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Seedling Diversity in Hydatellaceae: Implications for the Evolution of Angiosperm Cotyledons

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• *Background and Aims* Cotyledon number has long been a primary morphological feature distinguishing monocots from other angiosperms. Recent placement of Hydatellaceae near the early-divergent angiosperm order Nymphaeales, rather than in the monocot order Poales, has prompted reassessment of seedling morphology in this poorly known family.
• *Methods* Seedlings of six species representing all eco-geographical groups of Hydatellaceae are described using light and scanning electron microscopy.
• *Key Results* Two seedling types were discovered. Material examined of *Trithuria submersa*, *T. bibracteata*, *T. austinensis* and *T. filamentosa* possess a transparent bilobed sheathing structure that surrounds the main axis below the first foliage leaf. The seed coat is attached to the sheathing structure. Seedlings of *Trithuria lanterna* and *T. konkanensis* lack a sheathing structure, and the seed coat is attached to a short, narrow lateral outgrowth on the main axis of the seedling.
• *Conclusions* The sheathing structure that is present in seedlings of some Hydatellaceae could be homologized with the two united cotyledons of water lilies. It also resembles the single cotyledon of some monocots, and hence demonstrates a possible pathway of the origin of a monocot-like embryo, though no homology is implied. The sheathing structure is reduced in *Trithuria lanterna* and *T. konkanensis*, and the short, narrow outgrowth of its seedling could represent a single cotyledon. This synapomorphy suggests that the only Indian species of Hydatellaceae, *T. konkanensis*, is closer to the northern Australian *T. lanterna* than to the south-western Australian *T. bibracteata*.

Key words: Seedling, cotyledon, monocot, dicot, Hydatellaceae, *Trithuria*.

INTRODUCTION

The recent discovery that Hydatellaceae are closely related to the early-divergent angiosperm order Nymphaeales rather than (as previously assigned) to the monocot order Poales (Saarela *et al.*, 2007) has highlighted our inadequate knowledge of many significant morphological characters in this intriguing family. Cotyledon number has long been considered a primary morphological feature distinguishing monocots from other angiosperms, but was not hitherto properly documented in Hydatellaceae (Rudall *et al.*, 2007).

As recently recircumscribed (Sokoloff *et al.*, in press), Hydatellaceae consist of a single genus, *Trithuria*, with ten species in Australia, one species in New Zealand and one species in India. All species are aquatics with linear leaves in basal rosettes. Two species are perennials, but all other Hydatellaceae are annuals adapted mostly for seasonally wet habitats (Cooke, 1987; Yadav and Janarthanam, 1994; Gaikwad and Yadav, 2003; Sokoloff *et al.*, in press). Mature seeds of *Trithuria* possess a seed coat with an operculum and contain copious starchy perisperm, a small endosperm and a tiny, few-celled embryo not differentiated into organs (Hamann, 1975; Hamann *et al.*, 1979; P. J. Rudall *et al.*, unpubl. res.).

The only previous investigation of seedlings in Hydatellaceae was that of Cooke (1983*a*) for *Trithuria submersa*, who described germination as hypogeal. The green linear primary leaf and the radicle erupt between the operculum and the rest of the seed coat through the upper and lower sides, respectively. The cotyledon (not illustrated by Cooke) forms part of the tissue that remains inside the testa and does not become green. Cooke's report of a single cotyledon agreed with the traditional placement of Hydatellaceae in monocots, but contradicts their novel and robust placement among early-divergent angiosperms. A monocotylar embryo has sometimes been reported for Nymphaeaceae, but closer examination revealed the presence of two cotyledons (for discussion and references to earlier literature, see Tillich, 1990). Thus, since no other early-divergent angiosperm unequivocally possesses embryos with a single cotyledon, more data on seedlings of *Trithuria* will elucidate the controversy over cotyledon number in Nymphaeales, and improve our understanding of seedling evolution in angiosperms.

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MATERIALS AND METHODS

Seedlings of five species of Hydatellaceae from Australia and one species from India were examined. Two were from alcohol-fixed material from the Kew Herbarium (K): (1) *T. filamentosa* Rodway (K 28269: *Talbot de Malahide s.n.*), a perennial plant from Tasmania; this species was previously placed in a segregate genus, *Hydatella* (e.g. Cooke, 1987); (2) *T. lanterna* D.A. Cooke (K 47115: *C.R. Dunlop 4704A*), an annual from northern Australia. Material of *T. submersa* Hook. f. (the type species of *Trithuria*) was obtained from two sources: (1) seedlings grown at the Royal Botanic Gardens, Kew (RBGK) from seeds collected by one of us (R.E.T.) in south-west Western Australia; (2) FAA-fixed material collected in South Australia (*J. G. Conran 961 and P. J. Rudall*, voucher deposited at ADU; this material contains late stages only). *Trithuria submersa* is an annual with disjunct distribution between Tasmania and south-eastern and south-western parts of Australia. Material of two annuals endemic to south-west Western Australia (*T. bibracteata* Stapf ex D.A. Cooke and *T. austinensis* D.D. Sokoloff, Remizowa, T.D. Macfarl. & Rudall) was collected in natural populations by R.E.T. and seedlings were grown and fixed in FAA at Perth. Finally, the annual *T. konkanensis* Yadav & Janarth. was fixed in 70 % ethanol in a natural population near Kolhapur in India by one of us (S.R.Y.).

The six species investigated represent all eco-geographical groups of Hydatellaceae: (1) *T. filamentosa* grows in permanent lakes of temperate climates (the New Zealand *T. filamentosa* also falls in this group); (2) *T. submersa*, *T. bibracteata* and *T. austinensis* occur in mostly ephemeral pools in southern parts of Australia; (3) *T. lanterna* and *T. konkanensis* grow in tropical climates in ephemeral pools of northern Australia and India, respectively.

For investigation with the scanning electron microscope (SEM), material was dissected in 70 % ethanol, then dehydrated through absolute ethanol and critical-point dried using a Balzers CPD 020 (BALTEC AG, Liechtenstein) at RBGK. Dried material was further dissected and mounted onto specimen stubs using double-sided tape, coated with platinum using an Emitech K550 sputter coater (Emitech, Ashford, UK), and examined using a Hitachi cold-field emission SEM S-4700-II (Hitachi High Technologies Corp., Tokyo, Japan) at RBGK. For the investigation with the light microscope, material was embedded in paraffin or Tecnovit resin, microtome sections were produced using a Leica rotary microtome and stained with Delafields Haematoxylin and Eosin or Toluidine Blue. Seedlings were drawn by Anton Beer (Figs 5, 8, 10).

RESULTS

Trithuria submersa

The one-seeded fruit dehisces by separating three longitudinal ribs from the rest of the pericarp. The three ribs of the fruit, as well as the three faces between them, remain connected to each other and to the seed at the top of the fruit. The pericarp and the seed coat remain associated with the seedling (Fig. 1A, B). The seedling emerges between the operculum and the rest of the seed coat (the operculum is attached to the pericarp; Fig. 1C). The radicle appears on one side of the operculum (towards the substrate), while the first green leaf appears on the opposite side.

