-shaped one, as the pollinium may await the pollinator for many days. As in *Loroglossum hircinum*, much time elapses between pollen landing on the stigma and pollen tube emission (Johri & al. 1992). This delay in completion of male gametophyte development is not surprising as the female gametophyte is not yet formed and meiosis only occurs when the pollen tubes are close to the ovules (Johri & al. 1992). In *Loroglossum hircinum* the generative cell is already slightly elongated when the pollen tube is emitted; shortly afterwards it becomes spindle-shaped like generative cells of other species, and undergoes the second haploid mitosis before the pollen tubes enter the stigma. In general, this division marks male gametophyte maturation and may occur before opening of the anther (as is the case for all pollen grains that are tricelled at maturity), on the stigma before germination (as is the case in *Loroglossum hircinum*), in the style when the pollen tube grows towards the ovary (as is most common), or near the ovule (Maheshwari 1950).

Conclusion

A variable number of pollen grains reaches orchid stigmas, depending on the type of the pollen dispersing unit (Brown & Lemmon 1994). The organization of the pollen dispersing unit also determines the manner in which the grains must become spaced in order to emit pollen tubes, the path which the tubes follow to reach the stigma and the time necessary for them to emerge.

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