ON THE BRANCH PRIMORDIA IN NECKERA AND RELATED GENERA (BRYOPHYTA)

О ЗАЧАТКАХ ВЕТОЧЕК У *NECKERA* И БЛИЗКИХ К НЕЙ РОДОВ (BRYOPHYTA)

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Abstract

Foliose and filamentose structures around branch primordia in *Neckera* and related genera were described by different authors using different terms. This study applied both morphological and physiological criteria to elucidation homology of structures around dormant branch buds. An effect of exogenous abscisic acid was studied on *Exsertotheca crispa*, and morphological studies were conducted on species of *Neckera* with and without paraphyllia. A specifics of Neckeraceae is that the dormant branch buds are large and not very clearly delimited. Proximal branch leaves are often spaced from the inner part of primordium and subdivided into 2–4 separate parts, which are very similar to paraphyllia. However, unlike paraphyllia, the proximal branch leaves are defined as descendants of branch apical cell, possess phylotaxis and are arranged in a pellucid zone around branch primordium. A large size of dormant branch buds in *Neckera* and related genera is associated or maybe even defined by shape of the apical cell. The apical cell is relatively small and more deeply sinked in the stem tissue than in most other pleurocarpous mosses. The homology and terminology of various foliose and filamentose structures around branch primordia are discussed.

Резюме

Листовидные и нитевидные структуры, окружающие зачатки веточек *Neckera* и близких родов разные авторы понимали по-разному и описывали разными терминами. Для уточнения их гомологии мы использовали морфологические и физиологические критерии. Эффект воздействия экзогенной абсцизовой кислоты изучен на *Exsertotheca crispa*, а морфологическое изучение включало виды с парафиллиями и без них. Особенность многих видов Neckeraceae состоит в крупном размере зачатков веточек и не вполне четких их границах. Проксимальные веточные листья часто далеко отстоят от центральной части зачатка веточки, а также разделены на 2–4 отдельные доли, сходные с парафиллиями. В отличие от парафиллий, они происходят от апикальной клетки веточки, имеют филлотаксис и располагаются вокруг зоны тонкостенных клеток, окружающих зачаток веточки. Крупные размеры зачатков веточек *Neckera* и близких родов связаны, а возможно, и определяются формой апикальной клетки. Их апикальная клетка имеет сравнительно мелкие размеры и более глубоко погружена в ткань апикальной части стебля, чем у большинства бокоплодных мхов. Обсуждается гомология и терминология листовидных и нитевидных структур вокруг зачатков веточек.

KEYWORDS: bryophytes, branch development, Neckeraceae, apical cell, axillary hairs, paraphyllia, abscisic acid

INTRODUCTION

One-third century ago Rudolf M. Schuster (1988) published his view on the achievements and aims of bryologists. This overview was started with the question from his daugther, 18 years old, asking him: "In a world where so many exciting things are going on in science, how can you be bogged down in an eighteen century discipline?". Schuster's reply, the content of the paper, pointed how little we still know about bryophytes. Among others, Schuster noted a better understanding of main groups of Hepaticae rather than Musci due to more thorough morphological studies. The discordance of opinions on moss taxonomy Schuster explained as follows: "It is possible, due to the fact that such seemingly fundamental criteria as ramification pattern, branch origin and merophyte development and sequencing, criteria repeatedly analysed in the Hepaticae, have received rather little attention in mosses".

After a one-third century and already in twenty first century, Schuster's statement remains valid. The designation of the branching pattern, cauline vs. axillary, as a fundamental character for the differentiation mosses into acrocarpous and pleurocarpous (Buck & Vitt, 1986) was

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not continued, and not mentioned in, e.g., general moss classification (cf. Goffinet *et al.*, 2009). Similarly, the classification of branching patterns suggested by Akiyama (1990) and Akiyama & Nishimura (1993), with subdivision of branch buds into *Bryum*-type and *Climacium*-type is almost never used in taxonomic descriptions.

A big step forward in the study of regulation of branching in mosses has been done by Bell & Newton (2007), Bell *et al*. (2007), and Coudereit *et al*., (2017), who developed a terminology for the description of the moss architectural diversity. The latter however did not spread widely through taxa circumscriptions.

Spirina *et al*. (2015, 2020) recently demonstrated how unstable foliose structures around branch primordia in some genera of mosses are. Interestingly, the better development of paraphyllia appeared to be accelerated by simple phytohormone, the abscisic acid (ABA) (Spirina *et al*., 2020).

The present study continues such observations, with the focus on the genus *Neckera* s.l. It was selected for these studies because it includes species with abundant paraphyllia, e.g. *Neckera menziesii* Drumm.*,* whereas for most species of this genus there is no consistency between authors regarding paraphyllia and pseudoparaphyllia. For example, *Neckera pennata* Hedw. is described as lacking paraphyllia (Limpricht, 1895), but as "paraphyllia present or absent" (Allen, 2014), or "usually absent", but without any descriptions (Smith, 2004; Sastre-De Jesús, 2014). Even for *Neckera menziesii,* Guerra (2014) described it as having numerous branched pseudoparaphyllia, and illustrated them by structures subidentical to those named paraphyllia by Lawton (1971), Sastre-De Jesús (2014) and many other bryologists in the past.