The radicle is always shorter than the green leaf in young seedlings. Numerous root hairs develop on the radicle. The hypocotyl is extremely short and almost exclusively represented by the collar (i.e. the transitional region between the hypocotyl and the primary root: terminology after Tillich, 1995). Numerous collar rhizoids develop on the collar (Fig. 2A); these are longer than root hairs. There is a time interval between the development of collar rhizoids and the first appearance of root hairs on the main root. In older seedlings shoot-borne roots develop on the hypocotyl. These also possess root hairs.

The first green leaf is filiform, without a distinct sheathing base or auricles, typically with water stomata at the tip and some normal stomata along the surface. The basal part of the first green leaf is enclosed by a thin, transparent, membranous, non-photosynthetic sheathing structure (Figs 1–3). We believe the sheathing structure has cotyledonary identity (see Discussion), but because other interpretations could be proposed and because the number of cotyledons involved (two or one) is debatable, a morphologically neutral term 'sheathing structure' was used here.

The sheathing structure is mostly two cell layers thick (i.e. with inner and outer epidermises only). It is closely appressed to the first green leaf, and is discernible only on careful examination using a dissecting microscope, but is clearly visible in critical-point dried material using SEM. It consists of two transversely oriented parts that are invariably basally connected to form a transparent sheathing tube, which encloses the first green leaf. The seed coat is attached to the base of the sheathing structure on the radius just between the two parts of the sheathing structure (Figs 1–3). The degree of fusion between these two parts on the side opposite seed attachment (i.e. on the side of the operculum) is variable; in some cases they are almost completely united up to the top, and thus appear as a single unit.

The basal part of the sheathing structure is enclosed by a distinct tissue belt (labelled 'e' in Fig 2B and C) that is connected on one side to the seed coat. In some cases the belt is incomplete, and the tissue is restricted to a region around the opening of the testa (Fig. 3B). This belt of tissue (here interpreted as endosperm; see Discussion) has no organic connection with the seedling and on careful preparation can be readily separated from it (Fig. 2D, E). Its cells are different in morphology from the testal cells; they are apparently living at this stage, and are of irregular, often amoeboid shape (Fig. 2B, C), in contrast to the dead, rhomboidal testal cells. At this stage the perisperm is completely consumed by the growing seedling.

In young seedlings (Figs 1–3), orientation of the first green leaf with respect to the symmetry plane of the sheathing structure cannot be traced because the leaf base has radial structure and the shoot apex cannot be detected at this stage. Older seedlings (Fig. 4A–C) show that the shoot apex lies between the first green leaf and the side of the sheathing structure to which the seed coat is attached.
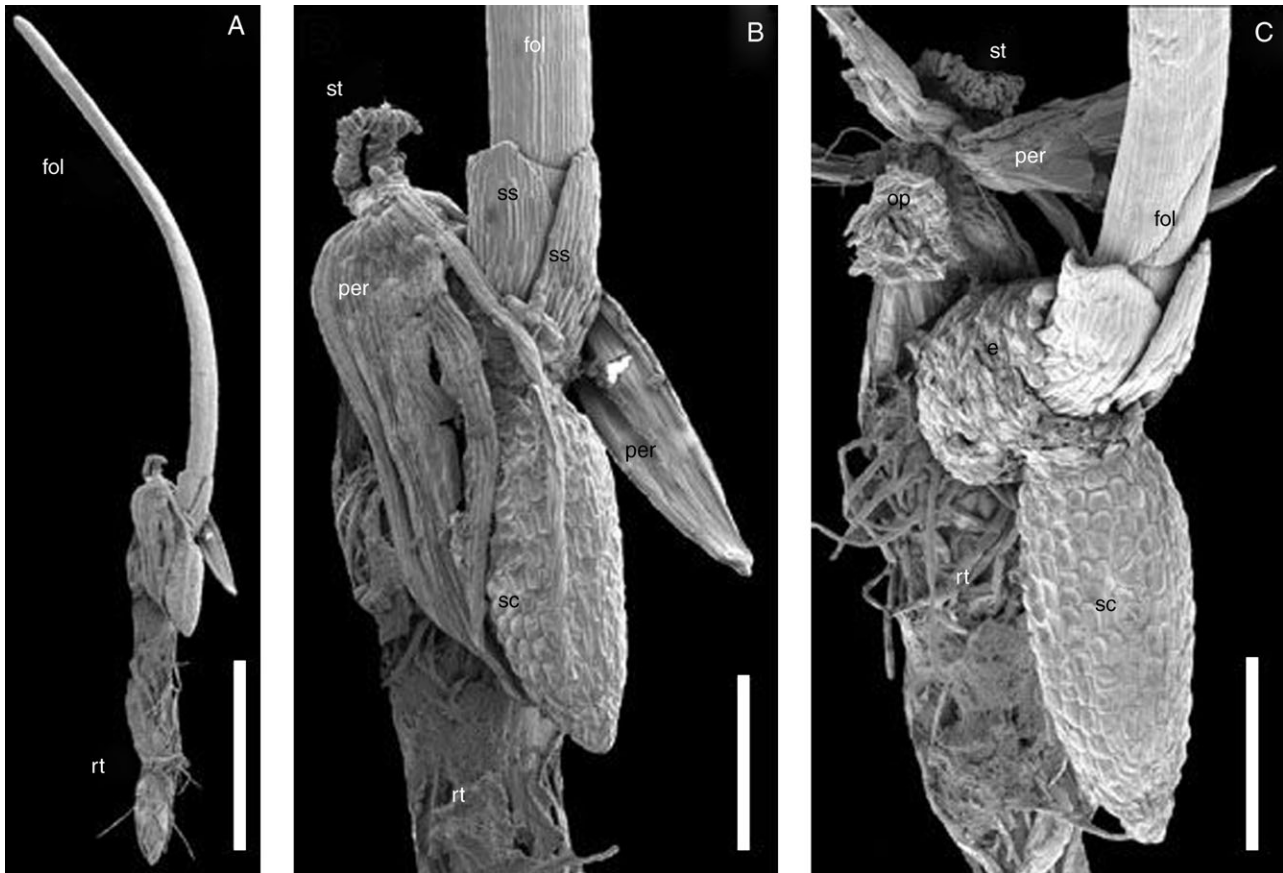
FIG. 1. Seedling of *Trithuria submersa* (SEM). (A) Entire seedling with pericarp and seed coat attached. (B) Detail of (A). (C) The same seedling separated from pericarp; note that the operculum remains attached to the inner surface of the pericarp. Labels: e, endosperm; fol, foliage leaf; op, operculum; per, pericarp; rt, root (note: fragments of agar on which the seedling was cultivated remain attached); sc, seed coat; ss, sheathing structure; st, stigma remaining attached to the fruit apex. Scale bars: A = 1 mm; B, C = 250 μm.

The divergence angle between the first and the second green leaf is <180°, so that the radius of the second leaf is slightly shifted from the radius of the seed coat attachment (Fig. 4B, C). In the latest available stage, three meristematic bulges are visible after formation of the first two green leaves. If our interpretation of primordia of the third leaf and the fourth leaf (Fig. 4C) is correct, then the radius of seed coat attachment and the radii of the first four leaves are arranged in a manner similar to a Fibonacci spiral.

Trithuria bibracteata

This species is similar to *T. submersa* in its ecology and fruit/seed structure. Germination and seedling structure are also similar to those of *T. submersa*, though a distinct (endospermous) tissue belt surrounding the base of the sheathing structure was not observed.

