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Pegged and smooth rhizoids in complex thalloid liverworts (Marchantiopsida): structure, function and evolution

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Rhizoids played essential roles in the early evolution of land plants. All liverworts, the closest living relatives of the first land plants, produce unicellular rhizoids, except for *Haplomitrium*. The complex thalloids are uniquely characterized by dimorphic rhizoids: smooth rhizoids like those also produced by the simple thalloid and leafy clades and pegged rhizoids. Although this dimorphism has been long and widely recognized, considerations of its functional basis are few and contradictory. Here we present conclusive cytological and experimental evidence that the function of smooth and pegged rhizoids is markedly different, as reflected by major differences in their structure, physiology and vital status. Mature smooth rhizoids are alive (indeed their main functions in nutrition, anchorage and as conduits for mycobiont entry all depend on living cytoplasm) and dehydration causes irreversible collapse of their cell walls, but pegged rhizoids, which are dead at maturity, function as a highly effective internalized external water-conducting system, especially within carpocephala. Their cavitation-resistant, elastic walls ensure retention of functional integrity during periods of desiccation. Our structural and functional data now raise novel hypotheses on patterns of rhizoid evolution in Marchantiopsida and open the way for dissecting the molecular basis of rhizoid morphogenesis in liverworts. *Botanical Journal of the Linnean Society*, 2014, **174**, 68–92.

ADDITIONAL KEYWORDS: carpocephala – cryo-scanning electron microscopy – desiccation biology – rhizoid dimorphism – root hairs – water conduction – X-ray microanalysis.

INTRODUCTION

One of the most striking features of marchantialean or complex thalloid liverworts is the occurrence of smooth and pegged (otherwise known as tuberculate or trabeculate) unicellular rhizoids. Although their presence has been cited in virtually every text that considers bryophytes since the late 19th century (e.g. Parihar, 1956; Schofield, 1985; Crum, 2001) considerations of the functional basis for rhizoid dimorphism are conspicuously absent (Duckett *et al*., 2000).

Roles for smooth rhizoids in anchorage, nutrition and as conduits from soil to endophytic fungi are suggested by branched tips in contact with the substrate (Cavers, 1904; Pocock & Duckett, 1985), disposition at right angles to the surface (Burgeff, 1943; Schuster, 1984a, b, 1992) and frequent presence of fungal hyphae (Pocock & Duckett, 1985; Duckett, Renzaglia & Pell, 1991; Read *et al*., 2000). Although Schuster (1966, 1984a, b) provided comparative information on the structure and distribution of pegged rhizoids in complex thalloid liverworts, their function was not considered. Indeed, the works that directly address function present a confused and contradictory

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picture. Leitgeb (1881) stated that both kinds of rhizoids attach plants to the soil, but added that pegged rhizoids also provide strength for the thallus; the latter claim was not confirmed by subsequent observations (Kamerling, 1897). Whereas Kny (1890) believed that pegs prevent the rhizoid walls from collapse with water loss, Haberlandt (1896) wrote that the pegs facilitate absorption by increasing the surface area of the cytoplasm. Cavers (1904) stated that the majority of smooth rhizoids lack cytoplasmic contents, whereas Kamerling (1897) illustrated smooth rhizoids as living cells and pegged rhizoids without contents. Both Cavers (1904) and Clee (1943) found no evidence of collapse in either kind of rhizoids under dry conditions. Unlike the uncertainty about the vital status of the two kinds of rhizoids, there is a general consensus, supported by the results of dye movement experiments (Bowen, 1935; McConaha, 1939, 1941), that pegged rhizoids conduct water by capillarity and transpiration. In contrast to smooth rhizoids that enter the substrate directly, pegged rhizoids frequently run parallel to the ventral surface of the thallus, variously enclosed by ventral scales, and within the grooves in stalks of carpocephala (Table 1). Pertinent to this proposed role in water conduction is Goebel's (1905) observation that pegged rhizoids are most highly developed in taxa likely to have high transpiration rates compared with hygrophilous genera such as *Dumortiera* Nees and *Cyathodium* Kunze. If they do indeed lack contents, as stated by Kamerling (1897), pegged rhizoids would clearly fulfil Raven's (1993) three criteria for water-conducting elements, namely dead at maturity, specialized walls and preferential conduction of water. For bryophytes it is also necessary to add a fourth criterion, maintenance of functional integrity through periods of dehydration as in moss hydroids (Ligrone, Duckett & Renzaglia, 2000).

We therefore undertook a series of cytological and experimental studies to clarify structure/function relationships of the dimorphic rhizoids in marchantialean liverworts. Our specific aims were: (1) to provide the first full descriptions of marchantialean rhizoid ultrastructure and to determine their vital status; (2) to confirm whether pegged rhizoids and their immediate surroundings conduct water preferentially by comparison with water-conducting cells of other cryptogams; and (3) to explore the potential role of pegs in desiccation biology using novel approaches involving both optical and cryo-scanning electron microscopy. Because of the fragmentary and sometimes inconsistent information in the taxonomic literature, we present the first comparative survey of the distribution, disposition and sizes of rhizoids across all the genera in the complex thalloid lineage (Marchantiopsida; Crandall-Stotler, Stotler & Long, 2008, 2009). Evaluation of rhizoid characters based on recent liverwort phylogenetic analyses provides new insights into possible patterns of rhizoid evolution in this group.

MATERIAL AND METHODS

The taxa examined in this study are listed in Table 1, and the herbarium specimens of all the plants are detailed in the Appendix. Also included is *Pellia epiphylla* (L.) Corda to illustrate the monomorphic rhizoids typical of simple thalloid liverworts. Light microscope observations were made on wild specimens collected both fully hydrated and in naturally desiccated states. Hydrated plants were mounted in water and desiccated specimens in immersion oil and observed with differential interference contrast optics in a Leitz Dialux or a Zeiss Axioskop 2 microscope. Where fresh material was not available the data were obtained from herbarium specimens.

The effects of de- and rehydration were examined in taxa that represent the range of variability in rhizoids. Fully hydrated wild-collected specimens of *Marchantia foliacea* Mitt., *M. polymorpha* L., *Reboulia hemisphaerica* (L.) Raddi (three species with smooth and pegged rhizoids with a range of sizes), *Dumortiera hirsuta* (Sw.) Nees (with smooth and sparsely pegged rhizoids associated with the thalli but numerous and long pegs occurring in rhizoids within the carpocephalum grooves), *Monoclea forsteri* Hook. and *Neohodgsonia mirabilis* (Perss.) Perss. [with apparently smooth rhizoids only (Bischler-Causse, Glenny & Boisselier-Dubayle, 1995; Bischler-Causse *et al*., 2005)] were allowed to dry out over a 24-h period and observed at half hourly intervals for up to 24 h after rehydration to see which rhizoids recovered their cylindrical shape and which ones remained flattened. The invariable recovery of pegged rhizoids but not smooth ones suggested that the former have elastic walls. To investigate further these possible differences in rhizoid wall elasticity, samples of dried rhizoids were cut with a razor blade in air before placing them in immersion oil: we hypothesized that those with inelastic walls would remain flattened whereas those with elastic walls would regain their cylindrical shape and allow the ingress of air. Thus, the presence of air bubbles in cut rhizoids mounted in immersion oil would be indicative of elastic walls.

Specimens of *Marchantia foliacea*, *Neohodgsonia* Perss. and *Reboulia* Raddi were prepared for transmission electron microscopy as described previously (Ligrone & Duckett, 1994) and observed in a Jeol 120 EX2 microscope. Several taxa (as indicated in Table 1) were studied in a Hitachi S570 scanning electron microscope following the protocol of Duckett & Ligrone (1995).

Table 1. Features of the rhizoids in complex thalloid liverworts based on observations on fresh material except where indicated (*herbarium specimens only); diameters of pegged and smooth rhizoids are based on measurements of 50 rhizoids

			Pegged (μm)	Smooth (μm)	Peg morphology	% Pegged parallel to thallus surface	Pegged enclosed by ventral scales	Stalked carpocephala	Grooves in carpocephala
Marchantiopsida									
Blasiales	Blasiaceae	*Blasia pusilla* L., *Cavicularia densa* Steph.	–	18–30	–	–	–	–	–
Sphaerocarpales	Sphaerocarpaceae	*Sphaerocarpos michelii* Bellardi, *S. texanus* Austin	–	18–30	–	–	–	–	–
		Geothallus tuberosus Campb.*	–	18–30	–	–	–	–	–
	Riellaceae	*Riella americana* M.Howe&Underw., *R. helicophylla* (Bory&Mont.) Mont.	–	18–30	–	–	–	–	–
Neohodgsoniales	Neohodgsoniaceae	*Neohodgsonia mirabilis* (Perss.) Perss.‡	–	10–24	–	–	–	+	2
Lunulariales	Lunulariaceae	*Lunularia cruciata* (L.) Dumort. ex Lindb.‡	6–16	18–24	short and blunt to long and pointed	90	++	+	0
Marchantiales	Marchantiaceae	*Bucegia romanica* Radian*	10–20	20–24	long, often branched	90	++	+	2
		Marchantia berteroana Lehm. & Lindenb.‡, *M. debilis* K.I.Goebel‡, *M. foliacea* Mitt.‡, *M. paleacea* Bertol.‡, *M. pappeana* Lehm.‡, *M. polymorpha*† L.‡	6–24	18–28	short and blunt to long and branched, sometimes forming annular and spiral bands	90	++	+ both male and female	2–4
		Preissia quadrata (Scop.) Nees‡	6–14	18–26	short and blunt to long and pointed	90	++	+	2
	Aytoniaceae	*Asterella abyssinica* (Gottsche) Grolle, *A. australis* (Hook.f & Taylor) Verd.‡, *A. bachmannii* (Steph.) S.W.Arnell, *A. muscicola* (Steph.) S.W.Arnell, *A. tenera* (Mitt.) R.M.Schust.‡, *A. wilmsii* (Steph.) S.W.Arnell‡	8–16	12–26	short and blunt to long and pointed	50	–	+	1
		Cryptomitrium oreoides Perold‡	8–16	8–18	short, blunt and pointed	50	–	+	1
		Mannia androgyna (L.) A.Evans‡, *M. fragrans* (Balb.) Frye & L.Clarke	9–12	9–20	long, often hooked	50	+	+	1

	Plagiochasma appendiculatum Lehm. & Lindenb.‡ *P. eximium* (Schiffn.) Steph., *P. rupestre* (G.Forst.) Steph.‡	8–16	12–26	short and blunt to long and pointed	90	++	+	0	
	Reboulia hemisphaerica (L.) Raddi‡	6–20	14–22	short and blunt to long and branched	90	++	+	1 sometimes bifurcating	
Cleveaceae	*Athalamia hyalina* (Sommerf.)S.Hatt.‡, *A. pinguis* Falc.*	8–20	18–24	short and pointed	50		+	0	
	Peltolepis quadrata (Saut.) K.Müller*	8–20	12–22	short and pointed	80	–	+	2	
	Sauteria alpina (Nees) Nees‡	12–20	18–24	short, blunt and pointed	80		+	1	
Monosoleniaceae	*Monosolenium tenerum* Griff.*	6–24	20–24	short and blunt to long and branched	50	–	+	2	
Conocephalaceae	*Conocephalum conicum* (L.) Dumort.‡, *C. salebrosum* Szweyk., Buczk. & Odrzyk.‡, *C. supradecompositum* (Lindb.) Steph.‡	6–14	14–22	short and blunt to long and pointed	90	++	+	1	
Cyathodiaceae	*Cyathodium cavernarum* Kunze‡	–	10–18	–	–	–	–	–	
	C. foetidissimum Schiffn.‡	6–15	10–15	short and pointed	50	–	–	–	
Exormothecaceae	*Aitchisoniella himalayensis* Kashyap*	18–24	18–24	short and blunt	20	–	–	–	
	Exormotheca holstii Steph.‡, *E. pustulosa* Mitt.	14–20	14–20	short, blunt and pointed	25	–	+/–	1 if stalked	
	Stephensoniella brevipedunculata Kashyap *	20–24	20–24	short and blunt	90	–	+	1 very shallow	
Corsiniaceae	*Corsinia coriandrina* (Spreng.) Lindb.‡	6–22	16–22	short, blunt and pointed	70	–	–	–	
	Cronisia fimbriata (Nees) Whitten.&Bisch. *	18–24	18–24	short and blunt	90	–	–	–	
Monocarpaceae	*Monocarpus sphaerocarpus* D.J.Carr*	–	18–28	–	–	–	–	–	
Oxymitraceae	*Oxymitra cristata* Garside‡ *O. incrassata* (Broth.) Sérgio & Sim-Sim‡	10–24	16–24	short and blunt to long and pointed	70	–	–	–	

Table 1. *Continued*

			Pegged (μm)	Smooth (μm)	Peg morphology	% Pegged parallel to thallus surface	Pegged enclosed by ventral scales	Stalked carpocephala	Grooves in carpocephala
	Ricciaceae	*Riccia bifurca* Hoffm., *R. beyrichiana* Hampe ex Lehm.‡, *R. canaliculata* Hoffm., *R. cavernosa* Hoffm., *R. ciliifera* Link ex Lindenb., *R. crystallina* L., *R. crozalsii* Levier, *R. glauca* L., *R. gougetiana* Durieu & Mont., *R. huebeneriana* Lindenb., *R. nigrella* DC.‡, *R. okahandjana* S.W.Arnell, *R. sorocarpa* Bisch.‡	8–24	16–30	short and blunt to long and pointed	10–90	–	–	–
		R. fluitans L., *R. subbifurca* Warnst. ex Croz.	–	18–24	–	–	–	–	–
		Ricciocarpos natans (L.) Corda	–	–	– rhizoids absent	–	–	–	–
	Wiesnerellaceae	*Wiesnerella denudata* (Mitt.) Steph.	12–20	24–30	short, blunt and pointed	90	–	+	2
	Targioniaceae	*Targionia hypophylla* L.‡	6–18	14–26	short and blunt to long and pointed	90	++	–	–
	Monocleaceae	*Monoclea forsteri* Hook.‡	6–12§	20–24	very short and widely spaced	5	–	–	–
		M. gottschei Lindb.‡	–	20–24	–	–	–	–	–
	Dumortieraceae	*Dumortiera hirsuta* (Sw.) Nees‡, *D. hirsuta* subsp. *nepalense* (Taylor) R.M.Schust.‡	6–12	8–24	sparse, short and blunt to numerous, long and pointed	90	–	+	2
Jungermanniopsida									
Pelliales (control)	Pelliaceae	*Pellia epiphylla* (L.) Corda‡	–	15–22	–	–	–	–	–

†Includes the three subsp. *polymorpha* L., *ruderalis* Bischl. & Boisselier, and *montivagans* Bischl. & Boisselier.
‡Taxa studied by SEM; §narrow dead smooth rhizoids running parallel to the thallus surface; ++, bundles of almost exclusively pegged rhizoids enclosed by ventral scales; +, occasional pegged rhizoids between ventral scales and thallus.