Pseudoparaphyllia in Neckeraceae were in the focus of the study of Cubero *et al.* (2006) who provided a careful count of them for European and Macaronesian species of *Neckera* Hedw. These authors followed Akiyama (1990) and Akiyama & Nishimura (1993) in the separation of the foliose structures around branch primordia into two types: (1) pseudoaraphyllia, and (2) *scaly* (fide Akiyama & Nishimura, 1993) or *juvenile* (fide Enroth, 1994) leaves. Enroth (1994) defined the latter by the presence of phyllotaxis, unlike pseudoparaphyllia, a trichome-like structures, i.e. outgrowth of epidermis that lack phyllotaxis. For practical separation of the scaly leaves and pseudoparaphyllia, Cubero *et al.* (2006) used a criterion suggested by Newton & De Luna (1999), who assigned to pseudoparaphyllia structures originated from dark, thick-walled cells, whereas scaly leaves appear on the pale, thin-walled cells around a bud. In the present study we attempted to combine these two criteria: phyllotaxis and position in the area of thin-walled cells; for being short, the latter is called 'pellucid area' (Fig. 1A).

Since the phyllotaxis of foliose structures around branch primordia follows the sequence of divisions of the branch apical cell (Ruland, 1924; Bertier, 1971; Ignatov & Hedenäs, 2007; Ignatov & Spirina, 2012; Spirina & Ignatov, 2005, 2008; Spirina *et al.*, 2015, 2020), we see no reason to use additional terms, e.g. 'scaly' or 'juvenile' leaves, using just 'branch leaves'. However, as they are morphologically the earliest branch leaves, they are named proximal (to stem) branch leaves. Some of them are subdivided into several independent units in the course of their development, as was shown, e.g. for *Hypnum cupressiforme* Hedw. (Spirina & Ignatov, 2008). The proximal branch leaves that are composed of two or more lobes subdivided to the stem level are called here as compound proximal branch leaves (Fig. 1B).

The genus *Neckera* is understood here in a broad sense, including not only species approved in this genus by molecular phylogenetic studies. Olsson *et al*. (2011) segregated several lineages, but many species used for this study were not tested for DNA sequencing yet, thus their generic placement remains uncertain. One species, *Exsertotheca crispa* (Hedw.) S. Olsson, Enroth & D. Quandt (≡*Neckera crispa* Hedw.) was used for ABA experiments as living plants*,* while others were studied for the branch primordia morphology using herbarium material.

MATERIAL AND METHODS

ABA experiments

Exsertotheca crispa was collected in July 2021 in the Caucasus, near Black Sea coast, where this species is a

Fig. 1. Branch primordia of *Neckera douglasii* (A: USA, California, Ignatov, MHA 9065801) and *Exsertotheca crispa* (B: Russia, Caucasus, Kartasheva, MHA 9132141, specimen from ABA experiment). A: dormant branch bud is surrounded by pellucid area flanked with narrowly lanceolate foliose structures keeping standard phyllotaxis with first leaf in four o'clock position. B: dormant branch bud surrounded by compound proximal branch leaves. Numerals mark the order of proximal branch leaves, denoting whole leaves as well as parts of compound proximal branch leaves. Scale bars: 50 μm.
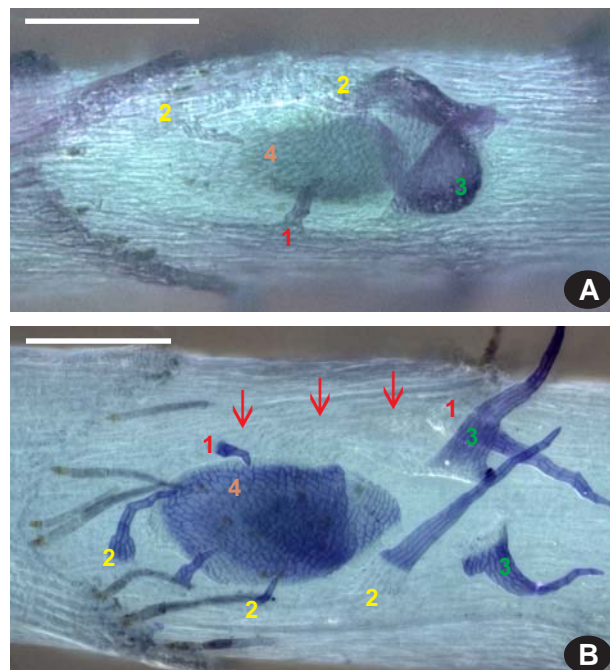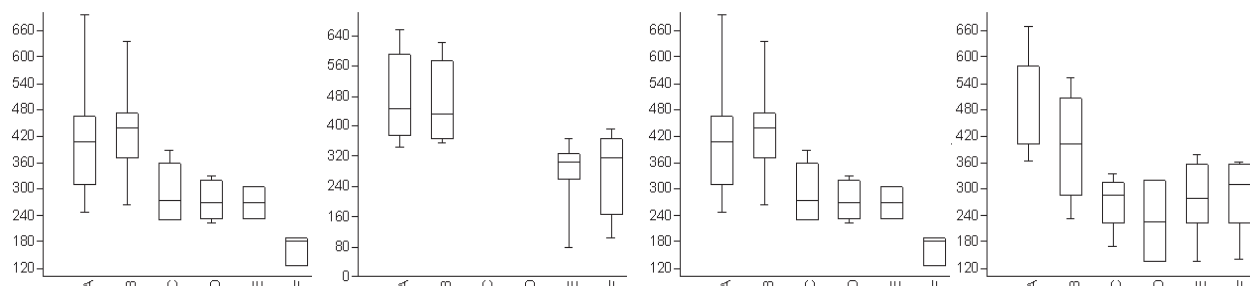
Fig. 2. *Exsertotheca crispa,* results of cultivation experiments with ABA (bars A,B), control (bars C, D) and ABA+fluridone (bars E, F). Two bars for each variant are resulted from two different Petri dishes. Four graphs show series with four groups of plants from different localities. Axis Y is the length of proximal branch leaves in μm.