Trithuria filamentosa

In the (very limited) material available (Fig. 5), seedling structure was similar to that of *T. submersa*, but a distinct (endospermous) tissue belt surrounding the base of the sheathing structure was not observed. Also, an elongate part of the hypocotyl was present above the collar. *Trithuria filamentosa* has indehiscent fruits, but in our material the seed coat attached to the sheathing structure was free from the pericarp. The opened operculum was still weakly attached on one side to the rest of the seed coat.

Trithuria austinensis

This species (Figs 4D, 6 and 7B, C) has indehiscent fruits. The very thin pericarp remains intact, surrounding the seed coat by the time of seed germination. It breaks together with seed coat when the operculum opens during germination. The operculum is still visible in some seedlings examined; it remains weakly attached by one side to the rest of the seed coat (Fig. 6E). The general bauplan of the seedling is close to that of *T. submersa* (Figs 4D and 6), though a distinct (endospermous) tissue belt surrounding the base of the sheathing structure was not observed. In addition, both hypocotyl length (Fig. 6) and presence/absence of root hairs (Fig. 7) are variable in the material of *T. austinensis* examined.

In the material examined, the hypocotyl length varied between approx. 0·2 mm and approx. 4 mm. Regardless of hypocotyl length, shoot-borne roots develop only near the
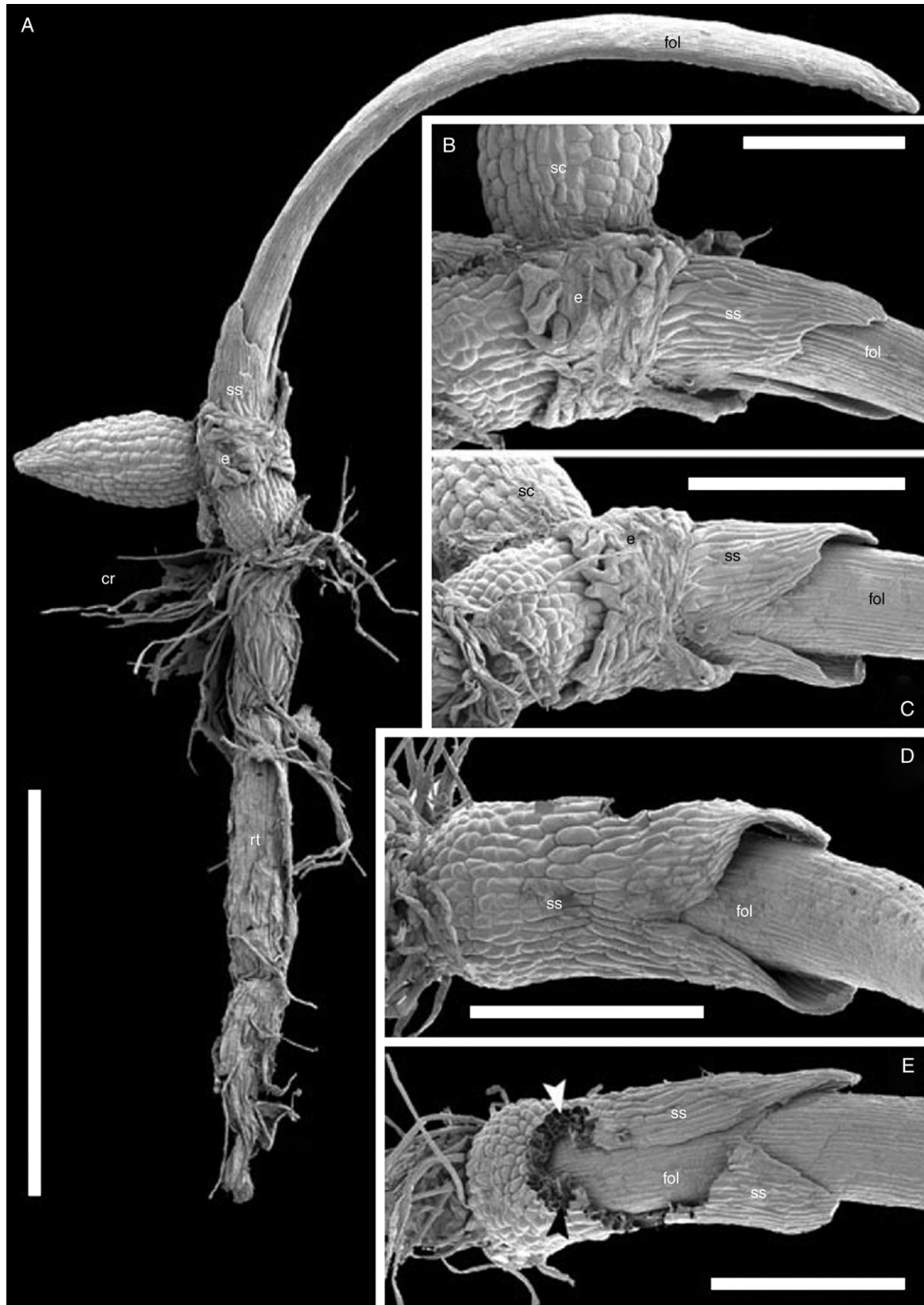
FIG. 2. Seedling of *Trithuria submersa* (SEM) with pericarp removed; all figures show the same individual seedling. (A) General view. (B) Portion of (A) to show sheathing structure. (C) View of sheathing structure from the side of the abscised operculum. (D, E) Seed coat and endosperm removed, viewed in (D) from the same angle as in (C), and in (E) from the side of attachment of the (removed) seed coat; the seed coat was attached in the space between the two arrowheads. Labels: cr, collar rhizoids; e, endosperm; fol, foliage leaf; rt, root; sc, seed coat; ss, sheathing structure. Scale bars: A = 1 mm; B = 200 µm; C − E = 300 µm.
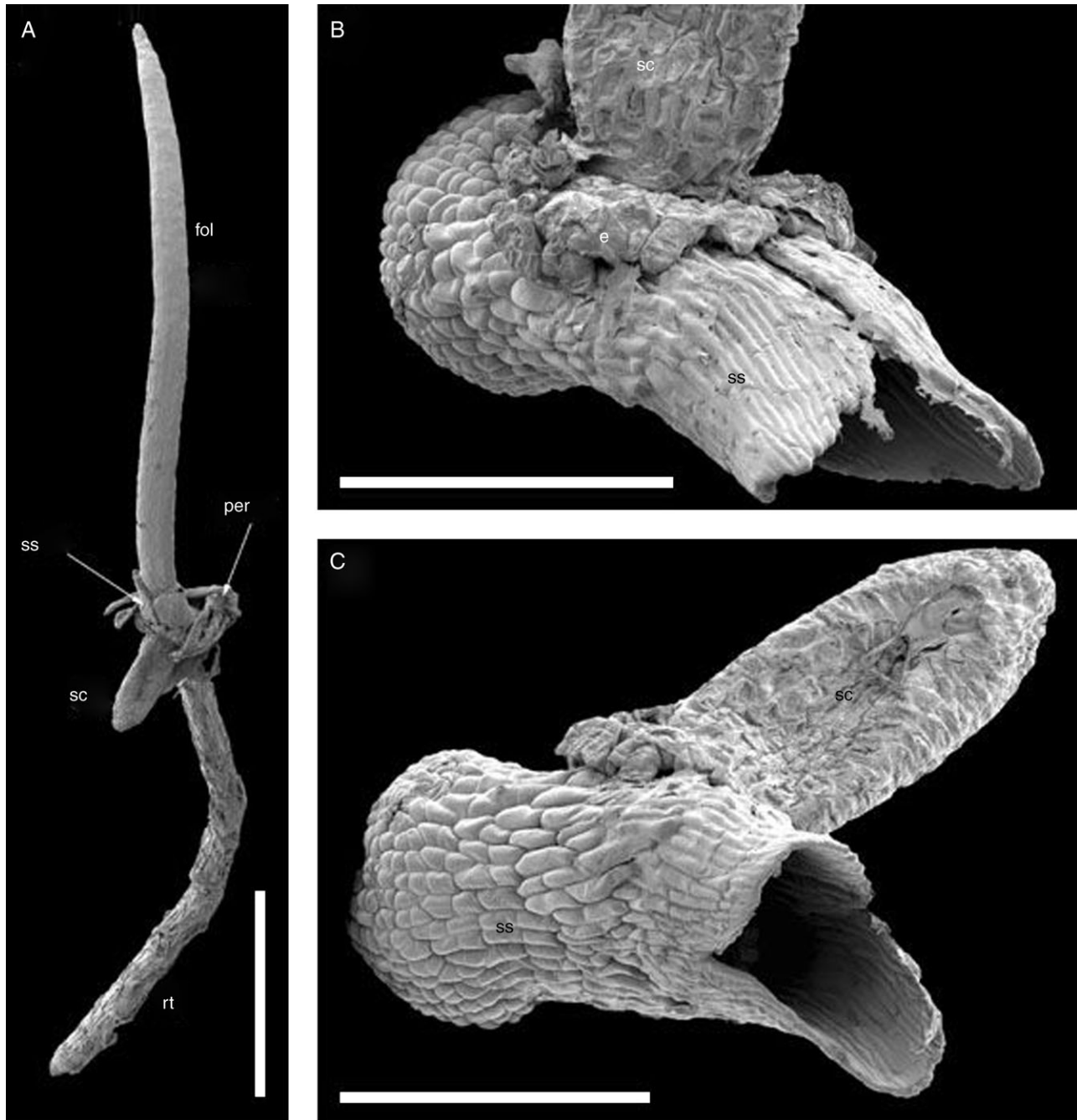
FIG. 3. SEM images of a seedling of *Trithuria submersa* (A) and details of its sheathing structure viewed from the side of the attached seed coat (B), and from the side of the abscised operculum (C). Labels: e, endosperm; fol, foliage leaf; op, operculum; per, pericarp; rt, root; sc, seed coat; ss, sheathing structure. Scale bars: A = 1 mm; B, C = 300 μm.

