Development of the exineless pollen wall in *Callitriche truncata* (Callitrichaceae) and the evolution of underwater pollination

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Abstract. The Callitrichaceae are a monogeneric family of aquatic angiosperms comprising approximately 50 terrestrial, amphibious, and obligately submersed species. *Callitriche* is unique in being the only known genus with co-occurring aerial and underwater pollination systems. Mature pollen structure is correlated with growth habit, pollination biology, and phylogeny within the genus. In the present study, development of exineless pollen in the obligately submersed species *Callitriche truncata* was examined, with particular emphasis on the tetrad stage. Pollen ontogeny occurred rapidly and non-synchronously; tetrads, free microspores, and two-celled pollen grains were identified within the same anthers. Formation of the intine also occurred relatively early, during the tetrad stage. Tetrads were surrounded by a structurally distinct envelope, and its ultrastructure and histochemistry indicate that this callose-like envelope is in a transitional state. Reduction or complete loss of the exine has evolved at least twice in Callitrichaceae, and the new ontogenetic data indicate that exine loss evolves more quickly than the loss of callose. In addition, developmental information on exineless pollen in *C. truncata* coupled with other palynological data for the exine-bearing terrestrial and amphibious growth forms provide support for the hypothesis that underwater pollination has had a relatively recent origin in the family.

The Callitrichaceae, or water starworts, are a monogeneric family of aquatic angiosperms comprising approximately 50 terrestrial, amphibious, and obligately submersed species (Philbrick and Les 2000). *Callitriche* is unique among angiosperms in being the only known genus with co-occurring aerial and underwater pollination systems. Anemophily, epihydrophily (pollination at the water surface), and hypohydrophily (pollination below the water surface) have all been reported (see Philbrick and Anderson 1992 and Osborn and Philbrick 1994). Pollen morphology and ultrastructure have also been shown to be correlated with growth habit and pollination biology. In general, terrestrial taxa have well-defined exines, amphibious taxa have thinner exines, and obligately submersed taxa have exineless pollen (Martinsson 1993, Osborn and Philbrick 1994, Cooper et al. 2000). This pattern of exine reduction and loss among species is also associated with the phylogenetic framework of the family (Cooper et al. 2000, Philbrick and Les 2000).

Significant reduction or complete loss of the exine is a common pollen character among hydrophilous angiosperms and appears to be a critical factor in the transition from aerial to submersed pollination systems (e.g. Cox 1988, Osborn and Philbrick 1994, Philbrick and Les 1996, Cooper et al. 2000). Given the uniqueness of Callitrichaceae, the family provides an excellent opportunity to study the evolution of hydrophily. Palynological data also indicate that underwater pollination in *Callitriche* may have evolved relatively recently (Cooper et al. 2000). Another line of evidence that may support a recent origin hypothesis comes from pollen ontogeny, particularly development of exineless pollen within the obligately submersed growth form.

The objective of this paper was to examine development of the exineless pollen wall in the obligately submersed species *Callitriche truncata* Gussone, with particular emphasis on tetrad structure. New ontogenetic data were compared to those of other hypohydrophilous angiosperms, particularly *Ceratophyllum*, in an effort to further interpret the evolution of underwater pollination in Callitrichaceae.

Material and methods

Plants of *Callitriche truncata* Gussone were collected in Spain (Cooper et al. 2000), and voucher specimens (4595, 4601) are deposited in the Western Connecticut State University (WCSU) herbarium.

Because of their extremely small size, whole plants were fixed in 3% glutaraldehyde (buffered in 0.2 M phosphate buffer; pH 7.4) for 24 hr and buffer-washed four times. Several anthers were dissected from the plants, post-fixed in 1% buffered osmium tetroxide for 3–6 hr, buffer-washed four times, and dehydrated in a graded ethanol series.

The dehydrated anthers were gradually infiltrated and embedded in Spurr epoxy resin and then thick- and thin-sectioned on an ultramicrotome using a diamond knife. Thick sections were collected on microscope slides and stained either with 1% toluidine blue for general histological study, or with 4′,6-diamidino-2-phenylindole (DAPI) for callose (Hansson and El-Ghazaly 2000). The thick sections were examined and imaged using a Zeiss Axioplan 2 light microscope either with brightfield or phase contrast illumination for histology, or with fluorescence for callose. Thin sections were either collected on copper grids and stained with 1% uranyl acetate (5 min) and lead citrate (5 min) for general ultrastructure, or collected on gold grids and stained with phosphotungstic acid in chromic acid (5 min) for acidic polysaccharides (i.e. primexine matrix, or glycocalyx coat). The thin sections were examined and imaged using a Zeiss EM 906 transmission electron microscopes at 80 kV.