common epiphyte (Appendix 1). Plants were brought to the laboratory, placed in Petri dishes (diameter 9 cm), ten well developed shoots in each dish. They were put on one layer of filter paper moistened with 5 ml of distilled water or an aqueous solution of ABA (Sigma, Germany), at a concentration of 0.1 mmol, which was found to be adequate for similar experiments by Spirina *et al.* (2020). Petri dishes were placed in a Sanyo Environmental Test Chamber MLR-352H: temperature + 7°C/+ 12°C (night/day), light period 10 hours, PPFD – 14 mmol/m^{-2}s-1, for 28 days.

After 28 days of cultivation, all plants where the shoot increment was clearly seen were selected for further morphological observations.

Fluridone Test

To make sure that exogenic ABA infuences the morphogenesis, we used a test with fluridone, an ABA biogenesis inhibitor (Popova, 1995; Velini *et al.*, 2010; Shu *et al.*, 2017) that may reduce the content of the free form of ABA by up to 40% (Stetsenko *et al.*, 2015). Fluridone (Sigma, Germany) at a concentration of 15 mmol was applied in 1 ml quantities to samples cultivated with ABA.

Statistics

The effects of ABA and fluridone were evaluated by an ANOVA test in PAST (Hammer *et al.*, 2001).

Morphological observation

Stems on a glass slide were covered by another slide, allowing us observations and photography of both sides of the stem, using objective lens 10x. All leaves were removed from the stems with thin tweezers. The stems were stained with fuchsine (Ziehl-Nelson carbol fuschine diluted 1:5 by distilled water, for 5–10 minutes). The stems were examined under the compound light microscope Olympus-CX41 and photographed with a digital camera Infinity 2-2.

Plants from herbaria were stained with fuchsine and indigocarmin as follows. Herbarium specimens were moistened in distilled water for 24 hours, then leaves were detached and stems were put in Ziehl-Nelson carbol fuschine diluted 1:5 by distilled water for 5–10 minutes. Then samples without washing were put in 0.5% solution of indigocarmine in saturated water solution of picrine acid, for 10–15 minutes. Then plants were washed

Table 1. Number of stems of *Exsertotheca crispa* from four localities in three series of experiment: with ABA, control (Ctrl), and ABA+fluridone (A+f).

Series	ABA	Ctrl	A+f
1	34	20	32
2	39	19	38
3	36	19	42
4	35	20	36

Table 2. Stem increment and mean number of newly formed branch buds per stem in *Exsertotheca crispa* in three series of experiment. Table shows T-test values of the difference between series.

Series	n	Mean	nABA	nCtrl
		stem increment, mm		
nABA	135	3,29		
nCtrl	73	2,36	9,5×10^{-5}	
nA+f	140	1,86	1,0×10^{-23}	0,0046
		number of newly formed buds		
nABA	135	2,49		
nCtrl	73	1,53	6,7×10^{-9}	
nA+f	140	1,00	4,7×10^{-15}	0,8033

Table 3. Number of parts of first and second proximal branch leaves (as explained in Fig. 1) in three variants of cultivation: with ABA, control (Ctrl), and ABA+fluridone (A+f); newly formed increment has prefix n, previously grown plant part with prefix o. Table shows T-test values of the difference between series.