level of attachment of the sheathing structure. In some seedlings the first shoot-borne root develops on the radius of the seed coat attachment (e.g. Fig. 6B, C) (and the second root is opposite it – Fig. 6C). In other seedlings the first shoot-borne root lies opposite the radius of the seed coat attachment (Fig. 6E). In all seedlings examined, the first shoot-borne root lies in the symmetry plane of the sheathing structure.

Some seedlings have well-developed root hairs on the main root, whereas in others roots hairs are either rare or absent. SEM investigation of entire roots, including their apices, has shown that absence of hairs is not due to hair abscision; also hairless roots were not too young to develop hairs. Most hairless roots of *T. austinensis* possess characteristic short cells, similar to those that develop hairs in roots of most Hydatellaceae. However, in *T. austinensis* some hairless roots, in which the surface was composed of long cells only, were also observed. Like the primary root, shoot-borne roots are variable with
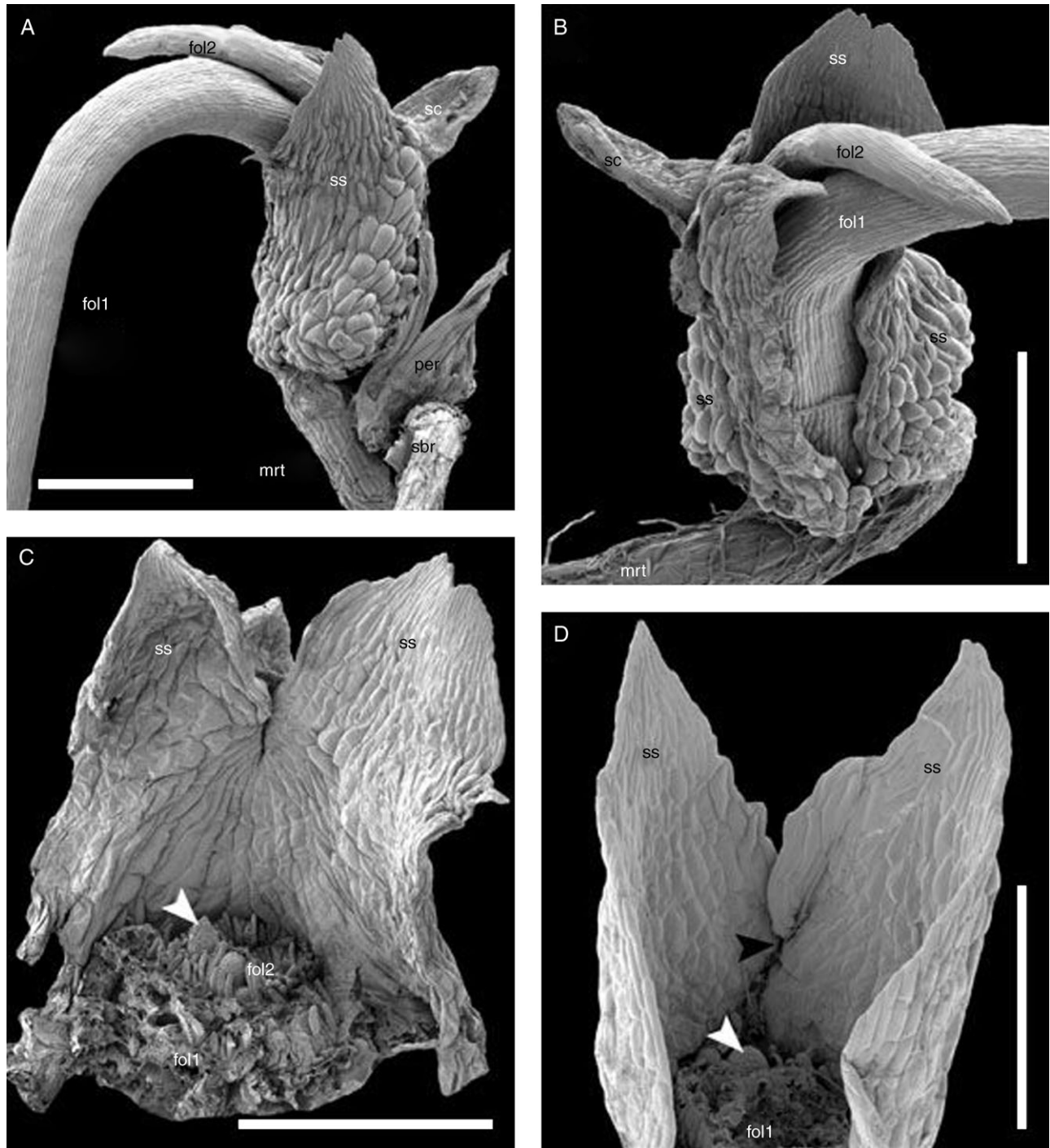
FIG. 4. SEM images of seedlings showing foliage leaf arrangement on the main axis. (A − C) *Trithuria submersa*, (D) *Trithuria austinensis*. (A, B) Different views of the same seedling. (C) The same seedling with the first and the second foliage leaves removed (labelled at their bases); arrowhead indicates the putative primordium of the third foliage leaf. (D) A seedling with the first foliage leaf removed (labelled at base); white arrowhead indicates the primordium of the second foliage leaf, black arrowhead indicates level of seed coat attachment to the reverse side of the sheathing structure. Labels: fol1, fol2, the first and the second foliage leaves; mrt, main root; per, pericarp; sbr, shoot-borne root; sc, seed coat; ss, sheathing structure. Scale bars: A − C = 500 μm; D = 300 μm.

respect to hair presence. One seedling with a hairy primary root and a hairless shoot-borne root was found; both roots were of the same length.