Table 2. Rates of methylene blue movement in marchantialean carpocephalum grooves, moss hydroids and fern xylem; the data for each taxon are based on at least 30 measurements

Taxon	Length of carpocephalum stalks (mm)	Time to reach carpocephalum caps (min)	Rate of dye movement (mm h^{-1})
CARPOCEPHALA			
Asterella australis	40	30–50	50–80
A. tenera	30	30–40	45–60
Conocephalum conicum	70–100	45–75	100–150
Dumortiera hirsuta	50	40–70	40–80
*Lunularia cruciata**	30	–	–
Marchantia foliacea	35–45	35–45	40–85
M. polymorpha	45–65	45–75	60–80
Neohodgsonia mirabilis	45	40–50	55–70
Preissia quadrata	20	30–40	30–40
Reboulia hemisphaerica	25	30–40	40–50
HYDROIDS			
Dawsonia superba Grev.	–	–	62 ± 14
Dendroligotrichum dendroides (Hedw.) Broth.	–	–	140 ± 24
Hypopterygium filiculaeforme (Hedw.) Brid.	–	–	24 ± 8
Pogonatum macrophyllum Dozy & Molk.	–	–	38 ± 16
Polytrichum commune Hedw.	–	–	118 ± 14
TRACHEIDS			
Nephrolepis sp.	–	–	141 ± 15
Polypodium vulgare L.	–	–	127 ± 19

*Grooves absent.

Living thalli were also observed in an FEI Quanta 3D FEG dual beam cryo-scanning electron microscope (cryo-SEM) either in the wet condition or in various states of de- and rehydration. These materials were mounted on an aluminium stub using Tissue-Tek OCT compound (Sakura Finetek) and plunged in liquid nitrogen slush to preserve their hydrated/dehydrated state in a frozen condition in a Gatan Alto 2500. Once frozen, they were vacuum-transferred to a high-vacuum cryogenic preparation chamber to prevent contamination and the build-up of ice. Ice was sublimed off the surface by raising the temperature to −90 °C for 5 min. The samples were then cooled to −130 °C, AuPd sputter-coated with a cold magnetron sputter coater and then inserted directly into the SEM via an airlock to avoid ice build-up and to maintain their frozen state. Inside the SEM, the samples rested on a cold stage with the temperature maintained at −130 °C.

To map the routes of apoplastic solute transport in the carpocephalum grooves and rhizoids, thalli of *Marchantia foliacea* and *Asterella australis* (Hook.f & Taylor) Verd. with mature carpocephala were floated in a 0.1% solution of lanthanum nitrate for 1 h and prepared for cryo-SEM as detailed above. The distribution of lanthanum was then determined by X-ray microanalysis to investigate which rhizoids are dead and which are living. Lanthanum salts do not cross intact membranes and are thus widely used to determine the vital status of cells (Peterson, Swanson & Hull, 1986; Carretero & Rodriguez-Garcia, 1995): absence of lanthanum within a cell is indicative of its vitality, whereas its presence throughout the lumen is a characteristic of dead cells.

To obtain rates of water movement, pieces of thalli with fully extended carpocephalum stalks from a range of species (Table 2) were floated on a 1% solution of either toluidine blue or methylene blue and the times taken to reach the caps were recorded. Comparative data in mosses with well-developed hydromes, *Dawsonia superba* Grev., *Dendoligotrichum dendroides* (Hedw.) Broth., *Hypopterygium filiculaeforme* (Hedw.) Brid., *Pogonatum macrophyllum* Dozy & Molk. and *Polytrichum commune* Hedw., and pinnae of the ferns *Nephrolepis* L. sp. and *Polypodium vulgare* L. were collected via parallel experiments on the rates of dye movement in cut stems and pinnae.

RESULTS

LIGHT MICROSCOPY

Figure 1 illustrates representative examples of rhizoid morphologies found in the complex thalloid

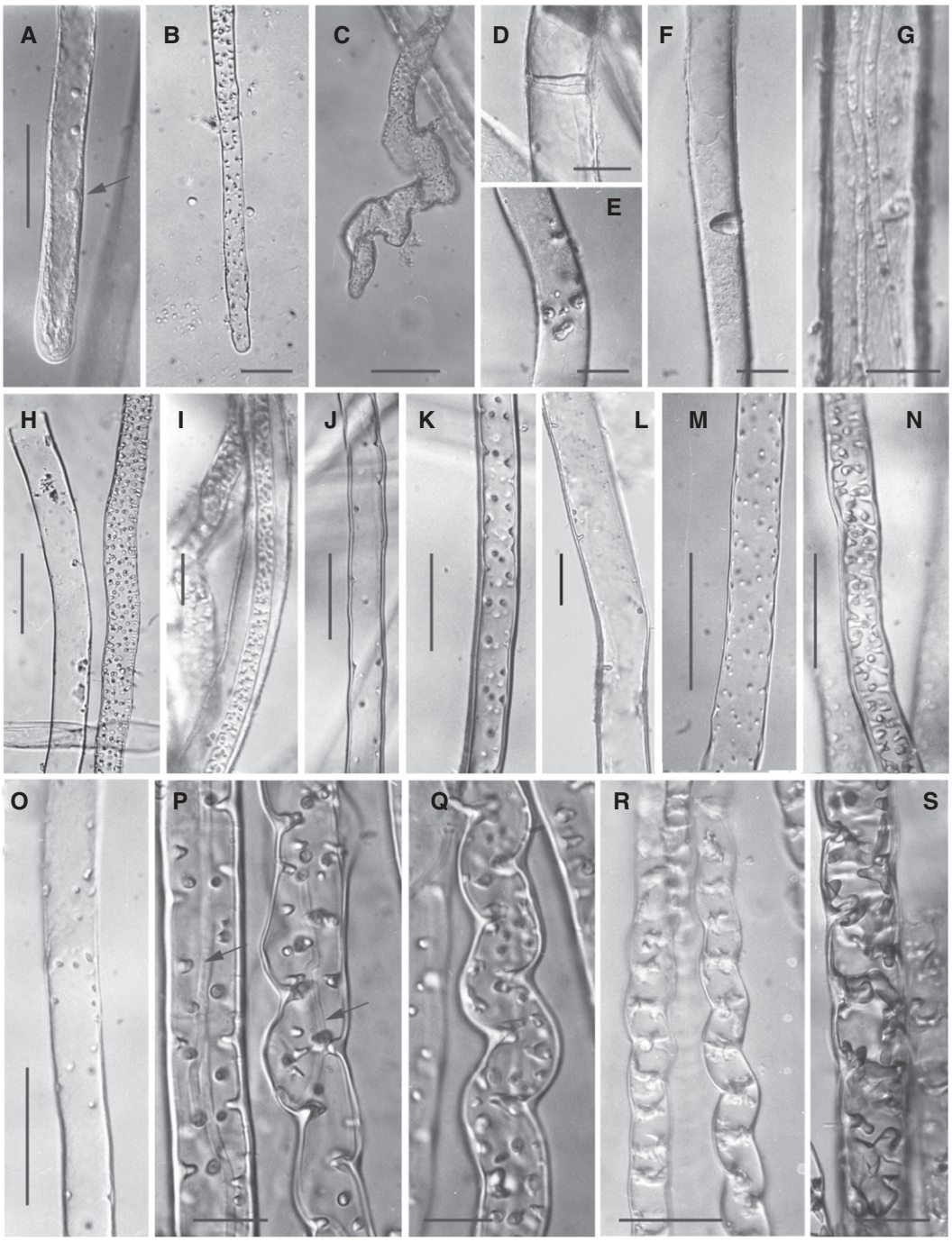

Figure 1. Light micrographs illustrating the range of morphologies in marchantialean rhizoids. A, *Corsinia coriandra*, smooth rhizoid showing the cytoplasmic cap and nucleus (arrowed) 30 μm from the tip. B, *Reboulia hemisphaerica*, tip of pegged rhizoid lacking cytoplasmic contents. C, *Plagiochasma rupestre*, tip of pegged rhizoid distorted by contact with irregularities in the substratum. D–F, false pegs associated with hyphal penetration sites in *Marchantia foliacea* (D) and *Monoclea forsteri* (E, F). G, aseptate glomeromycote hyphae in a smooth rhizoid of *Marchantia foliacea*. H, *Riccia gougetiana*, pegged and smooth rhizoids with the same diameter. I, *Corsinia coriandra*, pegged rhizoid growing inside a larger smooth one. J, K, *Dumortiera hirsuta*, rhizoids with sparse (J) and crowded (K) small pegs. L, *Monoclea forsteri*, small and widely scattered pegs in a rhizoid arranged parallel to the ventral surface of the thallus. M, N, *Corsinia coriandra*, small scattered pegs in a thin-walled rhizoid (M) and larger denser pegs in one with a thicker wall (N). O–S, *Marchantia foliacea*, the range of peg rhizoid morphologies from small sparse pegs (O) to spirally arranged branched pegs. Fungal hyphae are arrowed in P. Scale bars: D-G, L, P, Q, S, 20 μm; A–C, H–K, M–O, R, 50 μm.

lineage. Among all the 33 marchantialean genera, seven (*Blasia* L., *Sphaerocarpos* Boehm., *Geothallus* Campb., *Riella* Mont., *Monocarpos* D.J.Carr, *Neohodgsonia* and *Ricciocarpos* Corda) do not produce rhizoids with pegs; both smooth and pegged rhizoids are found in the remaining 26 (Table 1). Two genera (*Cyathodium* and *Riccia* L.) contain species with both mono- and dimorphic rhizoids. A further noteworthy feature of Ricciacaeae is that the proportions of smooth and pegged rhizoids vary considerably between taxa from 90% pegged in *R. bifurca* Hoffm., roughly equal numbers in *R. glauca* L., *R. okhandjana* S.W.Arnell, *R. cavernosa* Hoffm. and *R. crystallina* L., to 70% smooth in *R. nigrella* DC. and *R. crozalsii* Levier, 90% smooth in *R. ciliifera* Link ex Lindenb. and *R. beyrichiana* Hampe ex Lehm., to smooth alone in *R. subbifurca* Warnst. ex Croz. and *R. fluitans* L. (Alfano *et al*., 1993; Perold, 1999).

Examination of several specimens collected from different places and at different seasons indicates that the proportions of the two types seem to be constant for each species. In most genera the smallest pegged rhizoids are narrower than the smooth ones although there is always an overlap in the size range. Pegged and smooth with closely similar size ranges are found in *Cryptomitrium* Austin ex Underw., *Mannia* Opiz, *Aitchisoniella* Kashyap, *Exomortheca* Mitt., *Cronisia* Berk. and many *Riccia* spp. In *Pellia* Raddi, a typical exemplar of the simple thalloid liverworts, rhizoid diameters fall into a much narrower range than in the complex thalloids (Table 1).