| Series	n	meanN	nABA	nCtrl	nA+f	oABA	oCtrl
		first proximal branch leaf					
nABA	47	2,15					
nCtrl	24	1,29	1,6×10^{-5}				
nA+f	20	1,65	0,0162	0,0762			
oABA	77	1,28	1,3×10^{-7}	0,9446	0,0324		
oCtrl	61	1,35	1,8×10^{-6}	0,7207	0,0852	0,5364	
oA+f	77	1,22	1,8×10^{-8}	0,6045	0,0126	0,4628	0,2037
		second proximal branch leaf					
nABA	46	2,04					
nCtrl	19	1,37	0,0002				
nA+f	19	1,68	0,0730	0,1084			
oABA	77	1,27	2,9×10^{-7}	0,4606	0,0203		
oCtrl	56	1,27	4,7×10^{-7}	0,4723	0,0220	0,9971	
oA+f	62	1,22	9,2×10^{-8}	0,2836	0,0112	0,6129	0,6367
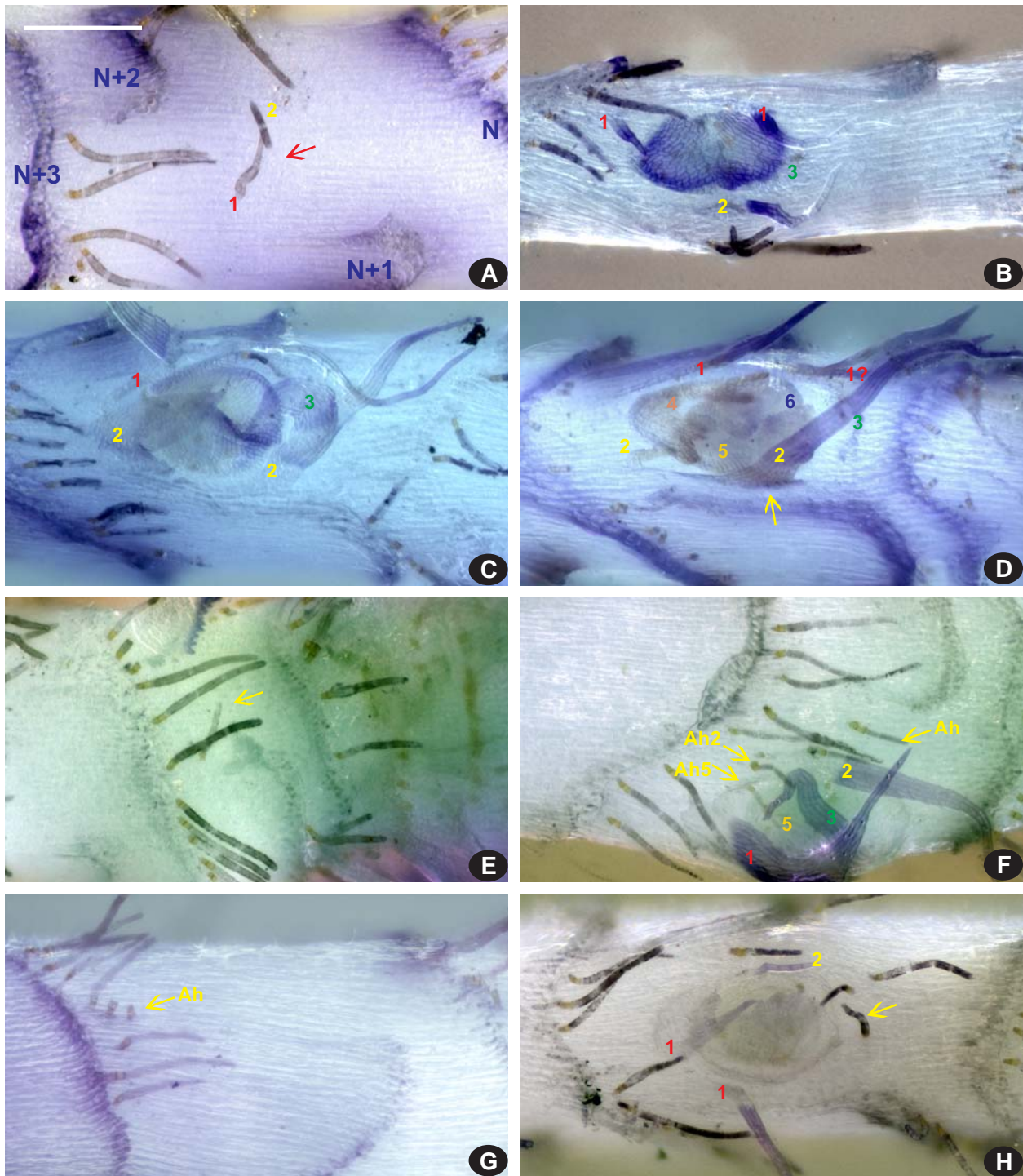
Fig. 3. *Exsertotheca crispa*: from various experiments of cultivation in Petri dishes (**A–E**: ABA; **F–H**: ABA+fluridone). Photographs from the apical part of stem grown during 28 days of the experiment. Numerous axillary hairs are well stained by fuchsine. Lower parts of leaves remain colorless. A: two- and three-celled uniseriate structures in a position, where branch primordium is normally developed (arrowed); B–D: proximal branch leaves are compound; F: proximal branch leaves are entire; F–H: anomalous position of axillary hairs (arrowed): in 'E': in a position, where branch primordium is normally developed; 'G–H': along the border of the bud (as it can be extrapolated from e.g. 1B). In 'F': Ah2 and Ah5 mean axillary hairs in axils of second and fifth leaves. Scale bar 50 µm for all. Sequence of stem leaves is shown in A (N to N+3).

by 30% ethanol up to light green color of subapical part of shoots, and saved in 30% glycerol with antiseptics, for subpermanent preservation and photographing in a same way as plants from ABA experiments. Measurements were done from digital images, consulting a respective shoots on glass slide in uncertain cases. Herbarium specimen studies were largely from MHA, their list is in Appendix 1.