The collar rhizoids are typically very long and conspicuous in *T. austinensis*, even in most seedlings with a hairless main root. However, one seedling with very short and inconspicuous (and less numerous) collar rhizoids was found (Fig. 6E). The collar was readily detectable in this seedling because of the characteristic thickening of this part of the main axis.
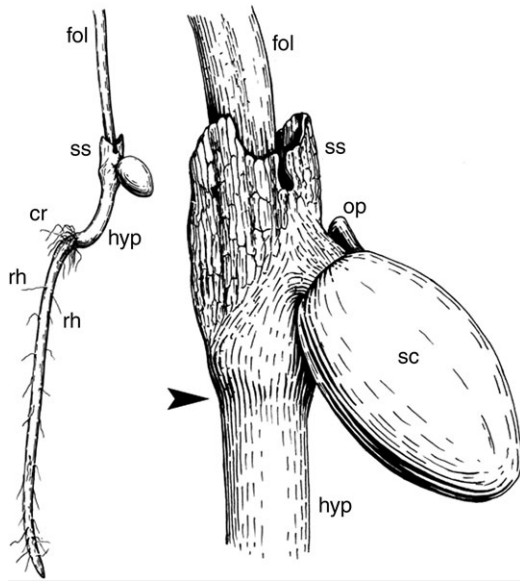
FIG. 5. Line drawing of a seedling of *Trithuria filamentosa*, with a detail of seed attachment to the sheathing structure. Labels: cr, collar rhizoids; fol, the first foliage leaf; op, operculum; rh, root hairs on the main root; sc, seed coat; ss, sheathing structure; arrowhead indicates level of attachment of sheathing structure.

Trithuria lanterna

All available seedlings were at later developmental stages than those described above for other species. They possess several roots with root hairs, three or four filiform leaves and a young reproductive unit. However, the seeds were in most cases still attached to the seedlings, and there was no evidence of abscission of any organ, an observation that was confirmed by careful examination of epidermal cells using SEM. The seedlings lack any trace of a sheathing structure near the point of seed coat attachment (Figs 8 and 9B). Above the attachment of the seed coat there is a portion of naked stem of variable length (here interpreted as epicotyl), and then the primary shoot possesses three or four filiform green leaves and finally a reproductive unit. In some seedlings, the internode above the first leaf was elongate, and then a rosette was formed; in other seedlings all leaves were arranged in a rosette. The shoot-borne roots are typically attached just below the leaves. Below the attachment of the seed coat, the seedling axis continues into a hypocotyl and a radicle (Fig. 8). The seed coat is attached to a short, nonvascularized and radially symmetrical outgrowth. Within the seed coat, the outgrowth is often surrounded by the remaining endosperm (Fig. 9A). No traces of perisperm can be found at this stage, and the space occupied by perisperm in the mature seed is empty (Fig. 9A). The hypocotyl has a single central vascular strand, which extends into the main axis of the plant above the nonvascularized outgrowth. The central vascular strand branches to supply a single vascular strand to each leaf and each shoot-borne root. Analysis of leaf traces in serial cross-sections suggests that leaves are arranged according to a Fibonacci spiral. The position of the outgrowth is such that it can be identified as the first member of this spiral.

Trithuria lanterna is apparently as variable as *T. austinensis* with respect to root hair and collar rhizoid presence, though only older stages in *T. lanterna* were available for examination.

Trithuria konkanensis

The seedlings available were at slightly younger stages than those of *T. lanterna*. The youngest seedlings examined possessed three green leaves and no mature reproductive unit (Fig. 10). Seedling morphology in this species is generally similar to that of *T. lanterna*. The sheathing structure is absent, and there is no trace of its having abscised; instead, the seed coat is attached to a very small lateral outgrowth of the main axis. The length of the main axis above the attachment of the seed coat (i.e. the presumed epicotyl length) is as variable as in *T. lanterna*. The seedling illustrated (Fig. 10) possesses an elongated epicotyl, but in many other seedlings of *T. konkanensis* the epicotyl was short. The hypocotyl was always elongated. The collar and collar rhizoids are clearly visible in all seedlings available. All roots examined possessed root hairs.

DISCUSSION

Seedling structure is variable in Hydatellaceae

Our data on seedlings of *T. submersa* differ significantly from Cooke's description (Cooke, 1983*a*). Cooke did not mention the sheathing structure that we found in all seedlings examined of this species. This discrepancy could be explained either because (*a*) the sheathing structure is transparent in living material, so it is difficult to discern without examination of critical-point dried material under SEM, or (*b*) *T. submersa* is variable in seedling structure (indeed, this is probably the most variable species in the family with respect to other characters; Cooke, 1987; Sokoloff *et al.*, in press). Interestingly, our material of *T. lanterna* lacks a sheathing structure entirely, and therefore corresponds closely to Cooke's description of *T. submersa*. We are confident of the precise taxonomic identification of all the material discussed here, because we have examined the voucher specimen of Cooke's study (adult plants), and our material of *T. lanterna* has reproductive units and is thus readily identifiable (reproductive morphology of plants from the same collection is illustrated in Rudall *et al.*, 2007). Furthermore, our material of *T. submersa* has the pericarp attached, and the pericarp structure of this species is quite distinctive (Sokoloff *et al.*, in press).