In nine genera (*Mannia*, *Plagiochasma* Lehm. & Lindenb., *Reboulia*, *Conocephalum* Hill, *Lunularia* Adans., *Bucegia* Radian, *Marchantia* L., *Preissia* Corda and *Targionia* Dumort.) the pegged rhizoids are typically underarched by ventral scales and most are orientated parallel to the thallus surface (Table 1). When not subtended by scales, at least 50% of the pegged rhizoids in most genera (*Asterella* P.Beauv., *Cryptomitrium*, *Dumortiera*, *Wiesnerella* Schiffn., *Monoselenium* Griff., *Athalamia* Falconer, *Corsinia* Raddi, *Cronisia*, *Targionia* and *Oxymitra* Bisch. ex Lindenb.) are still orientated parallel to the thallus. Exceptions where most of the pegged rhizoids, like the smooth, lie at right angles to the thallus are *Aitchisoniella*, *Exormotheca* and many *Riccia* spp. In taxa with lateral scales (e.g. *Athalamia*, *Oxymitra* and Ricciaceae) these are rarely associated with either pegged or smooth rhizoids. In *Dumortiera*, pegged rhizoids associated with the thalli have generally small, sparse pegs, whereas those occurring in the carpocephala are much more developed, on a par with those in *Marchantia*. Most *Riccia* spp. lack positional segregation of the two kinds of rhizoids but in a few, e.g. *R. okahandjana* and *R. glauca*, most of the pegged rhizoids lie along the centre of the thalli and in *R. sorocarpa* and *R. crozalsii* two clusters of pegged rhizoids line the ventral margins. Blasiales have smooth rhizoids that are not associated with ventral scales and the three sphaerocarpalean genera lack both scales and pegged rhizoids.

Monoclea Hook. lacks ventral scales, and small, sparse pegs occur only in a small proportion of the rhizoids. However, it has the same range of smooth rhizoid diameters as those in other taxa (e.g. *Marchantia*) with dimorphic rhizoids. Those with smaller diameters run parallel to the thallus surface (Fig. 2C). Those running parallel to the ventral surface of the thalli, and in the carpocephalum grooves in *Neohodgsonia*, have similar diameters to the pegged counterparts in other genera (10–20 μm) and are on average narrower and with thicker walls than the rhizoids orientated at right angles to the thalli.

Carpocephala occur in 19 genera (Table 1) and, with the exception of *Plagiochasma*, *Lunularia* and *Athalamia*, they always contain non-living rhizoids in one or more grooves of the inrolled stalk. The rhizoids in carpocephalum grooves of all 19 genera and in mature carpocephalum caps, with the exception of *Neohodgsonia*, are almost exclusively pegged.

When undamaged, mature smooth rhizoids are living with dense cytoplasm filling the apical dome (Fig. 1A) and a thin layer of peripheral cytoplasm around a large central vacuole accounting for > 90% of the volume of the cells. These cells may reach a length of 30 mm and they grow at their tips as long as the plants are fully hydrated. A large flattened nucleus is located in the peripheral cytoplasm 30–50 μm behind the apical dome. Small bumps, scattered in the peripheral cytoplasm along the entire length of the living rhizoids, and superficially resembling small pegs, are either plastids or mitochondria (cf. Figs 1A, 3B, C). In taxa that contain symbiotic fungal endophytes (see Ligrone *et al*., 2007; Bidartondo *et al*., 2011 for full listings), large aseptate hyphae are often visible along the entire length of rhizoids (Fig. 1G).

Entry points for other non-symbiotic fungi are marked by conspicuous ingrowths of host wall material (Fig. 1D–F) and have in the past sometimes been mistaken for and illustrated as true pegs (Proskauer, 1951; Schuster, 1966, 1984). In contrast to true pegs that they superficially resemble, these wall ingrowths are characterized by a tubular core marking the position of the invading hypha (Martinez-Abaigar *et al*., 2005).

Wall ingrowths in the pegged rhizoids vary from small protuberances only 2–3 μm in height (Fig. 1H, L, M, O) to branched structures up to 10 μm long that extend across more than half the diameter of the rhizoids (Fig. 1N, P–S). The frequency of pegs commonly varies from widely scattered and over 10 μm apart (Fig. 1J, L, O) to densely packed (Fig. 1P–S)

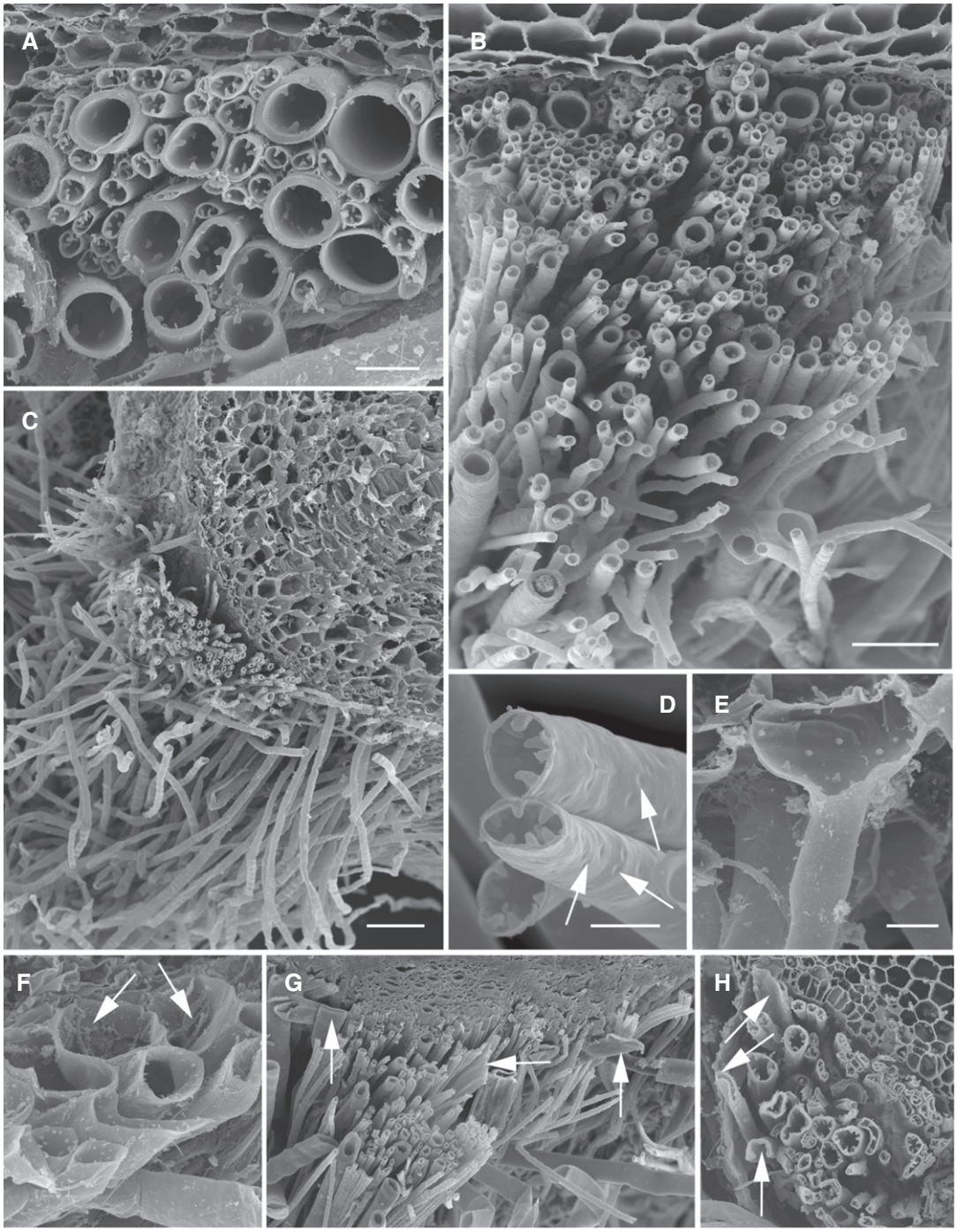

Figure 2. Scanning electron micrographs of critical point dried specimens. A, *Marchantia paleacea*, pegged rhizoids with a range of diameters on the ventral thallus surface. B, *Dumortiera hirsuta*, small pegged rhizoids and larger smooth ones with thick and thin walls. C, *Monoclea forsteri*, small rhizoids running parallel to the thallus and larger ones at right angles to it. D, *Corsinia coriandra*, shallow surface depressions (arrowed) mark the position of the pegs. E, *Oxymitra incrassata*, pegs do not extend onto the basal walls of the rhizoids. F, *Plagiochasma rupestre*, thin-walled pegged and smooth rhizoids of the same diameter. Note the fungal hyphae (arrowed) in the latter. G, *Dumortiera hirsuta*, rehydrated herbarium specimen. The pegged and large thick-walled rhizoids regain their shape but those with thin walls remain flattened (arrowed) as do the thallus cells. H, *Preissia quadrata*, collected in a dry state from nature and rehydrated. The pegged rhizoids have recovered their shape but the smooth ones (arrowed) remain flattened. Scale bars: D, 10 μm; A, E, F, 20 μm; B, H, 50 μm; C, G, 200 μm.

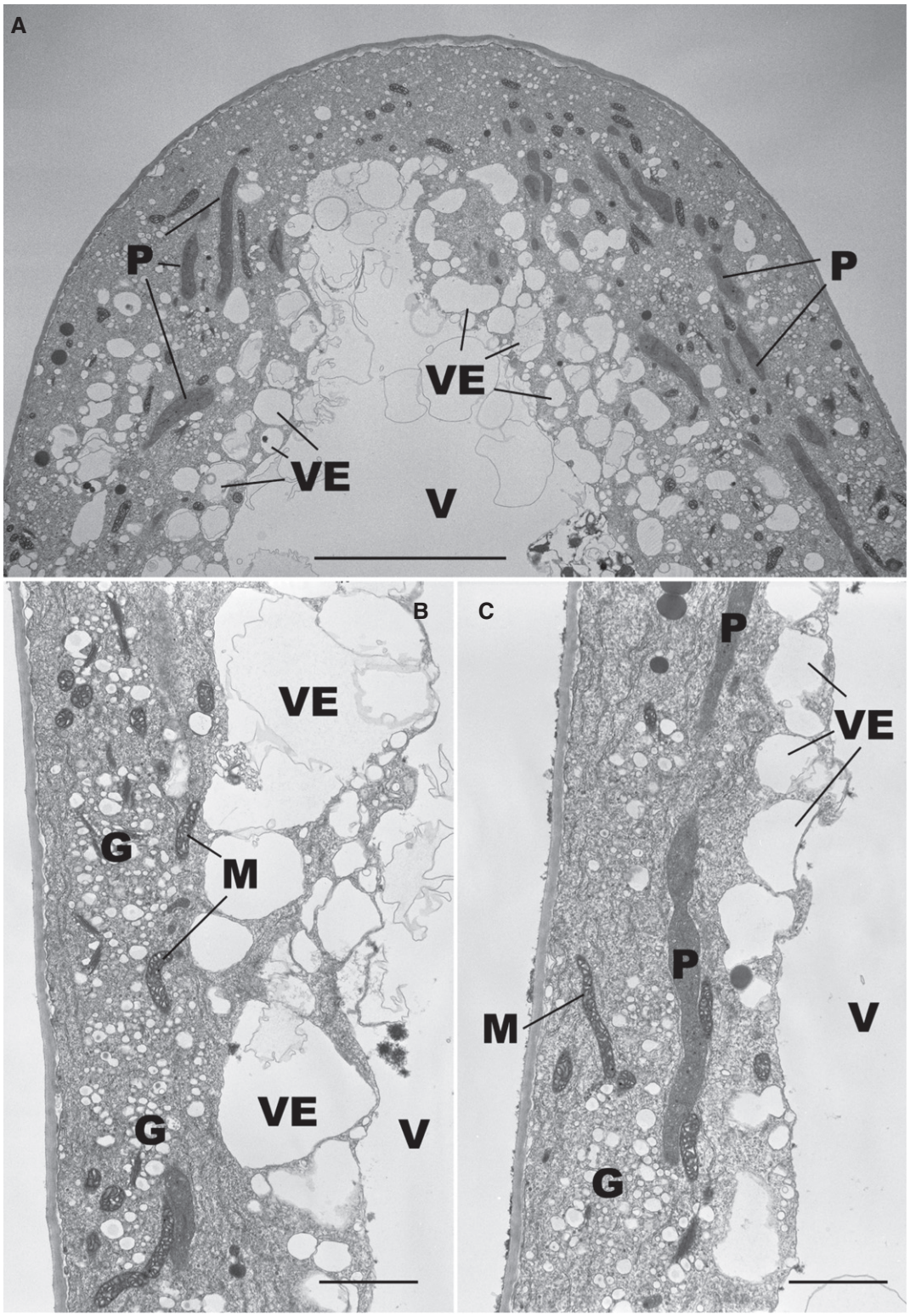

Figure 3. *Marchantia polymorpha*, transmission electron micrographs of smooth rhizoids. A, near median section of apex showing a large central vacuole extending into the apical dome. B, longitudinal section immediately behind the apical dome showing peripheral cytoplasm packed with Golgi bodies and large vacuoles adjacent to the central vacuole. C, peripheral cytoplasm 40 μm from the apex with fewer Golgi bodies and vesicles. G, Golgi bodies; M, mitochondria; P, plastids; V, vacuole; VE, vesicles. Scale bars: B, C, 2 μm; A, 5 μm.

within the same specimen. However, some taxa have short pegs only (Wiesnerellaceae, *Peltolepis* Lindb., *Cyathodium foetidissimum* Schiffn., Cleveaeae, Exormothecaceae, many *Riccia* spp. and *Monoclea*), whereas the most elaborate occur in *Bucegia*, *Reboulia*, *Marchantia* and *Dumortiera*, where they are restricted to the carpocephala. Sometimes the pegs are interconnected in a spiral arrangement along the entire length of the rhizoids (Fig. 1P–S). The pegs coat the inner surface of the apical domes of the rhizoids (Fig. 1C) but at their bases rarely extend onto the walls contiguous with other cells.