Anatomy sections

As *Exsertotheca crispa* was the only species studied by living plants, not as herbarium material, anatomical studies were conducted with this species to elucidate the structure of its apical cells and branch primordia. Anatomical sections were done in the upper parts of shoots after removal of leaves by thin tweezers, fixed in a 2.5% glutaraldehyde solution for 7 days, post-fixed in a 1% OsO_4 water solution for 3 hours, washed in water. Then they were dehydrated in an alcohol series (20%, 40%, 60%, 80% and 96% alcohol), alcohol+acetone mixture (1:1), and acetone for 1 hour in each solution, soaked in an acetone–resin mixture series (3:1, 1:1, 1:3) for 12, 24 and 3 hours respectively, and embedded in epon-araldite resin using the protocol of the manufacturer. The resin was polymerized at 60°C for 24 hours. Serial longitudinal, transverse, and oblique sections were cut 2 μm thick with glass knives, placed on glass slides without mounting medium, stained with 0.01% berberine or its combination with DAPI and scanned under a laser scanning confocal microscope Olympus FV-1000 based on Olympus BX61, using a combinations of 405 and 473 nm lasers. Z-stacks of several scans were usually obtained and are presented here.

Some specimens from previous projects were added to the morphological and anatomical studies.

Results

ABA effect on branch primordia in Exsertotheca

Collections of *Exsertotheca crispa* from Sochi area in the Caucasus, from two closely situated localities (listed in Appendix 1), two gatherings in each from a slightly different epiphytic habitats were included in the experimental studies (Table 1). They were studied separately, but gave a similar result (Fig. 2), thus other data on their measurements are presented for concatenated dataset.

Cultivation of *Exsertotheca* with ABA resulted in a longer stem increment compared to control and ABA +fluridone, thus the number of dormant buds on these newly grown stem portions was larger (Table 2).

Visual observations of plants taken after 28 days of cultivation with ABA revealed several differences in dormant bud structure from plants before cultivation, as well as from plants cultivated without ABA and with ABA+fluridone. This defined three characters for further quantitative studies. Measurements were conducted for: (1) length of longest separate foliose structures near branch primordia (proximal branch leaves or their separate parts, cf. Fig. 1B); (2) area of the branch primordium, approximated as ellipse of respective length and width, and (3) number of separate parts of proximal branch leaves (cf. Table 3).

The length of proximal branch leaves gave a statistically significant difference for all measurements. Moreover, in each series done for plants from four different localities, the plants cultivated with ABA had longer proximal branch leaves or their parts (Fig. 2).

The mean area of the bud in plants cultivated with ABA was larger, 74106 μm², against 65141 μm² in control and 68771 μm² in ABA+fluridone, however this difference was not statistically significant due to too broad variation, and in some series the mean area values were almost the same.

The number of parts into which the first (outermost) and the second proximal branch leaves are subdivided increased after ABA treatment (Table 3). The only case of the absence of differences was found between the series of ABA and ABA+fluridone. This exception however, can be explained: fluridone is easily decomposed by light, and in previous studies we added it several times during the experiment (Spirina *et al*., 2020). However, in this study the plants treated by ABA+fluridone had in the first week of cultivation so week increment that in view of risk to obtain a zero increment we cancelled adding fluridone to the medium. Thus the ABA +fluridone treatment shows inhibiting effect for some characters (e.g. for stem increment, Table 2), while for other characters it possesses just a weakened effect of ABA.

The proximal branch leaves were found quite variable in plants from each series of the experiment. They are either entire (Fig. 3F) or more commonly subdivided into separate parts, thus forming compound leaves (Fig. 3C–D). The fact that these parts belong to one leaf is sometimes obvious (e.g. leaf #2 in Fig. 1B, leaf #2 in 3C) as its parts are arranged one by one in a row along the border of the ovate area around the bud with more or less conspicuous outlines. In other cases, their belonging to one leaf is not so apparent (e.g. leaf #1 in Fig. 1B, leaf #1 in Fig. 3B), requiring tracing their position on the same line. The help for their interpretation often comes from the phyllotaxis which follows the divisions of the tetrahedral apical cell to three sides, with first outermost foliose structure being situated in a lateral position. The bases of parts of compound proximal branch leaves sometimes form a low ridge-like structure outlining the pellucid area around branch bud (Fig. 3D).