According to our data, seedling morphology varies within Hydatellaceae with respect to presence or absence of the sheathing structure. This structure is present in four of the six species examined here (*T. submersa, T. bibracteata, T. austinensis* and *T. filamentosa*) and absent from the other two (*T. konkanensis* and *T. lanterna*). Since the highly reduced morphology of Hydatellaceae offers very few useful taxonomic characters, seedling structure merits careful examination in a wider sampling of taxa. However, since we have sampled 50 % of the known species of
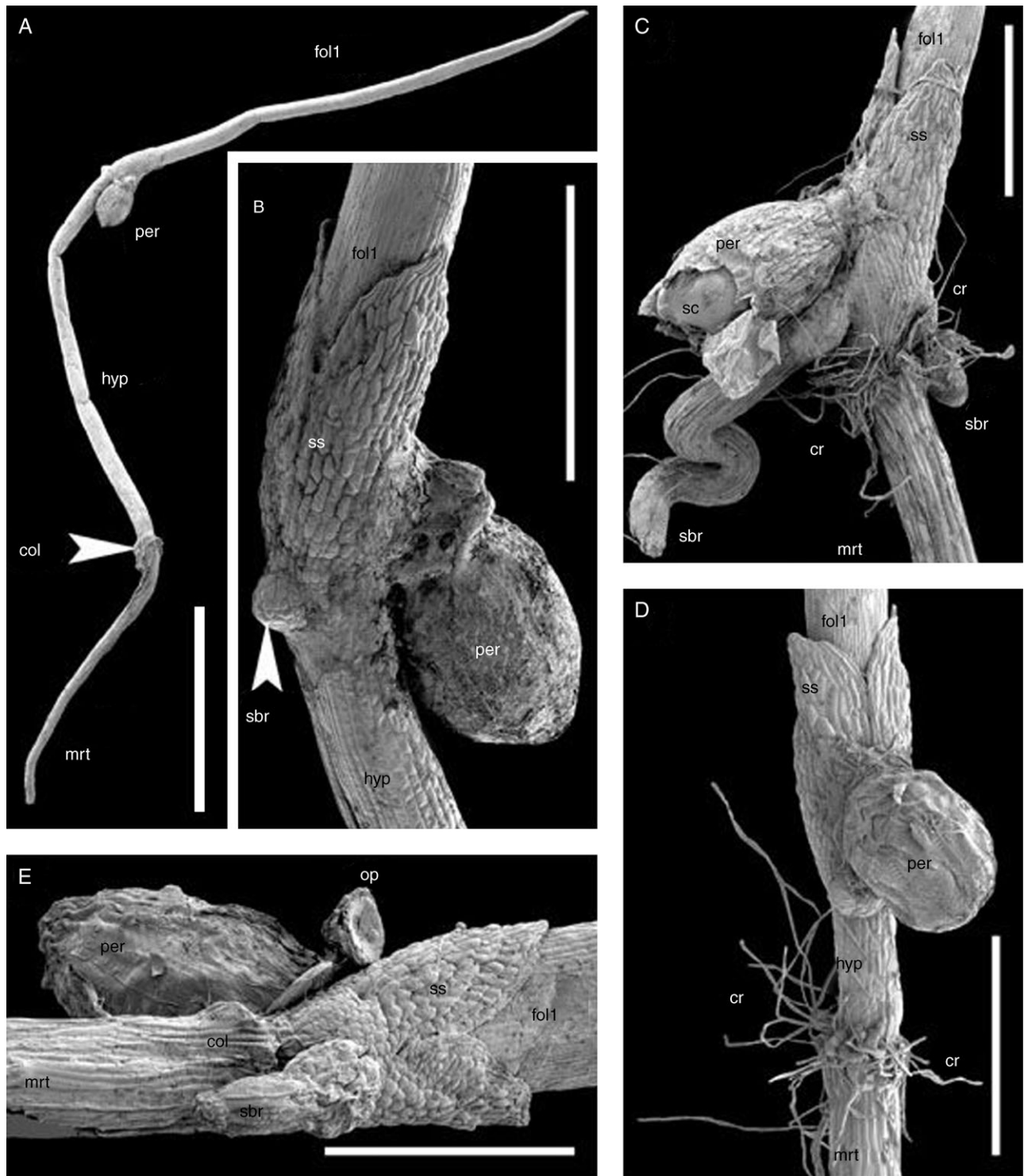
FIG. 6. SEM images of seedlings of *Trithuria austinensis*. (A) A seedling with very long hypocotyl; close examination of this seedling revealed the presence of collar rhizoids, but they are not clearly visible at this magnification. (B) The same seedling as in (A), detail from another angle showing sheathing structure. (C) Seedling with short hypocotyl and long collar rhizoids. (D) Seedling with short, but clearly visible hypocotyl and long collar rhizoids. (E) Seedling with short hypocotyl and inconspicuous collar rhizoids; close examination revealed that the collar rhizoids are present but rare and much shorter than in (A − D). Labels: col, collar; cr, collar rhizoids; fol1, first foliage leaf; hyp, hypocotyl; mrt, main root; op, operculum; per, pericarp; sbr, shoot-borne root; sc, seed coat; ss, sheathing structure. Scale bars: A = 2 mm; B − D = 500 μm; E = 400 μm.
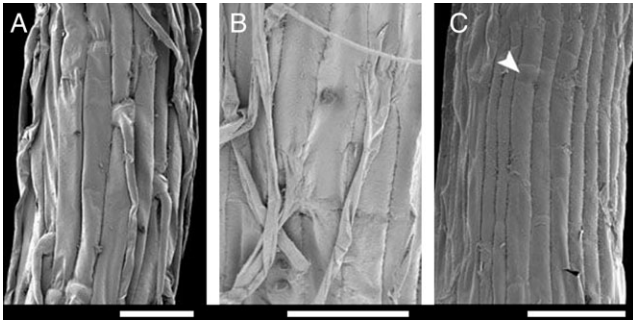
FIG. 7. Details of main root surface (SEM). (A) Hairy root of *Trithuria bibracteata*. (B) Hairy root of *T. austinensis*. (C) Hairless root of *T. austinensis*; arrowhead indicates short cell. Scale bars = 50 μm.

Hydatellaceae (i.e. 6 out of 12), we propose some preliminary conclusions on variation in this character.

(a) Presence/absence of the sheathing structure varies independently from growth form and seed/fruit morphology. Indeed, *T. filamentosa* is perennial with indehiscent fruits and smooth seeds, whereas *T. submersa* is an annual with dehiscent fruits and sculptured seeds, but both species possess the sheathing structure. In terms of fruit/seed morphology, *T. lanterna* is intermediate between *T. filamentosa* and *T. submersa* (Cooke 1981, 1983*b*, 1987; Sokoloff *et al.*, in press). However, seedlings of *T. lanterna* lack the sheathing structure and thus differ from both *T. filamentosa* and *T. submersa*.

(b) Seedling structure could be important for understanding the relationships of *T. konkanensis*, the only Indian species of Hydatellaceae (and therefore highly disjunct). This species has been compared with two Australian species, *T. lanterna* (northern Australia) and *T. bibracteata* (south-western Australia), based on
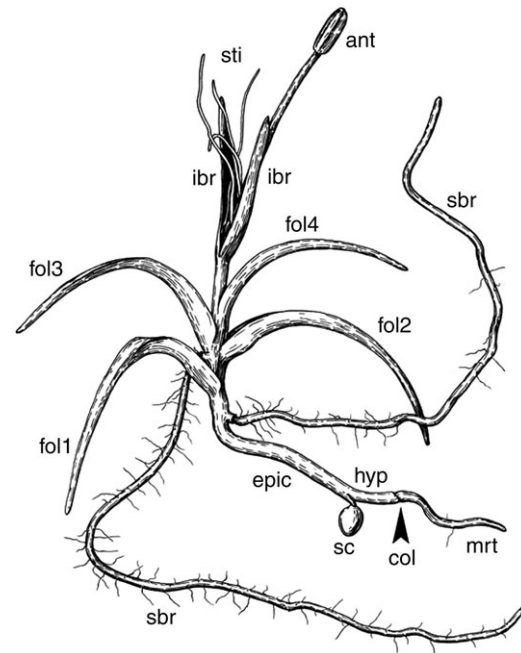