In contrast to smooth rhizoids, fully grown pegged rhizoids invariably lack living contents (Fig. 1B) and only occasionally contain fungal hyphae (Fig. 1P). Rhizoid development in *Conocephalum* underlines this difference. Pegged rhizoids are the first to appear. They are initiated beneath ventral scales 4–6 behind thallus apices. By the time the first smooth rhizoids appear in the vicinity of scales ten or more behind the apices, usually at the point where the growing thallus comes into direct contact with the substratum, the pegged ones are fully grown and dead.

Our measurements of pegged rhizoid lengths are generally in accordance with those previously given by McConaha (1941) [in brackets]: up to 8.5 [8.2] mm in *Preissia*, 2.0 mm [1.8] in *Reboulia*, 2.5 mm [2.5] in *Lunularia*, 8.0 mm [8.0] in *Marchantia* but up to 20.0 mm [16.5] in *Conocephalum*. Corresponding lengths for the smooth rhizoids, however, exceed those given by McConaha; *Preissia* 8.0 mm [3.7], *Reboulia* 6.0 mm [3.5], *Lunularia* 10.0 mm [3.2], *Marchantia* 30.0 mm [8.3] and *Conocephalum* 25.0 mm [6.9].

Scanning electron microscopy

Conventional scanning electron microscopy (Fig. 2) provides striking images of the disposition, dimensions and wall features of the rhizoids. Figure 2A illustrates the wide range of rhizoid diameters and peg frequencies typical of those enclosed by ventral scales in *Marchantia*. In *Dumortiera* (Fig. 2B), rhizoids running parallel to the thallus surface have a similar range of diameters to those in *Marchantia* and are both pegged and smooth. In *Monoclea* (Fig. 2C), the rhizoids running parallel to the surface tend to be narrower and have thicker walls than those extending at right angles. At higher magnification (Fig. 2D) the position of pegs is clearly marked by shallow depressions on the outer wall surface, as shown here in *Corsinia coriandrina* (Spreng.) Lindb., whereas pegs are not visible across the inner basal walls (Fig. 2E). Fungal hyphae are a frequent feature of smooth rhizoids (Fig. 2F). Rhizoids from specimens either collected from nature (Fig. 2G) in a dry condition and then rehydrated prior to critical point drying (CPD) or resurrected from herbaria (Fig. 2H) anticipate the results from the desiccation experiments (see below, Figs 6, 9). Whereas pegged and thick-walled smooth rhizoids in *Dumortiera* and *Monoclea* (and *Neohogdsonia* – not illustrated) have the same appearance as in fully hydrated specimens, smooth thin-walled rhizoids are invariably flattened.

Transmission electron microscopy

Cytoplasm at the apices of smooth rhizoids is packed with small vesicles, numerous elongate mitochondria and plastids (Fig. 3A). A large central vacuole extends well into the apical dome and is surrounded by numerous vesicles with transparent contents. For up to 30 μm behind the apex the peripheral cytoplasm is 7–9 μm deep and is stratified with Golgi bodies and sheets of rough endoplasmic reticulum (ER) near the wall, a mid-region containing mitochondria and plastids interspersed with small vacuoles, and an inner layer comprising larger vesicles with electron-transparent contents (Fig. 3B). Further from the apex the cytoplasmic layer decreases in thickness alongside a marked decrease in the density of the Golgi bodies and the vesicles lining the vacuole (Fig. 3C). Whereas the mitochondria are packed with saccate cristae in a dense stroma (Fig. 4B), the plastids contain scattered vesicles and lack flattened thylakoids (Fig. 3C). Longitudinally orientated endoplasmic microtubules and pleiomorphic microbodies up to 0.3 μm in diameter are visible in the vicinity of the mitochondria (Fig 4B). The Golgi bodies comprise stacks of six to eight central cisternae (Fig. 4C) with numerous peripheral vesicles of three kinds: coated vesicles up to 100 nm in diameter; smooth vesicles up to 200 nm in diameter with granular contents (Fig. 4D); and others with clear contents varying from 200 nm to > 1.0 μm in diameter (Fig. 4C). The first two are most frequent near the walls, the last adjacent to the vacuole.

The nucleus (Fig. 4A) is located 50–100 μm behind the apex. It is discoidal in shape, up to 30 μm in diameter and has an undulating envelope containing numerous pores. The nucleoplasm contains > 20 separate areas of nucleolar material. The thin layer of cytoplasm between the nucleus and vacuole contains sheets of ER, whereas a thicker external layer contains ER, scattered mitochondria and Golgi bodies. Behind the nucleus the rhizoids contain an extremely attenuated layer of peripheral cytoplasm that is scarcely 0.05 μm wide, alongside a thin, 0.5- to 0.8-μm-thick, non-stratified wall overlain by a thin cuticular layer (Fig. 4E).

Electron microscopy confirms that pegged rhizoids are devoid of contents (Fig. 5B) and the same is true for the narrow rhizoids running parallel to the thallus surface in *Monoclea* and those in the carpocephalum

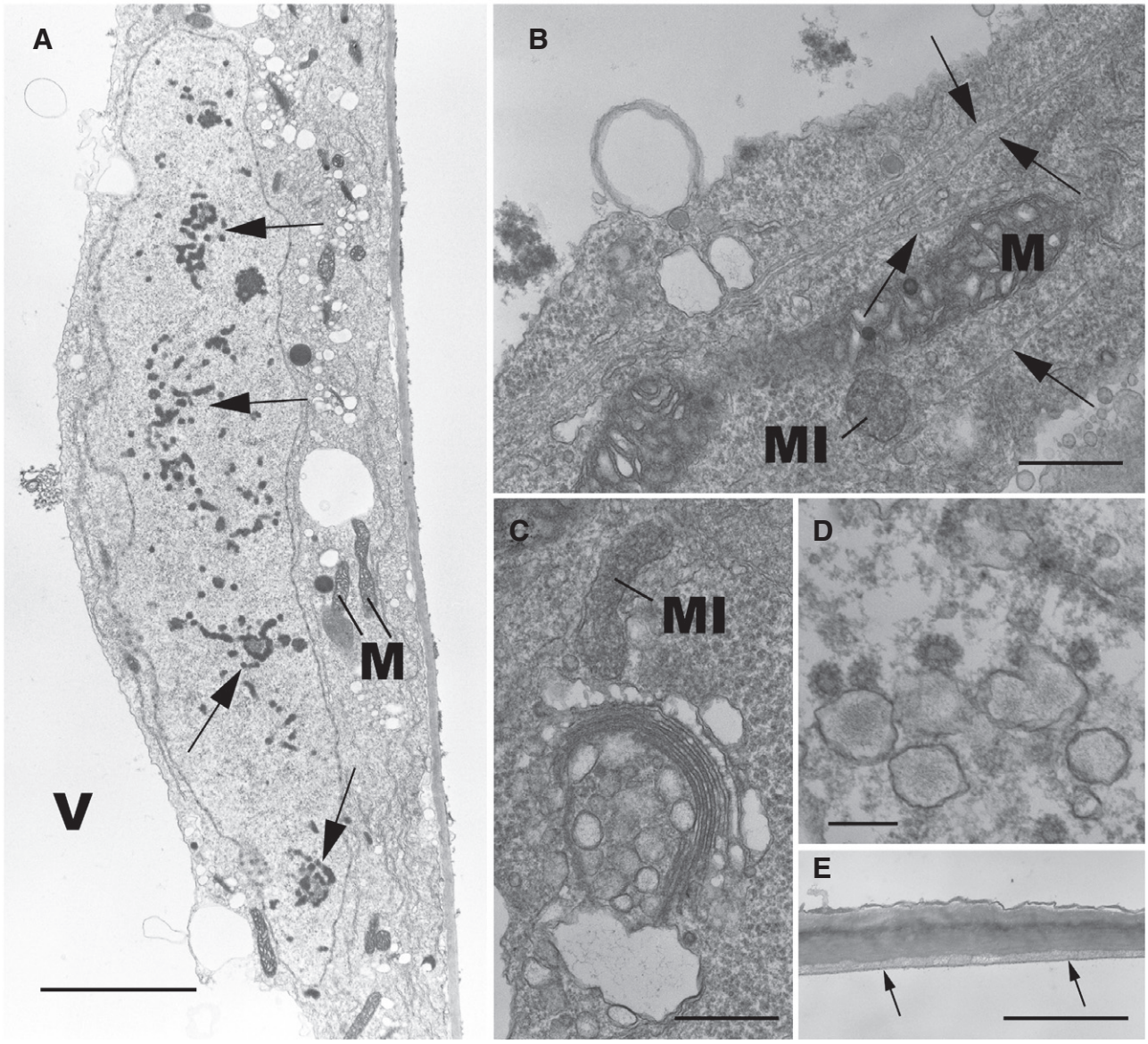

Figure 4. *Marchantia polymorpha*, transmission electron micrographs of smooth rhizoids. A, longitudinal section through the massive nucleus with several nucleolar regions (arrowed). B, longitudinally orientated endoplasmic microtubules (arrowed) associated with elongate mitochondria. C, Golgi body with large vesicles. D, coated vesicles and vesicles with granular contents. E, cell wall and attenuated peripheral cytoplasm (arrowed). M, mitochondria; MI, microbodies; V, vacuole. Scale bars: D, 0.2 μm; B, C, 0.5 μm; E, 2 μm; A, 5 μm.

grooves in *Neohodgsonia* (Fig. 5A). Their walls are 1–2 μm thick, lack stratification and have a fine fibrillar texture. The pegs, however, have a denser granular appearance which sometimes extends to the outside of the walls (Fig. 5B).

Desiccation experiments

Pegged and smooth rhizoids respond differently to de- and rehydration. When mounted intact either in water (not illustrated) or in immersion oil (Fig. 6A, B), smooth rhizoids of dry field-collected liverworts, herbarium specimens and specimens dried in the laboratory are completely flattened. When specimens that are dry when field-collected are then rehydrated, and grown in the laboratory the only smooth rhizoids with living contents are those produced anew following rehydration. In contrast, dehydrated pegged rhizoids have much the same appearance as those from fully hydrated specimens (Fig. 6A).

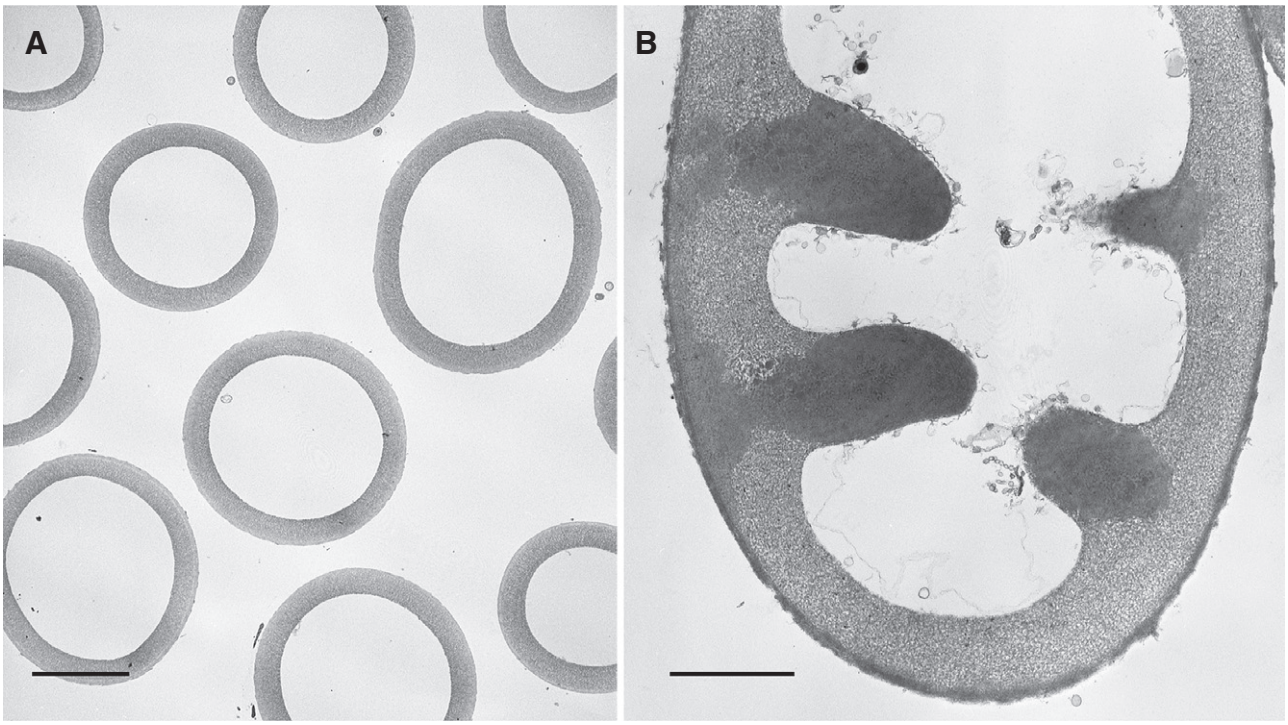

Figure 5. Transmission electron micrographs. A, *Neohodgsonia mirabilis*, exclusively smooth rhizoids in a carpocephalum groove. B, *Reboulia hemisphaerica*, pegged rhizoid. Scale bars: B, 2 μm; A, 5 μm.