Results

Anthers were observed that contained tetrads, free microspores, and two-celled pollen grains (Fig. 1). The tapetum remained intact at these stages and appeared to be of the secretory

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Figs. 1–6. *Callitriche truncata* – Anther structure and tetrad stage. **1.** Transverse section of an anther showing tetrads in the top locule, as well as free microspores and two-celled pollen grains in the bottom locule. Bar = 100 μm. **2.** Section through a tetrahedral tetrad showing a callose-like envelope (arrows) asymmetrically surrounding the microspores. There is a broad periplasmic space between the plasmalemmae (*P*) of the microspores and the developing pollen walls. Bar = 3 μm. **3.** Detail of the contact face between the three microspores of the tetrad depicted in Fig. 2 showing the developing intines (*I*) and central callose-like layer (*C*). Note the single, patchy white-line-centered lamella (arrow). Bar = 0.3 μm. **4.** Detail of the outer edges of four contiguous tetrads, including the top, right edge of the tetrad shown in Fig. 2. Note the two-zoned callose-like envelope (*C*), developing intines (arrowheads), and microspore plasmalemmae (*P*) separated from the intines by a periplasmic space. Bar = 1 μm. **5.** Outer edges of three contiguous tetrads showing intines (arrowheads), non-uniform distribution of lamellae (arrows), and surrounding callose-like layers (*C*). The electron-density of the lamellae indicates a positive histochemical reaction for acidic polysaccharides (stained with phosphotungstic acid in chromic acid). Bar = 0.5 μm. **6.** Detail of a developing microspore wall showing a white-line-centered lamella and intine (*I*). The lamella shows a positive histochemical reaction for acidic polysaccharides (stained with phosphotungstic acid in chromic acid). Bar = 0.11 μm