FIG. 8. Line drawing of a young plant of *Trithuria lanterna*. Labels: anth, anther; col, collar; epic, presumed epicotyl; fol1 − fol4, foliage leaves; hyp, hypocotyl; ibr, involucral bract of reproductive unit; sbr, shoot-borne root; sc, seed coat; sti, stigmatic papillae; mrt, main root.

reproductive morphology: all three species possess bisexual reproductive units with two involucral bracts (Yadav and Janarthanam, 1994). The similarity in seedling structure observed here suggests that *T. konkanensis* is much closer to *T. lanterna* than to *T. bibracteata*, and represents a potential synapomorphy that will be tested in a species-level cladistic analysis.
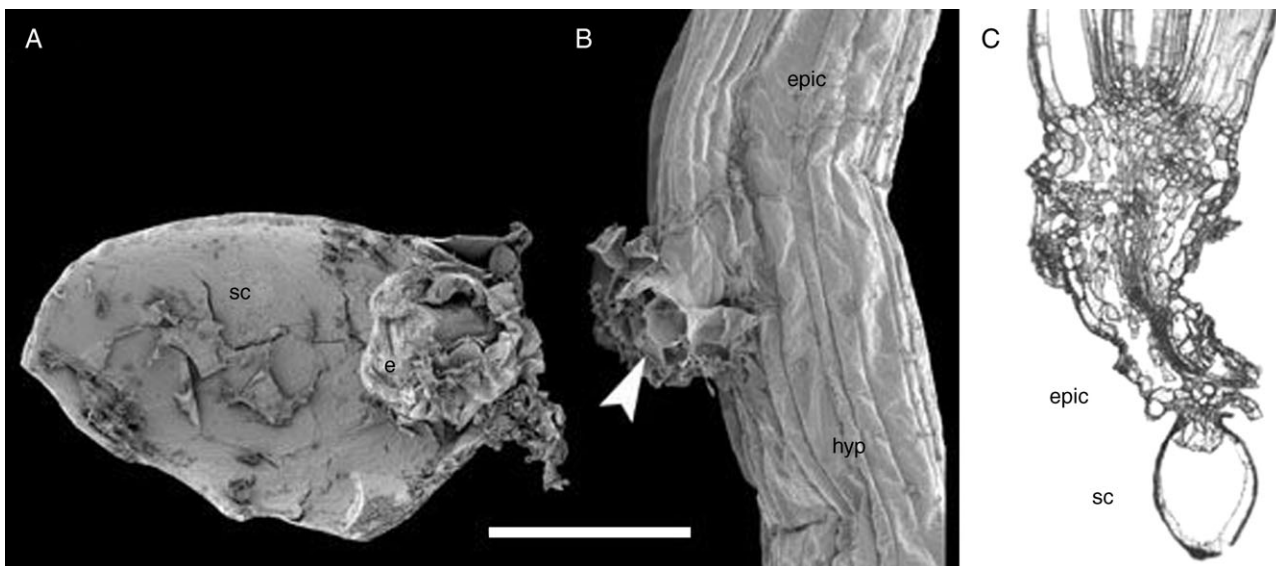

FIG. 9. *Trithuria lanterna*. (A) SEM image of longitudinally dissected germinated seed with perisperm consumed. The operculum was attached at the right side of the figure. (B) SEM image of the point of pericarp attachment to a young plant; arrowhead indicates the outgrowth (presumed cotyledon). Scale bar (common to A and B) = 100 μm. (C) Longitudinal section of young plant showing seed coat attachment under a light microscope. Labels: e, endosperm; epic, epicotyl; hyp, hypocotyl; sc, seed coat.
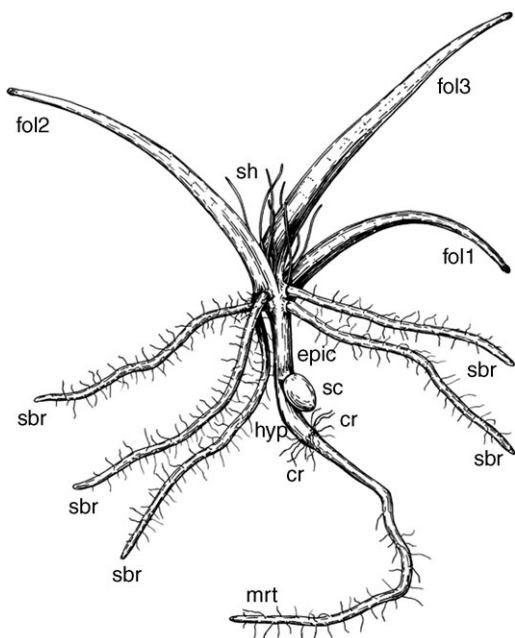
FIG. 10. Line drawing of a seedling of *Trithuria konkanensis*. Labels: cr, collar rhizoids; epic, epicotyl; fol1–fol3, foliage leaves; hyp, hypocotyl; mrt, main root; sbr, sc, seed coat; shoot-borne roots; sh, shoot hairs (very long hairs attached to the shortened stem internodes between foliage leaves).

Morphological interpretation of seedlings in Hydatellaceae

Trithuria lanterna and T. konkanensis. Seedlings of these two species possess a very short cylindrical outgrowth to which the seed coat is attached (arrowhead in Fig. 9B). The outgrowth is attached to the seedling axis below the attachment of the first leaf and above the main root – hypocotyl transition (the collar). Thus, by position in the seedling bauplan, the outgrowth can be interpreted as a cotyledon. The outgrowth is an entire structure and thus we have to postulate the presence of a single cotyledon in *T. lanterna* and *T. konkanensis*. The cotyledon is apparently haustorial. The absence of vasculature in this outgrowth (well documented so far only in *T. lanterna*) is intriguing for an organ of haustorial function (though the outgrowth is very short). Vasculature is generally poorly developed in *T. lanterna* and other Hydatellaceae; e.g. the hypocotyl bears a single tiny vascular strand.

The presumed cotyledon of *T. lanterna* and *T. konkanensis* has a very narrow base, whereas the single cotyledon of monocots has a broad base that completely surrounds the plumule (e.g. Lodkina, 1988; Tillich, 1995). In the few eudicots that possess a single cotyledon (which could be a fusion product of two cotyledons), the cotyledon also has a broad base (Haccius, 1952; Haccius and Hartl-Baude, 1957; Titova, 2000). Among monocots, a superficially similar condition occurs when the basal part of the coleoptile is congenitally fused with the epicotyl, forming a structure termed a mesocotyl (Tillich, 1995). For example, in some Cyperaceae, a cotyledonary haustorium with a narrow base is attached to the seedling axis below the mesocotyl (Tillich, 1995), which resembles the attachment of the presumed cotyledon to the seedling axis in *Trithuria lanterna* and *T. konkanensis*. However, *T. lanterna* and *T. konkanensis* have no trace of a coleoptile.

An alternative interpretation of the seedlings of *T. lanterna* and *T. konkanensis* is that the outgrowth to which the seed coat is attached represents a unique organ *sui generis*. There is currently no easy way of testing this hypothesis, which can be only accepted if all other possible hypotheses are refuted. It is possible that molecular genetics will help to solve the problem, as suggested by Burger (1998).