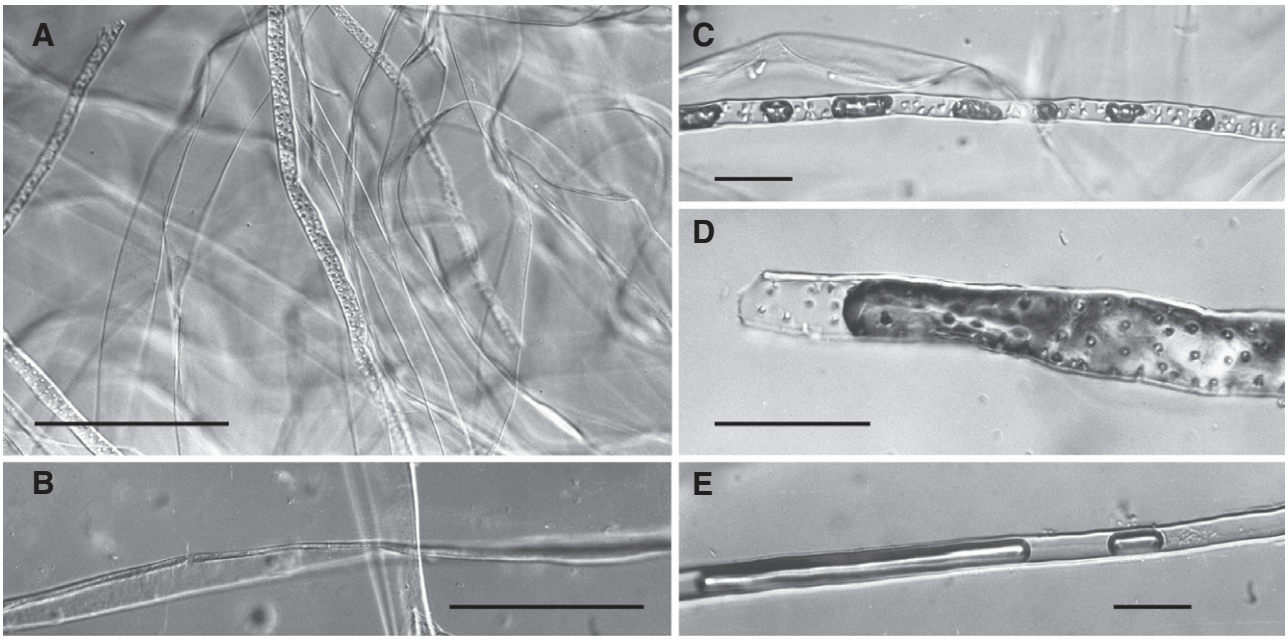

Figure 6. Light micrographs of desiccated rhizoids mounted in immersion oil. A, C, *Corsinia coriandra*. B, E, *Monoclea*, smooth large (B) and narrow rhizoid (E). *D*, *Riccia sorocarpa. A*, herbarium specimen; note the flattened smooth rhizoids and unchanged appearance of those with pegs compared with Figure 1. B, flattened smooth rhizoid. C–E, air bubbles in the lumina of dry rhizoids cut before placing them in oil. Scale bars: E, 20 μm; D, 50 μm; A–C, 100 μm.

Cutting the same sets of dry rhizoids (Fig. 6C, D) before placing them in immersion oil produces different results. The smooth rhizoids remain flattened and never contain air bubbles whereas the pegged rhizoids contain air bubbles, thus revealing that they have elastic walls and regained their original shape following cutting. The only exceptions to these results, obtained from a range of genera, were in *Monoclea*, *Dumortiera* and *Neohodgsonia.* In *Monoclea* the narrow rhizoids (some of which were observed, for the first time in the present study, to contain sparse pegs) cut in air often contained air bubbles (Fig. 6E) whereas the larger ones remained flattened. The same was also true in *Dumortiera* (not illustrated) and narrow thick-walled rhizoids in the carpocephalum grooves in *Neohodgsonia* remained largely non-collapsed in dried specimens and often contained air bubbles when rehydrated.

Cryo-SEM

Cryo-SEM analyses (Figs 7–9) permit an immediate and unequivocal diagnosis of the vital status of individual rhizoids and provide new insights into the functions of the carpocephalum grooves as internalized external water-conducting systems, pegged rhizoids as water-conducting cells, hitherto undetected cryptic rhizoid dimorphism in *Dumortiera*, *Neohogdsonia* and *Monoclea*, and further demonstrates the failure of smooth rhizoids to recover from desiccation.

Cross-sectional images through fresh hydrated rhizoids of *Pellia* (Fig. 7A) and *Marchantia* reveal their vital status (Fig. 7B). The presence of finely patterned ice eutectics (crystallization patterns) in pegged rhizoids identical to those in the surrounding liquid confirms their lack of living contents, whereas the much coarser ice patterns in all the *Pellia* rhizoids and the smooth ones of *Marchantia* are like those of typical thallus cells. Cryo-SEM of rhizoids of *Neohodgsonia* (Fig. 7F) and *Monoclea* (Fig. 8) reveals that the smaller rhizoids almost invariably lack living contents whereas the large ones are clearly alive. In *Dumortiera* (Fig. 7G, H) highly pegged rhizoids are associated preferentially with the carpocephalum grooves (Fig. 7G); young pegged rhizoids are alive and thus exhibit different ice eutectics from their mature and dead counterparts (Fig. 7H, but see also young pegged living rhizoid of *Marchantia* in Fig. 7C).

A further benefit from cryo-SEM is that it permits a distinction between hydrophilic and hydrophobic surfaces (Pressel, P'ng & Duckett, 2010a). The thin cuticle lining the entire external surface of *Marchantia* thalli is highly hydrophobic. This same layer also lines the walls of the carpocephalum grooves, as evidenced by a clear-cut separation between the walls and the liquid inside the grooves (Fig. 7D, E).

Turning to desiccation biology (Fig. 9), images of fully hydrated carpocephala in *Marchantia* clearly show that the spaces between the rhizoids are liquid-filled (see Fig. 7D, E). With mild dehydration (2 h in the laboratory), mucilage exudes from the groove (Fig. 9A) and, with complete dehydration, pegged rhizoids become irregular in outline (Fig. 9B). On adding water, pre-desiccation morphology is completely restored in the pegged rhizoids (Fig. 9C), but occasional smooth ones in the grooves remain flattened (Fig. 9D).

Dye and lanthanum experiments

When marchantialean liverworts are placed in methylene blue solution, within 5 min the dye completely fills the spaces delimited by the ventral scales containing the pegged rhizoids. Times for the dye to reach the carpocephalum caps (Table 2) range from 30 to 75 min, corresponding to rates of movement of between 30 and 150 mm h^{-1}. No relationship is apparent between the rates of movement and the presence of one or two grooves. Dye moves at a similar rate in the hydromes of *Hypopterygium* Brid., *Dawsonia* R.Br. and *Pogonatum* P.Beauv., but the faster rates in *Polytrichum* Hedw. and *Dendroligotrichum* (Müll. Hal.) Broth. match those in the xylem of the ferns.

Under our laboratory conditions, when left standing in water for 24 h the two ferns and the liverworts with carpocephalum grooves showed no signs of water stress, whereas all five mosses and *Lunularia* (grooves absent) became highly dehydrated within 1–2 h. In fact desiccation of the carpocephala in *Lunularia* was similar to that observed for mature sporophytes with undehisced capsules in *Monoclea* with setae of dimensions similar to the stalks. It took approximately 1 h for the capsules to dehisce and after 2 h both the setae of *Monoclea* and the stalks of *Lunularia* had collapsed.

Because it was impossible to see from light microscopy whether dye was present within the lumina of the pegged rhizoids, we mapped the distribution of lanthanum in the carpocephalum grooves 1 h after floating the thalli in a 0.1% solution of lanthanum nitrate. Typical X-ray microanalysis spectra for *Asterella australis* are shown in Figure 10. The results with *Marchantia foliacea* (not illustrated) were almost identical. High lanthanum peaks were detected in the mucilage filling the groove (Fig. 10A), in the mucilage along the channel closing the groove (similar to Fig. 10A – not illustrated) and within the lumina of the pegged rhizoids (Fig. 10B). Lanthanum was still detectable in the walls of some of the cells near the grooves (Fig. 10C), but not inside any of the living carpocephala cells (Fig. 10D) or within the lumina of smooth rhizoids (not illustrated).

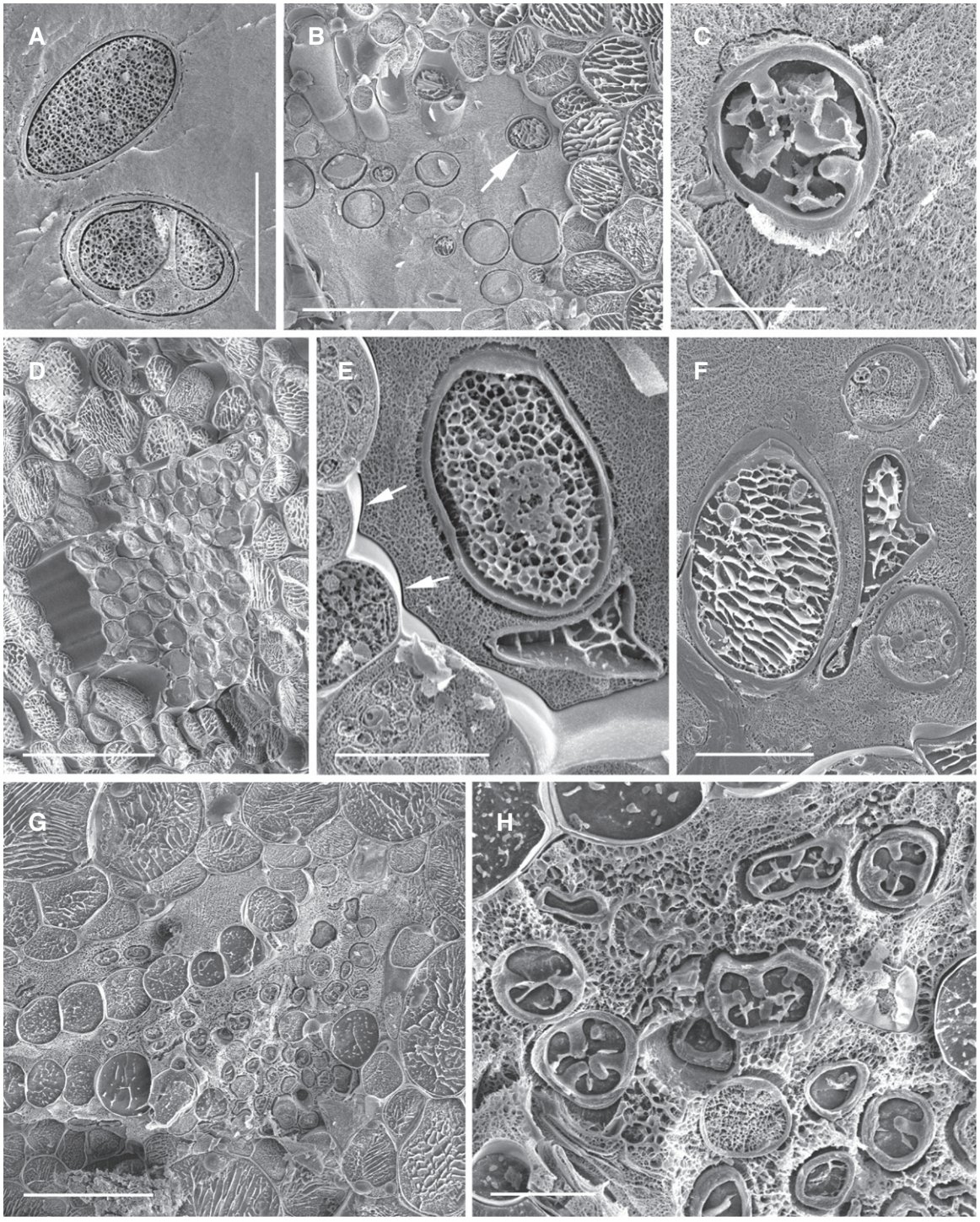

Figure 7. Cryo-scanning electron micrographs. A, *Pellia epiphylla*; B–E, *Marchantia polymorpha*; F, *Neohodgsonia mirabilis*; G, H, *Dumortiera hirsuta*. A–c, differences in ice eutectics of frozen rhizoids; the frozen vacuolar contents of the living monomorphic rhizoids of *Pellia* (A) and the smooth rhizoids of *Marchantia* (B, arrowed) are like those of the living thallus cells; the same is true for those of young pegged rhizoids (C) whilst the much finer ice eutectics in mature pegged rhizoids are identical to those in the surrounding frozen liquid (B). D, E, clear-cut separation between the walls and the liquid inside the carpocephalum grooves of *Marchantia* (arrowed in E). F, large and living rhizoids next to smaller and dead ones in *Neohodgsonia*, as revealed by differences in ice eutectics between the contents of the two types. G, H, highly pegged rhizoids associated with the carpocephalum grooves in *Dumortiera*. Scale bars: C, E, H, 10 μm; A, F, 20 μm; B, D, G, 50 μm.