type (Fig. 1). A range of tetrad configurations were observed, but most were tetrahedral (Figs. 1–2). Each tetrad was surrounded by a thin envelope that averaged 0.73 μm in thickness on the sides (Figs. 2, 4). This layer was callose-like in both position and general ultrastructure; it asymmetrically enclosed the entire tetrad (Figs. 2, 4), separated each of the constituent microspores (Figs. 1–3), and was two-zoned, with the outer zone being more dense (Fig. 4). When stained with DAPI, the tetrad envelope exhibited only a faint positive reaction. The developing sporoderm was directly beneath and contiguous with the callose-like layer (Figs. 3–4). At this stage, the sporoderm consisted of thin, discontinuous lamellae underlain by a fibrillar intine that averaged 0.10 μm in thickness (Figs. 3–6). The lamellae stained positive for acidic polysaccharides; however, a comprehensive
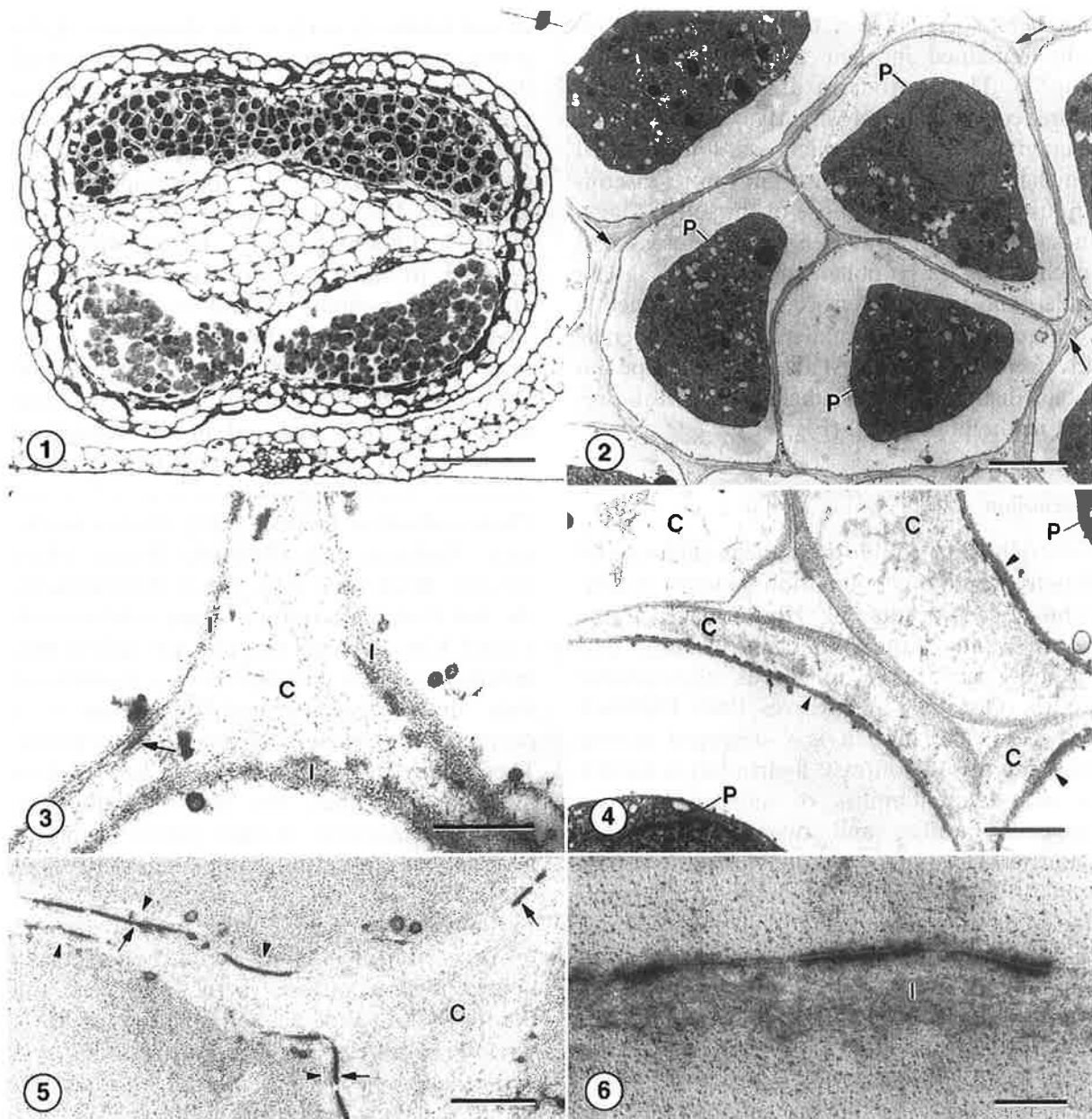
primexine matrix was not present (Figs. 5–6). A relatively broad periplasmic space was present between the intine and microspore plasmalemma at the tetrad stage (Figs. 2, 4). This space was interpreted as an artifact, with the microspore protoplasts having plasmolyzed within the tetrads. The free microspores and pollen grains within the same anthers did not exhibit as broad of a periplasmic space, however.

At the free microspore and early pollen grain stages, the callose-like layer had completely dissociated, but the microspores typically remained in their tetrad configuration (Fig. 7). The sporoderm of each free microspore consisted of a slightly thicker intine, averaging 0.13 µm in thickness, and residual lamellae that were thinner and more discontinuous (Fig. 8) than those of the tetrad stage. In addition, many free microspores had reached the early pollen grain stage, having undergone mitotic division, and contained a vegetative cell and a smaller, peripheral generative cell (Figs. 7, 9). At the two-celled pollen stage, distinguishable lamellae were not present in the pollen wall (Fig. 9).

Discussion

Callitrichaceae exhibit both anemophilous and hypohydrophilous pollination systems. Anemophily also occurs in Hippuridaceae, the closest related family to Callitrichaceae and the outgroup taxon in recent phylogenetic studies (Olmstead and Reeves 1995, Philbrick and Les 2000), and it is widespread among angiosperms. In contrast, hydrophily is known in only eight families of angiosperms: six monocot families and two dicot families, Callitrichaceae and Ceratophyllaceae (see Les et al. 1997). Given the phylogenetic distribution of these systems, anemophily is thought to be primitive within Callitrichaceae. What clues can palynological data contribute to understanding the origin of underwater pollination in *Callitriche*?