Trithuria submersa, T. bibracteata, T. filamentosa and T. austinensis. The most unexpected finding of this study is the bilobed sheathing structure present in seedlings of these species. This structure could be cotyledonary, representing either a fused pair of cotyledons or a single cotyledon. With respect to position, the sheathing structure resembles a pair of cotyledons in seedlings of Nymphaeaceae. Many authors have reported that the cotyledons of water lilies are slightly united at the base, which is more clearly visible at younger stages (reviewed by Haines and Lye, 1975; Titova and Batygina, 1996); hence the embryo of water lilies has often been described as monocotylar with a bifid cotyledon. On the other hand, in favour of its interpretation as a single cotyledon, seedling topography in *T. submersa* resembles that of some monocots (e.g. *Schizocarpa* in Taccaceae, *Heliconia* in Heliconiaceae), which possess a sheathing base continued into two lateral lobes with the haustorial part attached on the radius between the lobes (Tillich, 1995). Taccaceae and Heliconiaceae are not early-divergent monocots, but a bilobed cotyledonary hypophyll is also present in some members of the relatively basal monocot family Araceae (Tillich, 2003). The hypophyll lobes, when present, are typically green and photosynthetic in Araceae, but at least in *Dracontioides* and *Cyrtosperma* the cotyledon bears small paired sheath lobes (Tillich, 2003), so that this structure resembles the sheathing structure in *Trithuria*.

Based on current evidence, a cotyledonary interpretation is our preferred hypothesis. However, possible alternative interpretations are that the sheathing structure represents either (*a*) the first leaf of the plumule, or (*b*) two united opposite leaves. The second case is unlikely because fusion of opposite leaves was otherwise never observed in Hydatellaceae (though opposite leaves sometimes occur in reproductive units of many species; Cooke, 1987; Rudall et al., 2007; Sokoloff et al., in press). To interpret the sheathing structure as a single leaf, it should be compared with the sheathing base of foliage leaves. In some species, including *T. filamentosa*, the sheathing base of some foliage leaves possesses two lateral membranous stipule-like auricles (illustrated for *T. australis* in Rudall et al., 2007). The two parts of the sheathing structure could be compared with these auricles, and the entire sheathing structure could be viewed as a bladeless leaf. However, this interpretation is also problematic for several reasons. (*a*) Vegetative leaves of Hydatellaceae never possess a closed sheath; their base is typically narrower than the circumference of the stem. (*b*) The first green leaf of the seedling lacks a sheathing base, though

some subsequent leaves possess one; this would indicate a highly unusual bimodal change of leaf base structure in the leaf series of the shoot. (*c*) In seedlings of water lilies, the closest relatives to Hydatellaceae, the first leaf after the cotyledons has a filiform blade and scarcely recognizable stipules (summarized by Tillich, 1990). The axillary sheathing stipule is present only from the second leaf onwards. The first leaf of water lilies is closely similar to the first green leaf of Hydatellaceae (and to other leaves of Hydatellaceae; Rudall *et al.*, 2007), but has nothing in common with the sheathing structure of *T. submersa* and *T. filamentosa*.

Interpretation of the sheathing structure as the first leaf of the plumule would not in itself contradict a homology between the sheathing structure of *Trithuria* and the cotyledon of monocots. Indeed, several authors have discussed a hypothesis that the cotyledon of monocots is homologous with the first leaf of the plumule of water lilies (Meyer, 1960; Kudryashov, 1964; Valtzeva and Savich, 1965; Burger, 1998).

Endosperm behaviour in germinating seeds

In most species examined, the endosperm remains within the testa during seed germination. As in water lilies, it apparently helps to transfer nutrients from the copious perisperm to the growing seedling. In our material of *T. submersa*, the tissue belt surrounding the sheathing structure must be interpreted as endosperm, since these cells have no organic connection with the seedling. Apart from endosperm cells, there are no other living cells surrounding the embryo. Similar endosperm release from the seed coat during germination has been described for some other early-divergent angiosperms, such as Myristicaceae, Saururaceae and Piperaceae (Takhtajan, 1988). Like Hydatellaceae, both Saururaceae and Piperaceae possess a well-developed perisperm, and thus similar endosperm behaviour could be of adaptive significance.

CONCLUSIONS

The unusual bilobed sheathing structure – described here for the first time – below the first foliage leaf in four species of *Trithuria*, is probably cotyledonary. There is evidence to homologize each lobe of the sheathing structure with one of a pair of cotyledons in Nymphaeaceae, though it also resembles the single cotyledon in some monocot seedlings. Since Hydatellaceae are now robustly resolved as the closest relatives of water lilies, the first interpretation is most parsimonious.

In Nymphaeaceae each cotyledon has its own haustorium at the tip (Tillich, 1990), in contrast to *Trithuria*, in which the tips of the lobes of the sheathing structure are clearly not haustorial. This could mean that the cotyledon tips in water lilies are homologous to the place of testa attachment in *Trithuria* (which is probably haustorial). If so, the two lobes of the sheathing structure appear to be organs *sui generis*.

It is possible that the sheathing structure of *Trithuria* is homologous to the cotyledons of water lilies *and* represents a single cotyledon. In other words, this could be a case of true syncotyly. The hypothesis that the single cotyledon of monocots is homologous to two united cotyledons has been proposed by many authors, but disputed by others (reviewed by Haines and Lye, 1979). Syncotyly has been reported from several scattered eudicot lineages (reviewed by Titova, 2000), but these cases represent derived transitions that are irrelevant in this context. Reports on syncotyly in water lilies are more relevant here, because of the phylogenetic placement of water lilies far below the major monocot/dicot divergence. However, the degree of syncotyly is weak in water lilies and the general morphology of their cotyledons is very different from that of monocots. If our interpretation proves correct, the sheathing structure of the *Trithuria* seedling demonstrates how a monocotyledonous condition could arise from the condition that is present in water lilies. It is unlikely that cotyledon structure is synapomorphic between Hydatellaceae and monocots, but shows a possible evolutionary scenario by analogy.

Reduction clearly played an important role in the evolution of seedlings of Hydatellaceae. Their cotyledons are very simple structures in terms of histology. In *Trithuria lanterna* and *T. konkanensis*, the sheathing part of the cotyledon is presumably reduced and thus the seedling is very different from that of other species examined. No other family of early-divergent angiosperms shows similar range diversity in seedling morphology. However, seedling diversity in Hydatellaceae does not necessarily represent a primitive polymorphism that elucidates ancestral angiosperm seedling morphology. Rather, it could be caused by a loss of body plan stability, as in some other specialized aquatics, such as Lentibulariaceae (Kondo *et al.*, 1978; Rutishauser and Isler, 2001; Degtjareva *et al.*, 2006). Leaves of many aquatics, especially among early-divergent monocots, show a high degree of developmental plasticity, often dependent on environmental factors (reviewed by Rudall and Buzgo, 2002).

Some authors have suggested that a monocotylar embryo represents the primitive angiosperm condition (reviewed by Tzvelev, 2000). However, we consider it most likely that the dicotylar condition was ancestral for Hydatellaceae, because this condition exists in most other early-divergent angiosperms and in most extant gymnosperms, though little is known about seedlings in extinct stem-group angiosperms. Our observations raise many questions about the evolution of the body plan of the earliest angiosperms. Future work on Hydatellaceae will focus on the development of the embryo from fertilization onwards.

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NOTE ADDED IN PROOF

Tillich (2007) has recently described seedlings of Eriocaulaceae and Mayacaceae (Poales), in which cotyledon structure closely resembles that described here for *Trithuria lanterna* and *T. konkanensis*.

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