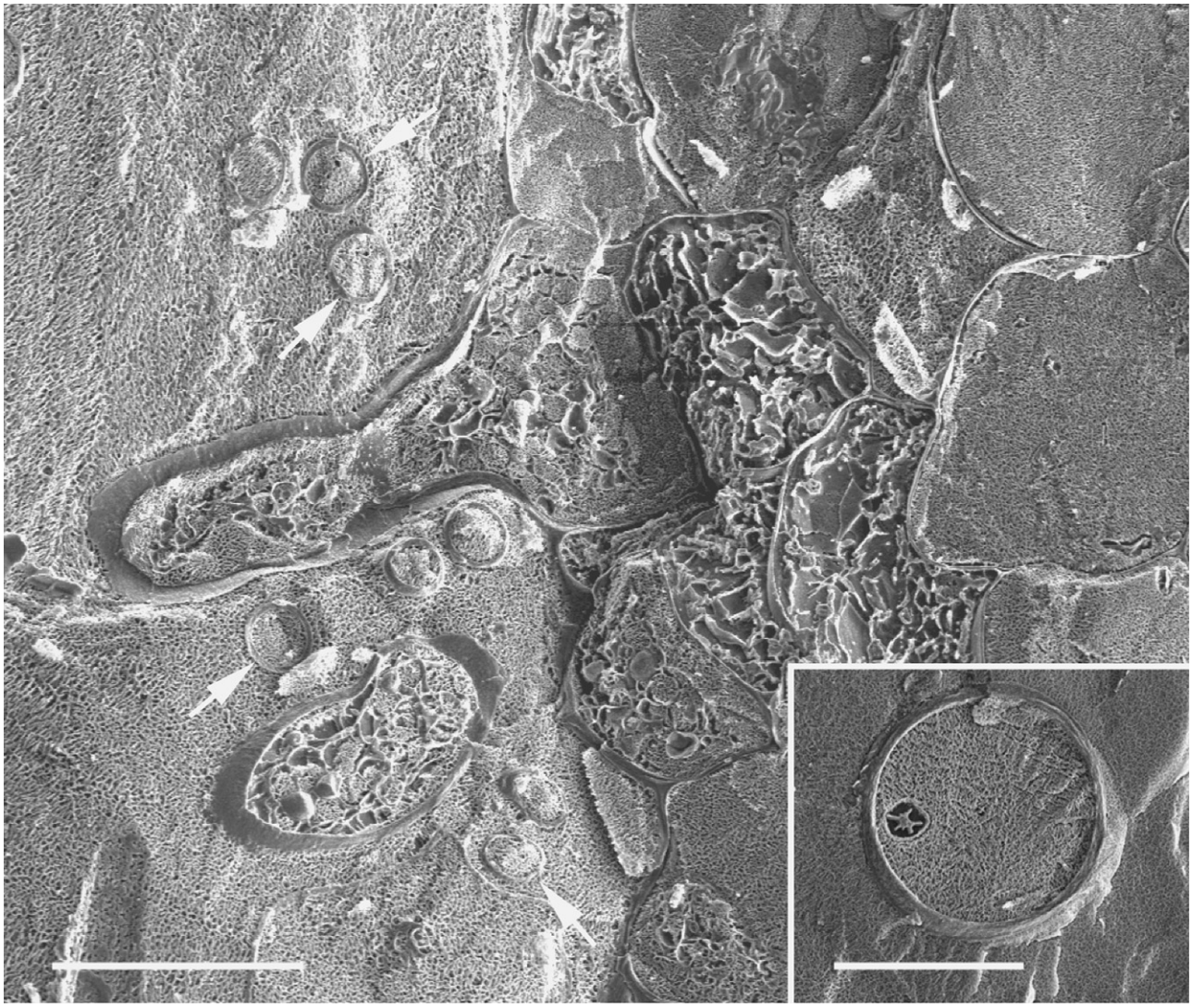

Figure 8. Cryo-scanning electron micrographs; *Monoclea forsteri*. Longitudinal cross-section through a thallus showing a large rhizoid with living content positioned at right angles to the thallus and numerous small dead rhizoids with parallel disposition, one of which is enlarged in the inset. Scale bars: 50 μm; inset, 20 μm.

DISCUSSION

Pegged and smooth rhizoids of marchantialean liverworts are markedly different from each other in structure, physiology and function. Cytological and experimental data indicate that the structural integrity of smooth rhizoids in these liverworts depends on living cytoplasm and the turgor pressure of the vacuolar contents. Dehydration results in death and collapse of the cell wall, which is irreversible. A similar response to desiccation is true of unicellular rhizoids in simple thalloid and leafy liverworts and on fern gametophytes (Pressel, 2007; S. Pressel & J. G. Duckett, unpublished data). In contrast, multicellular protonemata and rhizoids of mosses can recover from desiccation, apart from their apical cells (Pressel, 2007; Rowntree *et al*., 2007; Pressel, Ligrone & Duckett, 2008a).

In contrast, the structural integrity of the pegged rhizoids depends on wall elasticity (as revealed by the ingress of air bubbles following cutting and mounting in immersion oil; see Fig. 6) with the pegs preventing complete collapse during dehydration. Thick-walled smooth and dead rhizoids in *Dumortiera*, *Neohogdsonia* and *Monoclea* have similarly elastic walls. Absence of air bubbles inside intact desiccated pegged rhizoids also demonstrates that these are highly resistant to cavitation, a feature shared with the water-conducting hydroids in mosses (Ligrone *et al*., 2000).

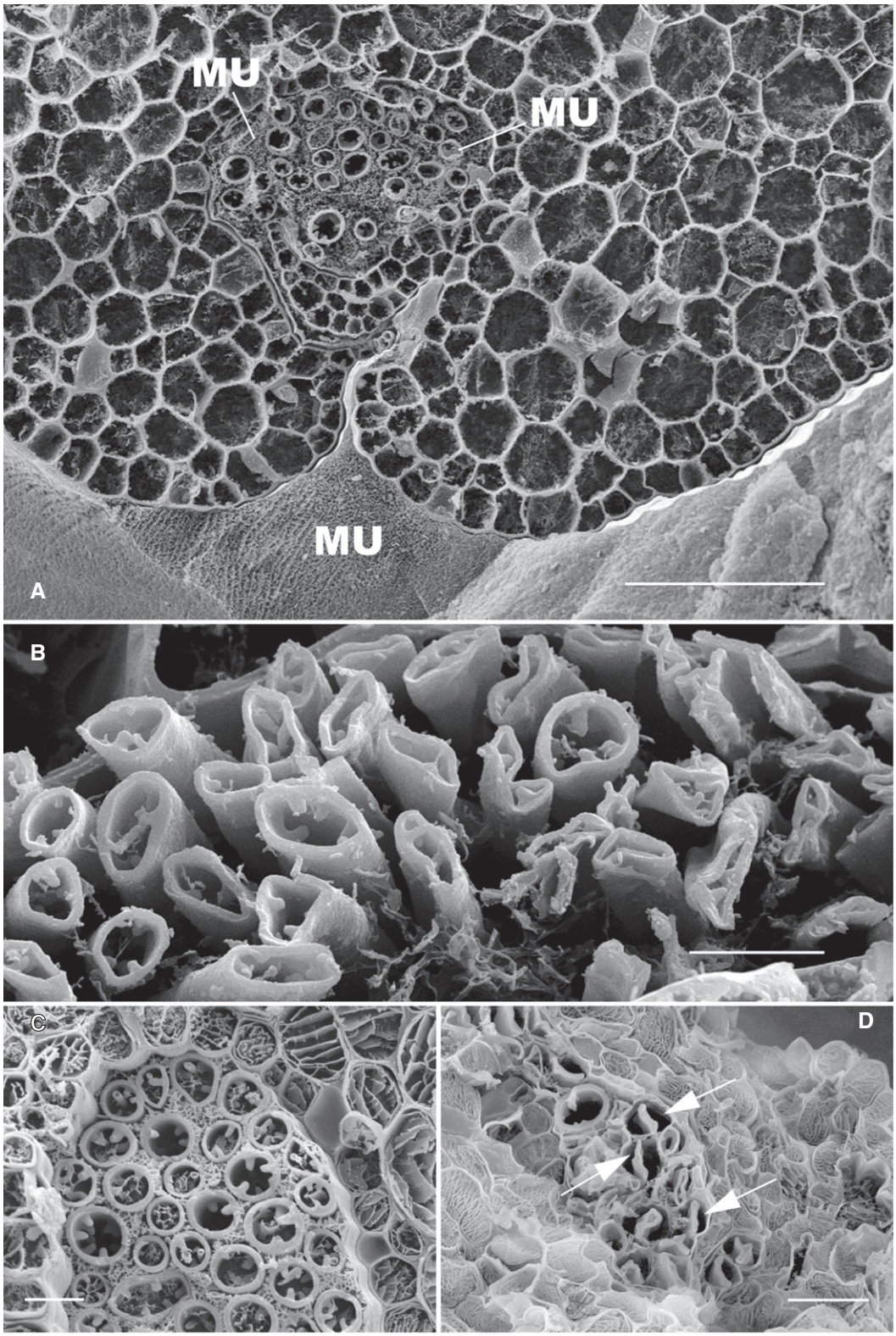

Figure 9. Cryo-scanning electron micrographs; carpocephalum stalks of *Marchantia foliacea*. A, partially desiccated specimen. Note the plug of mucilage (MU) sealing the groove. B, desiccated specimen showing pegged rhizoids with lumina still open. C, rehydrated specimen with fully recovered pegged rhizoids. D, rehydrated specimen with flattened smooth rhizoids (arrowed). Scale bars: B, C, 20 μm; D, 50 μm; A, 100 μm.

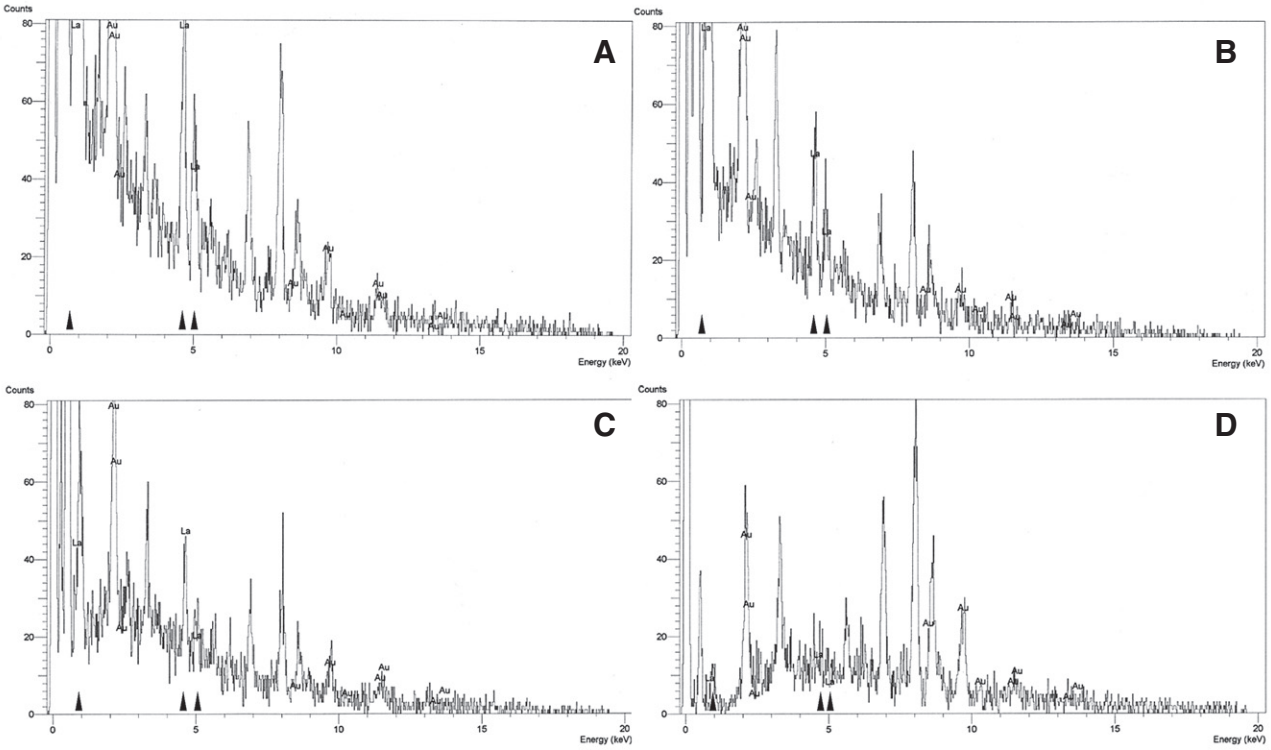

Figure 10. X-ray microanalysis spectra of lanthanum (arrows) in carpocephalum stalks of *Asterella australis*. Note the prominent lanthanum peaks in the mucilage around the rhizoids within the groove (A), the lumen of a rhizoid (B) and in the wall of cell bordering the groove (C). Lanthanum is undetectable in the lumen of cells bordering the groove (D).