A callosic 'special wall' is believed to play an important role in protection for early deposition of the microspore wall, as well as exine patterning during the tetrad stage in angiosperms (e.g. El-Ghazaly and Jensen 1986, Blackmore and Barnes 1990, Schmid et al. 1996). The tetrads of *Callitriche truncata* Gussone are surrounded by a structurally distinct envelope, but this layer is relatively thin and not as well-defined in ultrastructure as most callose special walls. As such, this enveloping layer has been described as "callose-like" in the present paper. If the origin of underwater pollination in *Callitriche* had occurred relatively early in the divergence of the genus, one might expect a callose layer around the tetrads to be completely absent in an exineless species such as *C. truncata*. This is the case during pollen ontogeny in *Ceratophyllum demersum* L. (Ceratophyllaceae; Takahashi 1995), the only other dicot known to have hypohydrophily. *Ceratophyllum* is believed to have a more ancient origin of underwater pollination and to be primitive among angiosperms (Les et al. 1991, Soltis et al. 1999, Qiu et al. 2000, Mathews and Donoghue 2000). Callose layers are also absent or greatly reduced in the tetrads of several hydrophilous monocots (e.g. Cymodoceaceae: *Amphibolis* [Ducker et al. 1978] and *Thalassodendron* [Pettitt 1981]; Hydrocharitaceae: *Thalassia* and *Halophila* [Pettitt 1981]; see also Blackmore et al. 1987). Furthermore, the fact that the tetrad envelope exhibits only a weak histochemical reaction for callose may indicate that this envelope is in a transitional state in *C. truncata* and that there is a progression toward complete loss occurring. These data indicate that exine loss evolves more quickly than the loss of callose in *Callitriche*, because mature pollen grains of *C. truncata* are completely exineless (Cooper et al. 2000).

Pollen ontogeny in *C. truncata* also appears to proceed quite rapidly and non-synchronously, both in meiotic (between locules) and mitotic (within a locule) activity. Tetrads, early free microspores, late free microspores, and two-celled pollen grains can all be identified within the same diminutive anthers.
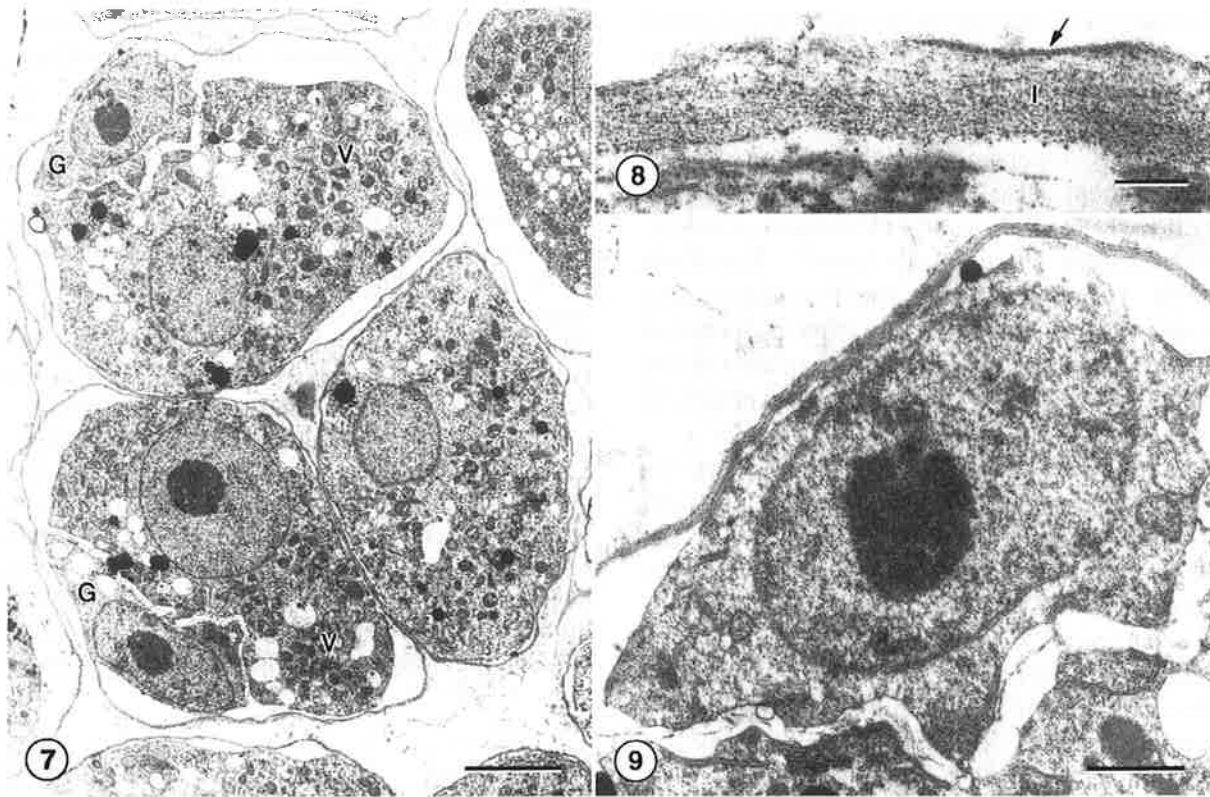
Figs. 7–9. *Callitriche truncata* – Free microspore and two-celled pollen stages. **7.** Three, free microspores still in a tetrad configuration. Note that the two microspores on the left have undergone mitotic division, reached the two-celled pollen stage, and each contains a larger vegetative cell (*V*) and smaller generative cell (*G*). Bar = 3 μm. **8.** Detail of the wall from a late free microspore showing fibrillar intine (*I*) and residual lamella (arrow). Bar = 0.1 μm. **9.** Detail of the topmost pollen grain depicted in Fig. 7 showing the peripheral generative cell and the young pollen wall, which lacks distinguishable lamellae. Bar = 1 μm