Despite of this variation, all clear views of foliose structures around branch primordia show the arrangement which agrees with its interpretation as a phyllotaxis. No paraphyllia with indefinite position as e.g. in Fig. 4E were noticed.

The only example of "generation" of paraphyllia is shown in Fig. 3A. Two small filamentous structures appeared in a position normal for branch primordia, but
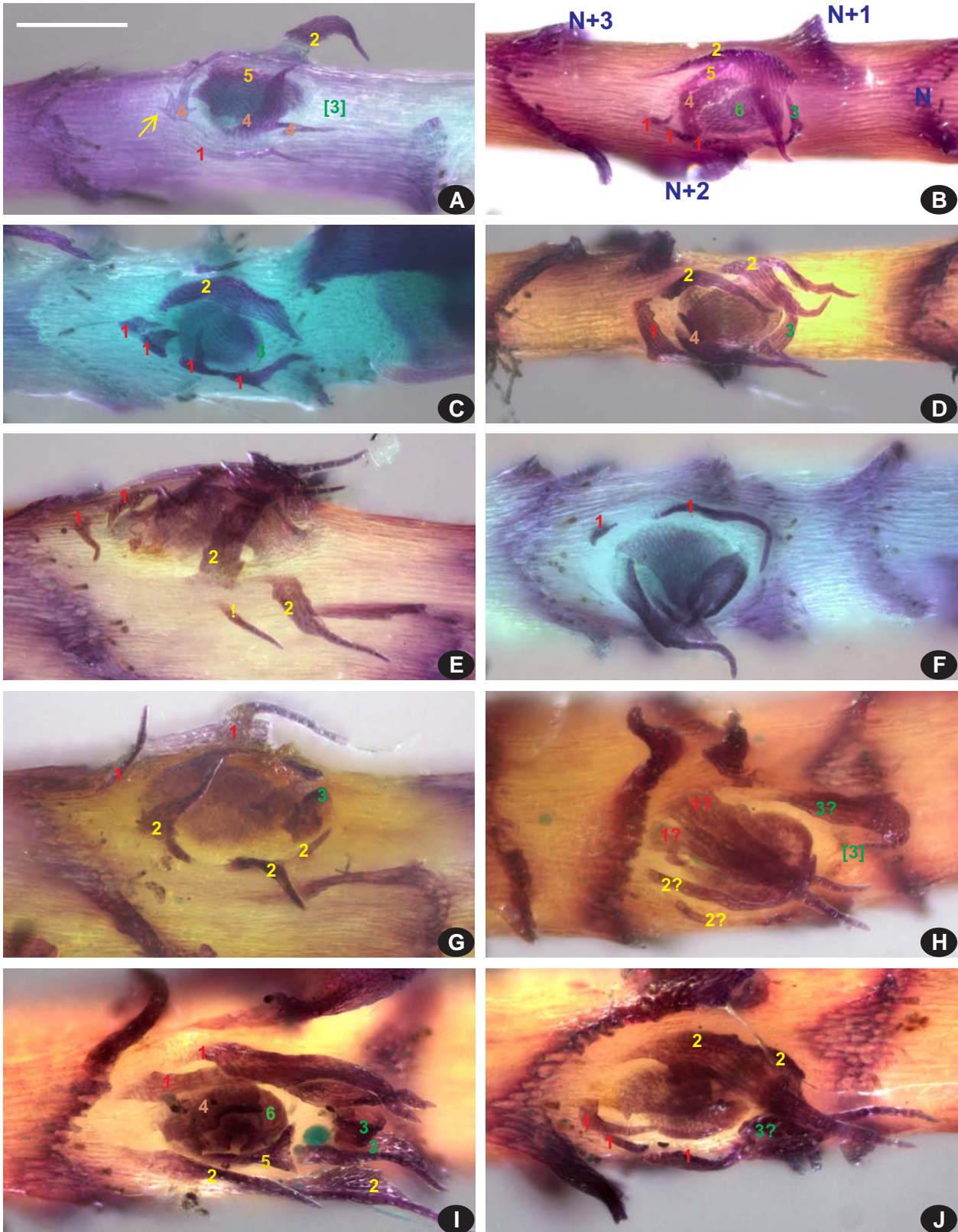
Fig. 4. Dormant buds of *Neckera douglasii* (A–D) and *Neckera humilis* (E–J) showing variation. Numerals mark the order of proximal branch leaves, denoting whole leaves as well as their parts. Sequence of stem leaves is shown in B (N to N+3). Scale bar 50 µm for all. (A–C: USA, California, Ignatov, MHA9065796; D: USA, California, Ignatov, MHA9065795; E–G: Japan, Honshu, Deguchi 38346, MHA9065813; H–J: Japan, Nichinan, Ignatov & Ignatova 98-546, MHA9065812). Scale bar 50 µm for all.

without anything on the stem surface in between them (Fig. 3A). Noteworthy in this case is that these structures are arranged in positions about four o'clock and eleven o'clock, common for first and second proximal leaves in pleurocarpous mosses (Ignatov & Hedenäs, 2007). Although this is the only one observation in the ABA series and none in other series, a similar case in previous studies (Spirina *et al.*, 2020) makes it worth to mention and it will be discussed below.