This is the first unequivocal documentation that smooth rhizoids in marchantialean liverworts are alive and almost certainly grow at their tips as do root hairs, pollen tubes, fungal hyphae and moss protonemata. This process is cytologically identical in all cases, including the presence of numerous highly active Golgi bodies, associated with proplastids and mitochondria with prominent saccate cristae and longitudinally orientated endoplamic microtubules at the apex (Bartnik & Sievers, 1988; Heath, 1990; Ryan, Steer & Dolan, 2001). Unlike gravitropic moss caulonemata and rhizoids that contain prominent amyloplasts in their apices, starch and a similar response are both lacking in liverwort rhizoids (von Lehmann & Schulz, 1982) and fern rhizoids (Parton *et al*., 2000). Smooth rhizoid orientation appears to be fixed at right angles to the thalli at their initiation. Similarly, pegged rhizoids initially extend at right angles but their orientation is then changed either by an investiture of ventral scales or the narrow confines of the carpocephalum grooves. Other differences between rhizoid and caulonemal apices (Pressel, 2007; Pressel *et al*., 2008a) is the peripheral rather than central location of the nucleus and the distribution of active Golgi bodies in the peripheral cytoplasm well behind the apical dome. The simplest explanation for the production of two kinds of vesicles by the Golgi bodies and their disposition is that those with granular contents contribute to the growing cell walls, whereas the larger ones with clear contents are added to the central vacuole.

By far the most remarkable feature of the smooth rhizoids is their massive nucleus containing numerous nucleolar fragments like those in fully differentiated moss caulonemal cells (Kingham *et al*., 1995). Microfluorometric measurements, following DAPI staining (J. G. Duckett, unpublished data), reveal considerable endoreduplication paralleling that seen during moss caulonemal differentiation (Kingham *et al*., 1995) and in animal salivary glands that also contain large numbers of hypertrophied Golgi bodies (Hand, 1971). This profound nuclear differentiation may explain why marchantialean rhizoids, commonly exceeding 10–20 mm in length to a maximum of *c*. 30 mm in *Marchantia polymorpha*, are the longest cells in liverworts and, like differentiated caulonemal cells, lack the ability to regenerate. Even where fungal infections stimulate cell divisions in the tips of the rhizoids in Schistochilaceae, these remain incapable of regenerating new plants (Pressel *et al*., 2008b). Thus, this study confirms previous suggestions that their principal functions, all dependent on

the rhizoids having living contents from the outset, are in nutrition and anchorage and as fungal conduits.

Pegged rhizoids, by contrast, are dead at maturity. Their cavitation-resistant, elastic walls ensure retention of functional integrity through periods of desiccation which may last for several months in taxa that experience seasonal drought and is still retained in herbarium specimens. The pegs prevent complete occlusion of the lumina in the dry state and thus facilitate refilling with water. Kamerling (1897) correctly concluded that pegged rhizoids have a key role in water conduction, but, in contrast to the present study, failed to recognize the importance of the pegs and wall elasticity in this process.

The dye experiments clearly demonstrate that the pegged rhizoid systems of marchantialean liverworts, running parallel to the lower surface of the thalli and particularly within the carpocephalum grooves, function effectively as internalized external water-conducting systems. The same is true for the smooth but dead rhizoids in *Neohodgsonia*.

Not only are individual pegged rhizoids desiccation-resistant but the carpocephalum grooves function in a similar manner, with mucilage-invested flaps forming an effective seal preventing the formation of air bubbles within the grooves. Water movement, as revealed by the dye and lanthanum data, is strictly channelled within the grooves and within the lumina of the pegged rhizoids.

In terms of maintaining water balance in the carpocephalum caps the grooves appear to be much more efficient, albeit over shorter distances, than moss hydroid systems, presumably because of a lower resistance to water flow, a feature enhanced by the hydrophobic internal surface of the grooves. The bundles of pegged rhizoids extending almost to the tips of the arms on the carpocephalum caps are crucial to maintaining the sex organs in a fully hydrated state.

Under laboratory conditions (temperature *c*. 20 °C and relative humidity *c*. 70%) carpocephalum caps, like the fern pinnae, remain fully hydrated as long as the thalli are fully hydrated whereas the moss stems all show signs of drying out within 1–2 h. This mirrors the situation in nature with carpocephala of *Marchantia* remaining fully hydrated even after several hours of full sunlight and temperatures in the mid twenties, whereas the shoots of *Polytrichum commune* forming tussocks with their stem bases in standing water are severely wilted and require rain to recover.

Ecological considerations

Rhizoids played essential roles in the early evolution of land plants, especially in gametophyte diversification. Across bryophytes, except *Haplomitrium* Nees. which lacks rhizoids even during spore and gemma germination (Furuki, 1986; Bartholomew-Began, 1991), smooth rhizoids are produced early in spore-ling growth, even when these are lacking in the mature plants (e.g. *Pleurozia purpurea* Lindb.), and anchor germinating spores to the substrate. Beyond the role in affixing the plant to the substrate, rhizoids have been assumed to function as absorptive structures similar to root hairs. In liverworts, following loss of the primeval endogonaceous fungal symbioses in *Treubia* K.I.Goebel and *Haplomitrium*, which do not involve rhizoids (Duckett, Carafa & Ligrone, 2006; Ligrone *et al*., 2007; Bidartondo *et al*., 2011), all the subsequent liverwort fungal associations have rhizoids as the conduits for mycobiont entry (Pressel *et al*., 2010b; Pressel, Duckett & Bidartondo, 2012) culminating in the exclusively rhizoidal infections in leafy liverworts with the ascomycete *Rhizoscyphus ericae* (D.J.Read) W.Y.Zhuang & Korf (Read *et al*., 2000; Pressel *et al*., 2008b).

Perhaps the most astonishing and unique function of rhizoids in any land plant is that of pegged rhizoids in complex thalloid liverworts. In these plants, bundles of elongated pegged rhizoids run along the ventral surface of the thallus to form a highly efficient external conduction system as demonstrated in this study. Each rhizoid is dead at maturity and contains invaginating pegs of highly reinforced wall material. The ability to conduct is not disrupted by dehydration and the pegs prevent the cells from collapsing during dry periods. Thus, the evolution of these cells is inextricably linked to water availability. More conventional completely internal water-conducting systems are rare in liverworts; they occur in the early-divergent lineage *Haplomitrium*, and in only one derived family of simple thalloids, Pallaviciniaceae (Ligrone & Duckett, 1996; Ligrone *et al*., 2000). However, the water-conducting cells here, unlike pegged rhizoids, are not desiccation-resistant.

It is assumed that the origin of pegged rhizoids was instrumental in the evolution of carpocephala and a unique means of elevating sporophytes above the thallus surface. Unlike the vegetative thallus, carpocephala enclose their pegged rhizoids in grooves. This functionally internalized water-conducting system maintains water balance and the carpocephalum provides a stable and reliable way of getting spores into turbulent air. Capsule elevation in other liverworts relies entirely on hydrostatic pressure in its highly elongate thin-walled seta cells. Spore discharge from one carpocephalum involves several sporophytes, all genetically different, that are protected by a water-repellent cap and may extend over several weeks, instead of 1–3 days for solitary sporophytes in other liverworts. In this context it is significant that

in *Marchantia*, which has carpocephala that can maintain their functional integrity for up to 2 or 3 months (Duckett & Pressel, 2009), the rhizoid conducting system extends well beyond the archegonia into the finger-like arms of the archegoniophores.

Protracted spore liberation, plus small spore size (10–16 μm) compared with other Marchantiales (Longton & Schuster, 1983), may well be a key factor contributing to the effectiveness of the members of the genus *Marchantia* as primary colonists (Duckett & Pressel, 2009). *Marchantia* is the only complex thalloid genus with stalked antheridiophores. Not only are these much shorter, rarely exceeding 30 mm, than the archegoniophores (Table 2) but they also function differently (Duckett & Pressel, 2009). Their caps are highly hydrophilic and, unlike those of the archegoniophores, absorb raindrops like a sponge. Upwards water movement during dry periods is punctuated by downwards flow taking the motile sperm with it during rain.

Seta lengths in liverworts lacking carpocephala mostly fall in the range of 10–20 mm but in a few may reach 100 mm (*Pellia*) or even 200 mm (*Noteroclada confluens* Taylor ex Hook. & Wilson). Ultimately the length of carpocephalum stalks (Table 2), to a maximum of 70–100 mm in *Conocephalum*, is most likely limited by avoidance of air bubble formation in the mucilaginous matrix around the pegged rhizoids in the grooves (also see Raven, 1993), a situation analogous to embolism in vessels (Canny, 2001a, b). Thus, the basic construction of rhizoid grooves, although highly effective for relatively short-distance water transport, severely limits the extent to which liverworts are able to extend their reproductive organs above the ground.

Evolutionary considerations

Across Marchantiopsida (Table 1), highly pegged rhizoids are predominantly found in taxa with ventral scales that experience periodic and often prolonged desiccation (e.g. *Mannia*, *Plagiochasma*, *Reboulia*, *Lunularia*, *Bucegia*, *Marchantia*, *Preissia*, *Targionia* and some *Riccia* spp.) or are largely restricted to the carpocephala in the hygrophilous genus *Dumortiera*. Thus, the role demonstrated in this study as a means to resist desiccation is reinforced by habitat preference and the need to maintain water balance of carpocephala.

The earliest divergent clade Blasiales (Forrest *et al*., 2006; Crandall-Stotler *et al*., 2008, 2009) produces ventral scales and smooth rhizoids only. In this strictly mesic group, ventral scales develop from the lateral derivative and rhizoids from the ventral derivative of the wedge-shaped apical cell (Renzaglia, 1982). Ventral scales have a similar origin in Marchantiidae, but the pegged rhizoids are central and presumably derived from the ventral merophyte. Smooth rhizoids, except in derived taxa (Ricciaceae), are in contrast lateral in origin. The logical conclusion from these developmental considerations is that pegged rhizoids evolved from smooth rhizoids.

When the data from this study are superimposed onto the latest liverwort phylogenetic trees (Forrest *et al*., 2006; Crandall-Stotler *et al*., 2008, 2009), the cryptic and hitherto undetected rhizoidal dimorphism in the presumed early-divergent genus *Neohogdsonia*, in which the rhizoids within the carpocephalum grooves are by and large, albeit smooth, of smaller diameter, thick-walled, dead at maturity and with cavitation-resistant walls, strongly suggests that pegged rhizoids arose from these and that their evolution was intimately linked to that of stalked carpocephala. Of course the placement of *Neohodgsonia* as an earlier divergent lineage than *Lunularia* (Forrest *et al*., 2006; Crandall-Stotler *et al*., 2008, 2009) is highly problematic, given that *Lunularia* lacks carpocephalum grooves and has pegged rhizoids associated exclusively with ventral scales. However, the order of divergence of *Neohogdsonia* and *Lunularia* is anything but resolved (Forrest *et al*., 2006; Crandall-Stotler *et al*., 2008, 2009). An alternative hypothesis, which requires the placement of *Neohogdsonia* as a later divergent genus, would be that pegged rhizoids first appeared in *Lunularia*, their absence in *Neohogdsonia* being a secondary loss.

Indeed, pegs have been lost in *Cyathodium* and some *Riccia* spp., taxa in every case associated with continuously wet conditions where retention of pegs designed to withstand desiccation is superfluous. In the two derived and closely aligned hygrophilous genera *Monoclea* and *Dumortiera* (Forrest *et al*., 2006; Crandall-Stotler *et al*., 2008, 2009), rhizoidal dimorphism in the former is reflected mainly by the presence of smooth rhizoids that are dead at maturity orientated parallel to the thalli and which recover from dehydration, and living smooth rhizoids, orientated at right angles, that irreversibly collapse during drying, with small and sparse pegs present in only a small subset of the total rhizoid population. In *Dumortiera*, pegged rhizoids are present, but whereas those occurring within the carpocephala often have long and crowded pegs, like those in *Marchantia*, rhizoids associated with the thalli are characterized by short and widely spaced pegs with the occurrence also of a few thick, elastic-walled smooth, dead rhizoids. Taken together, these observations suggest that *Dumortiera* and *Monoclea* represent intermediate stages in the disappearance of more highly pegged rhizoids and point to pegged ancestry.

Ricciaceae are a reduced, derived yet species-rich clade that represents the most recent radiation in

complex thalloid liverworts. Much variability in this group reflects its more recent ancestry. In this family we see a trend from well-developed pegged rhizoid systems and scales in perennials (e.g. *R. crozalsii*, *R. nigrella*, *R. okhandjana*) to their gradual loss associated with temporary (e.g. arable land) and finally aquatic habitats (*R. fluitans*). Moreover, there is no evidence that, once lost, pegs may be acquired again, suggesting that the potential for future radiation into drier environments for these taxa is limited.

Schuster (1966, 1984b) argued, on the basis of comparative morphology, that the primitive marchantialean taxa were mesic and gave rise to progressively more xeromorphic forms. In support of his case, the pegs in *Monoclea* are considered a rudimentary condition in the evolution of more highly pegged rhizoids. All the data presented here suggest that the opposite might be true with the small and sparse pegs of *Monoclea* indicative of reduction processes leading to secondary loss. That the rhizoid system of *Monoclea* is derived is clearly reinforced by the position of this genus in the latest total evidence liverwort phylogenetic analyses (Forrest *et al*., 2006; Crandall-Stotler *et al*., 2008, 2009).