Formation of the intine occurs relatively early in *C. truncata*, during the tetrad stage. This is unusual among angiosperms, in which the intine typically develops in the early pollen grain stage after the first mitotic division (see El-Ghazaly et al. 2001). However, early intine development has been reported in several taxonomically disparate taxa, including *Zingiber spectabile* Griff (Zingiberaceae; Theilade and Theilade 1996) and *Canna generalis* Bailey (Cannaceae; Rowley and Skvarla 1986). The mature pollen of these two Zingiberalean monocots remains hydrated at anthesis, and it has a reduced exine and a thickened intine. These two pollen wall character states appear to be correlated with non-desiccating environments (Kress 1986, Furness and Rudall 1999).

Pollen of the obligately submersed *C. truncata* is not subject to desiccation either. Early initiation of the intine in *C. truncata* may be an ontogenetic adaptation for underwater dehiscence/pollination, thereby providing more time for intine development given the absence of exine deposition.

Although the earliest tetrad stage has not been identified in *C. truncata*, the lamellae observed in later tetrads more than likely develop before the intine. As indicated by a positive histochemical reaction for acidic poly-saccharides (i.e. a primexine matrix), the lamellae represent rudimentary endexine units on which sporopollenin is never deposited to form an exine. The lamellae are thin and non-uniform in distribution in the tetrad stage,

become more discontinuous and thinner in the free microspore stage, and are virtually absent in the two-celled pollen stage. Lamellae are absent in the mature pollen wall of *C. truncata* as well (Cooper et al. 2000). Rapid pollen development also occurs in *Ceratophyllum* with early formation of well-defined, continuous lamellae ("trilaminar layer"; Takahashi 1995). However, during the free microspore stage in *Ceratophyllum* a thin exine is deposited over the lamellae (which persists on the mature pollen) and is followed by intine development after microspore mitosis (Takahashi 1995).

Significant reduction and/or loss of the exine has evolved at least twice in Callitrichaceae (Cooper et al. 2000, Philbrick and Les 2000), and four species of *Callitriche* are known to have exineless pollen. These include *C. truncata*, *C. lusitanica* Schotsman, *C. brutia* Petagna (Cooper et al. 2000), and *C. hermaphroditica* L. (Osborn and Philbrick 1994). However, variability occurs in the mature pollen wall of *C. hermaphroditica*, in which a rudimentary exine has been reported in some collections (Martinsson 1993, Osborn and Philbrick 1994). The differences in pollen wall ultrastructure between the perennial vs. annual growth forms of *C. hermaphroditica* (Osborn and Philbrick 1994), coupled with ontogenetic plasticity in the extremely reduced exine of the amphibious taxon *C. hamulata* Kütz. (Cooper et al. 2000), and the new data on development of exineless pollen in *C. truncata* presented in the current paper provide different lines of support for the hypothesis that underwater pollination has had a relatively recent origin in Callitrichaceae.

Although *Callitriche* is an ideal system to study evolutionary queries concerning hypohydrophily, it is difficult to investigate pollen development because of the overall small size of plants, greatly reduced flower and anther sizes (anthers are less than 0.5 mm in diameter), and the ontogenetic rapidity in pollen formation. Nevertheless, studies of pollen ontogeny of the amphibious and terrestrial growth forms, including taxa that have the range of exine types present in Callitrichaceae,

should provide significant insight into understanding the evolution of underwater pollination within this unique family, as well as among angiosperms overall.

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