Interesting is the fact that axillary hairs occasionally occur in positions others than axillary (Fig. 3F–I) in all series of experiment, in near ca. 5% of the studied buds. They are usually arranged in the periphery of the 'internode', farer from the branch apical cell than the 'ring' (imperfect one) of the proximal branch leaves (Fig. 3E, H).

Size and shape of the buds in *Exsertotheca* differs from that in most pleurocarpous mosses in being larger and more or less flat, thus their edges are not definite. However, our observation did not reveal structures other than proximal branch leaves. No paraphyllia were detected, despite recent literature does not state that they are absent, describing situation for the whole genus *Neckera* (including *Exsertotheca*) as only "usually absent" (Smith, 2004) or "from lanceolate to absent" (Guerra, 2014). Old literature described species of *Neckera* (except *N. menziesii*) as lacking paraphyllia (Limpricht, 1895).

Morphology of branch primordia and other stem surface structures in other species of *Neckera*

For comparison with *Exsertotheca* we took seven species (cf. Appendix 1), for which the dormant buds were photographed and analyzed for the position of the proximal branch leaves (Figs. 4–5). Selected illustrations are provided and commented here to present the whole range of intraspecific variation of structures called "paraphyllia", "pseudoparaphyllia", "scaly leaves", juvenile leaves", which we call proximal branch leaves. Two of studied species are commonly described as having paraphyllia – *N. menziesii* and *N. andina* Mitt. One species, *N. douglasii*, was reported by Lawton (1971) as lacking paraphyllia, and another species, *N. humilis* Mitt*.,* was described as having pseudoparaphyllia, sometimes numerous (Wu, 2011).

Neckera douglasii (Fig. 4 A–D)

Pellucid area around the bud is rather clear (Fig. 4B) or only moderately so (Fig. 4E) and the branch area thus falls inside the ring of outermost proximal branch leaves. The latter are either entire (e.g. #2 in Fig. 4A and Fig. 4C), or subdivided into two to four parts. More parts are usually seen in the first and second proximal branch leaves, although the progressive diminishing of the number of leaf parts is not a strict rule: bud shown in Fig. 4A has more strongly divided fourth leaf, and in addition to being compound, one of its part (arrowed) is strongly incised.

Neckera humilis (Fig. 4 E–J)

Pellucid area around the bud is usually apparent, in some cases almost reaching the axil of the N+3 leaf (Figs.

4E, I). Outermost proximal branch leaves are mainly arranged along the border of the pellucid area. They are often compound and their parts are well spaced one from another, so it is sometimes difficult to find out that two or more foliose structures are parts of the same leaf, and some assignings can be challenged (Fig. 4H). One foliose structure marked '!' in Fig. 4E stands behind the circle in which other proximal branch leaves are arranged, thus representing paraphyllium of *Leskea*-type as was defined by Spirina *et al.* (2020). Dormant branch buds in *Neckera humilis* look more variable in shape and size of proximal branch leaves compared to *N. douglasii*: some of them are very long and their phyllotaxis is sometimes questionable.

Neckera andina (Fig. 5A–D)