Ultimately, clarification of the pattern of pegged rhizoid evolution awaits further resolution of phylogenetic relationships in Marchantiideae. The branching order of the three earliest divergent lineages, Sphaerocarpales, Neohodgsoniales and Lunulariales, varies according to the analytical criteria used and support for the different topologies is low (Forrest *et al*., 2006; Crandall-Stotler *et al*., 2008, 2009). Stronger hypotheses on these early evolutionary events are also needed to understand whether the evolution of pegs preceded that of grooves or vice versa. Resolving the position of *Neohogdsonia*, the only liverwort that produces carpocephala grooves containing only smooth, albeit dead at maturity, rhizoids, is thus crucial. Our data clearly indicate that pegged rhizoids are intimately associated with carpocephala, where they function as a highly effective internalized external water-conducting system capable of maintaining hydration of carpocephala during even prolonged periods of dehydration.

Another important evolutionary issue that remains unresolved is whether carpocephalum stalks evolved only once or had multiple origins. Development considerations (Crum, 2001) suggest multiple origins: separately as dorsal outgrowths from the thallus in *Lunularia*, *Plagiochasma* and *Athalamia*, the three unrelated genera lacking grooves, and once in the ancestor of the remaining genera as modified terminal branch systems, with the grooves reflecting this history. On the other hand, disregarding these developmental considerations, it could be argued that stalks first arose in *Lunularia*, acquired grooves in the *Marchantia*/*Preissia* lineage and then subsequently lost these in *Plagiochasma* and *Athalamia*. Whatever the answer, it would seem beyond reasonable doubt that the absence of stalks in several crown groups, namely Cyathodiaceae, Corsineaceae, Targioniaceae, Oxymitraceae and Ricciaceae, is secondary loss. *Exomortheca* with its mixture of stalked and non-stalked taxa illustrates an intermediate condition.

Besides raising novel hypotheses as to the evolution of pegged rhizoids and carpocephalum grooves in Marchantiopsida, the structural and functional data presented here now open the way for dissecting the molecular basis of rhizoid morphogenesis in liverworts and exploring how far this matches that in other tip-growing filaments in land plants and, all the more so, with the current foci on *Marchantia* and the moss *Physcomitrella* Bruch & Schimp. as model organisms. A key question is homology across the different lineages of a cell type that almost certainly evolved first in liverworts, the group firmly anchored at the base of the land plant tree of life (Forrest *et al*., 2006; Crandall-Stotler *et al*., 2008, 2009). Apoptotic pegged rhizoids are certainly unique to complex thalloid liverworts, but do the huge endoreduplicated nuclei in the smooth rhizoids also occur in hornworts and in the root hairs of vascular plants? In hornworts, there is a similar lack of regenerative ability and a tendency for rhizoids to be lost in aquatic and highly mesic (*Megaceros* Campbell) and epiphytic (*Dendroceros* Nees) genera. Most problematic, however, is equating the unicellular rhizoids in liverworts, hornworts and vascular plants with the multicellular protonemal/rhizoid systems in mosses. Although numerous genes essential for rhizoid/root hair development are common to *Physcomitrella* and *Arabidopsis* L. (Jang, Manand & Dolan, 2011; Jones & Dolan, 2012) it remains difficult to explain why the three clades sister to the remaining mosses lack proper protonemal systems. Tip-growing filaments are absent in *Takakia* S.Hatt. & Inoue and Andreaeales (Newton *et al*., 2000) and the filaments sometimes present in the juvenile stages in *Sphagnum* L. lack the growth patterns and hormonal responses found in other mosses (Goode, Duckett & Stead, 1993). Regardless of the genes involved in their development, current moss phylogenetic analyses (Cox *et al*., 2004) point to multicellular protonemata/rhizoid systems as an independent innovation.

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APPENDIX

Voucher information for all taxa used in this study. Voucher specimens are deposited in the following herbaria: NHM, Natural History Museum, London; DGL, private herbarium, D. G. Long, Royal Botanic Garden Edinburgh; JGD, private herbarium, J. G.

Duckett at the Natural History Museum, London. SP, S. Pressel, private herbarium at the Natural History Museum, London. SP/JGD, S. Pressel & J. G. Duckett private herbarium at the Natural History Museum, London.

Liverworts

Asterella abyssinica JGD; JGD & H W Matcham 6049 Jan 1995 South Africa.

A. australis JGD Oct Nov Dec 1999, Sept 2001 New Zealand.

A. bachmanii JGD Jan 1995, Jan 2012 South Africa.

A. muscicola JGD Jan 1995, Jan 2012 Lesotho.

A. tenera JGD Oct Nov Dec 1999, Sept 2001 New Zealand. SP/JGD Aug 2011 New Zealand.

A. wilmsii JGD Jan 1995, Jan 2012 Lesotho. JGD Jan 1992 South Africa.

Aitchisoniella himalayensis NHM July 1933 India.

Athalamia hyalina JGD June 2004 Italy. JGD 3 Aug 2005 USA. DGL 30889 India. SP/JGD Oct 2012 India.

A. pinguis DGL 30889 India. SP/JGD Oct 2012 India.

Blasia pusilla JGD Oct 1980, Aug 1990, 11 Nov 2006, Feb 2007 UK. JGD Aug 1995 USA.

Bucegia romanica NHM 10 Sept 1940, 19 Nov 1909 Rumania.

Cavicularia densa JGD Apr 2006 USA (ex *in vitro* culture).

Cyathodium cavernarum JGD Aug 1998 Uganda. SP/JGD Oct 2012 India.

C. foetidissimum JGD June 2003 Italy.

Conocephalum conicum JGD Aug 1972 France. JGD 5 Nov 1996, 24 Feb 2006 Italy. JGD 8 Apr 1965, 24 July 1967, 1 Apr 1973, 21 Jan 2002, 2 Apr 2004 UK. JGD 28 Mar 2007, 3 Apr 2007 USA.

C. salebrosum JGD 11 Nov 2006, 8 Dec 2006, 28 Mar 2007 UK. JGD 3 Apr 2007 USA.

C. supradecompositum SP/JGD Oct 2012 India.

Corsinia coriandrina JGD 3 Nov 1996. JGD 24 Feb 2006 Italy.

Cronisia fimbriata NHM 8903 Brazil.

Cryptomitrium oreoides JGD Jan 1994, Jan 1995, Jan 2012 Lesotho.

Dumortiera hirsuta JGD 12 Sept 2006 Chile. JGD July 1973 France. JGD 18 May 2005 Venezuela. JGD Aug 1966 UK. SP/JGD Aug 2012 UK.

D. hirsuta subsp. *nepalense* SP Dec 2011 China. SP Feb 2012 Vietnam. SP/JGD Aug 2011 Malaysia. SP/JGD Oct 2013, India.

Exomotheca holstii JGD Jan 1995, 2012 Lesotho.

E. pustulosa JGD Jan 1994 Lesotho.

Geothallus tuberosus JGD Aug 1995 USA (ex *in vitro* culture).

Lunularia cruciata JGD 2 Jan 2007 France, 23 Feb 2006. JGD 2 Nov 1996 Italy. JGD Jan 1982, Sept 1968, 10 Jan 2007, Dec 2012 UK.

Mannia angrogyna JGD 25 Feb 2006 Italy.

M. fragrans DGL 27059 China. JGD 28 Oct 2005 Germany.

Marchantia berteroana JGD 12 Aug 2006 Chile. JGD 18 May 2005 Venezuela.

M. debilis JGD Jan 2012 South Africa.

M. foliacea JGD 9 Jan 2005 Chile. JGD Jan 2000, Sept 2001 New Zealand.

M. paleacea JGD Jan 2012 South Africa.

M. pappeana JGD Jan 1991, Jan 1995 Lesotho.

M. polymorpha subsp. *polymorpha* JGD 24 Aug 1966, 15 Apr 1967, 23 Aug 1969, 19 June 2007 UK.

M. polymorpha subsp. *ruderalis* JGD Sept 1994, 20 Sept 1999, 10 June 2007 UK.

M. polymorpha subsp. *montivagans* JGD 11 Nov 2006, 8 Dec 2006 UK.

Neohodgsonia mirabilis JGD Jan 2000, Sept 2001 New Zealand. SP/JGD Aug 2011 New Zealand.

Monocarpus sphaerocarpus JGD Aug 1981 Australia. NHM June 1971 Australia.

Monoclea forsteri JGD Oct Nov Dec 1999, Jan Feb 2000, Sept Oct 2001 New Zealand. SP/JGD Aug 2011 New Zealand.

M. gottschei JGD 16 Sept 2006 Chile. JGD June 1998 Mexico. 16 May 2005 Venezuela.

Monosolenium tenerum JGD 28 Oct 2005 Germany (from aquarium), JGD Nov 2006 Japan.

Neohodgsonia mirabilis JGD Jan 2000, Sept 2001 New Zealand. SP/JGD Aug 2011 New Zealand.

Oxymitra cristata JGD Jan 1992, Jan 2012 Lesotho.

O. incrassata JGD 26 Feb 2006 Italy.

Pellia epiphylla JGD 4 Apr 2004, Sept 2006, 8 Dec 2006, 2 Feb 2007 Dec 2012 UK. JGD 20 Mar 5 2007, 3 Apr 2007 USA.

Peltolepis quadrata NHM July 1882 Norway. NHM 2 Aug 1876 Russia (Siberia). NHM Aug 1906 Switzerland.

Plagiochasma appendiculatum SP/JGD Oct 2012 India.

P. eximium JGD Jan1993, Jan 1995, Jan 2012 Lesotho. JGD Jan 1992, Jan 1993 South Africa.

P. rupestre JGD Jan 1992, Jan 1994, Jan 1995 South Africa. JGD Jan 1989, Jan 1996, Jan 2012 Lesotho.

Preissia quadrata JGD 28 Feb 2006 Italy. JGD 6 Apr 1973, Aug 1979 11 Nov 2006, 8 Dec 2006 UK.

Reboulia hemispherica JGD 15 Jan 2005, 8 Sept 2006 Chile. JGD May 2003, 23 Feb 2006 Italy. 27 Aug 1964, 3 Apr 2004, 8 Dec 2006 UK.

Riccia bifurca JGD Mar 1968 UK.

R. beyrichiana JGD 8 May 1971 UK.

R. canaliculata JGD 10 Nov 1972, 1 Aug 1978 UK.

R. cavernosa JGD June 1989, Jan 1994 Lesotho. 22 Oct 1967, 12 Oct 1969, 16 Sept 1970 UK.

R. ciliifera JGD Feb 2006 Italy.

R. crozalsii JGD 22 Feb 2006 Italy. 19 Mar 1968, June 2004 UK.

R. crystallina JGD Jan 1994 Lesotho. 6 May 1968, June 1989 UK.

R. fluitans JGD 1 Dec 1968 12 Oct 1969, 7 Dec 1969, Dec 2006 UK.

R. glauca JGD Apr 1972, Sept 1994, Apr 2003, Nov 2005 UK.

R. gougetiana JGD Apr 1994 France.

R. huebeneriana JGD Feb 1967 UK.

R. nigrella JGD 24 Feb 2006 Italy. JGD June 1989, Jan 1995 Lesotho. JGD Sept 2001 New Zealand. JGD Apr 1967, 19 Mar 1968 UK.

R. okahandjana JGD 24 Nov 2005 Botswana.

R. sorocarpa JGD 18 Mar 1968, 11 Nov 2006 UK.

R. subbifurca JGD June 1968, Sept 2004, Nov 2006 UK.

Ricciocarpos natans JGD Feb 1964 UK.

Riella americana JGD Aug 1995 USA (ex *in vitro* culture).

R. helicophylla JGD Aug 1970 Greece.

Sauteria alpina NHM 30 June 1870, June 1880 Switzerland.

Sphaerocarpos michelii JGD Nov 1996 Italy. JGD 7 Apr 6 May 1968 UK.

S. texanus JGD 6 May 1968 UK.

Stephensoniella brevipedunculata NHM Nov 1934. DGL 30890. India.

Targionia hypophylla JGD 28 Dec 2006 France. JGD Nov 1996, 24 Feb 2006 Italy. JGD Oct 1999, Feb 2000, Sept 2001, Aug 2011 New Zealand. JGD 4 Apr 1967, 5 May 1968, 23 Mar 1969, UK Nov 1996.

Wiesnerella denudata NHM Apr 1951 Japan. NHM 27 July 1953 Java. NHM 11 Apr 1899 Sikkim. DGL 30673 Nepal.

Mosses

Dawsonia superba JGD Oct 1999, Feb 2000 Jan 2001 New Zealand.

Dendroligotrichum dendroides JGD Oct 1999, Feb 2000, Jan 2001 New Zealand.

Hypopterygium filiculaeforme JGD Oct 1999, Feb 2000, Jan 2001 New Zealand.

Pogonatum macrophyllum JGD Jan 2001 Malaysia.

Polytrichum commune JGD Apr 2009 UK.

Ferns

Nephrolepis sp. JGD Apr 2009 UK.

Polypodium vulgare JGD Apr 2009 UK.