This species has abundant paraphyllia, and their arrangement in many parts of stem is irregular. Paraphyllia are more numerous closer to branch primordia and some clusters of them occur in the place where the bud typically develops but is not always apparent. The pellucid area around branch primordia is inapparent. The transitions between branch leaves on the base of branch, compound proximal branch leaves and paraphyllia are gradual, thus in many cases separation of these structures is arbitrary. This can be demonstrated by a series shown in Figs. 5A–D. In buds with proximal branch leaves broadly ovate, the paraphyllia are often fewer and their arrangement more or less agrees with the usual phyllotaxis of proximal branch leaves (Fig. 5A). The bud with the narrower proximal branch leaves is shown in Fig. 5B. The proximal branch leaves have an apparent phyllotaxis and they are situated somewhat apart from the 'inner' part of the bud. A moderately abundant subfilamentous paraphyllia are situated mostly behind the large lanceolate proximal branch leaves (Fig. 5B). Further variant looks as a rather large cluster of thin filamentous to narrowly lanceolate paraphyllia, with broadest of them being in central position (Fig. 5C); sometimes a raised structure, apparently equivalent of the inner part of bud, is discernable between these broadest foliose structures, but sometimes it is hard to say if it exists. The phyllotaxis of such broadest foliose structures is usually inapparent, thus their identity and terminology are losing a rational explanation: such cases represent simply an intermediates between proximal branch leaves and paraphyllia. Finally, the area between corners of the leaves N+1 and N+2 possesses a number of filamentous structures (Fig. 5D). Sometimes some of them in the centre are slightly larger, while often they are all subidentical. A characteristic feature of such assemblages is that some paraphyllia are pointed upwards and some downwards. The same pattern is characteristic also for *Neckera californica* Hook. & Arn. (not shown here, but illustrated by Ignatov & Hedenäs, 2007), and sometimes for another genus of the Neckeraceae, *Leptodon smithii* (Dicks. ex Hedw.) F. Weber & D. Mohr (Fig. 6). Also worthy is a comparison with the case shown for
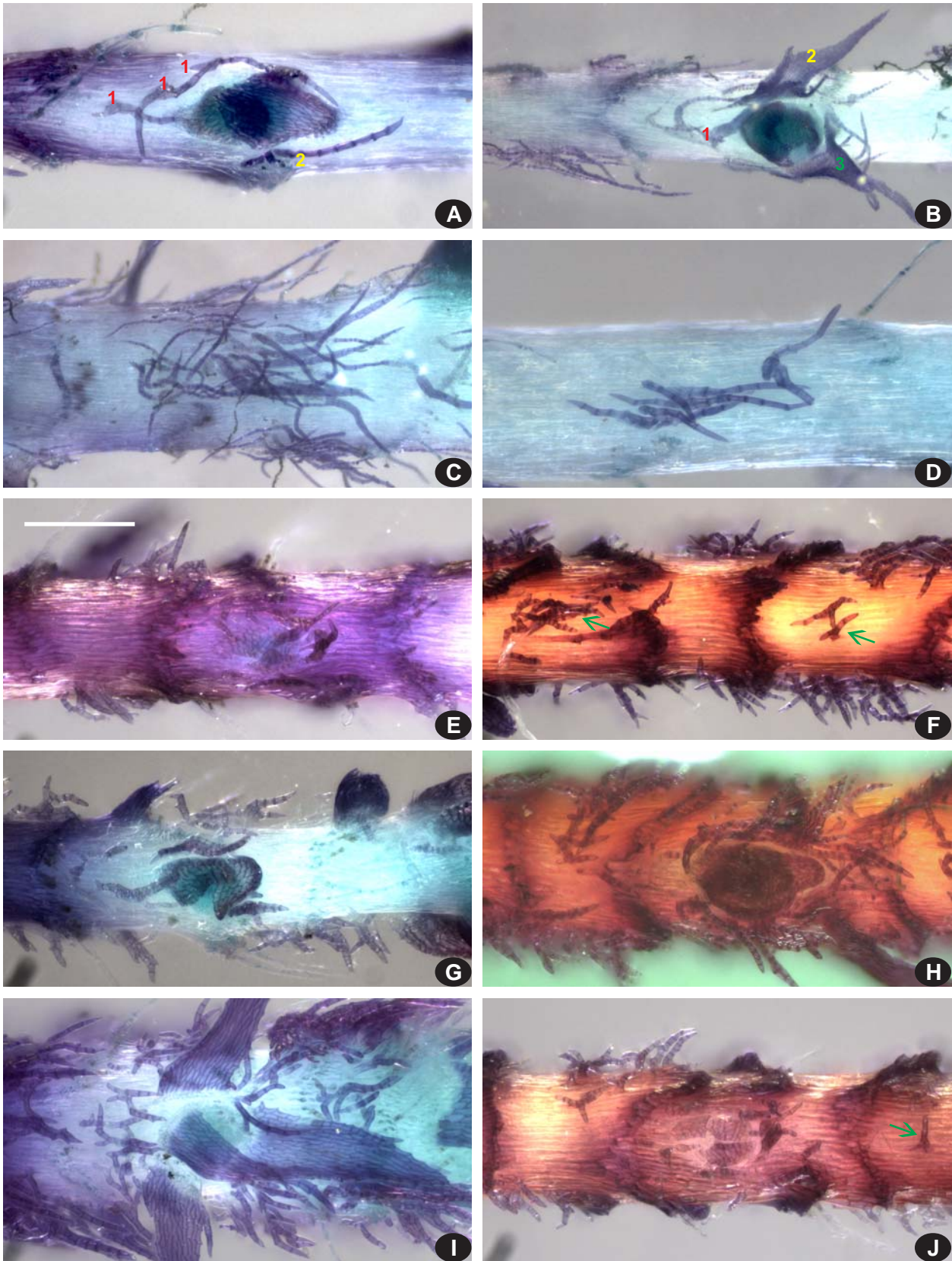
Fig. 5. Dormant branch buds and paraphyllia of *Leskea*-type in *Neckera andina* (A–D) and *Neckera menziesii* (E–J), showing variation. Numerals mark the order of proximal branch leaves where they are well seen. Scale bar 50 µm for all (A–D: Peru, Hegewald, Bryoph. Neotropica Exs. #120, MHA9065787; E, G, H: France, Gardet, MHA9065814; I: USA, California, Ignatov, MHA9062364; B, J: USA, California, Ignatov, MHA9062366). Note paraphyllia occurrence in the position where the branch primordium may occur in 'E' and 'J', arrowed.

Exsertotheca in Fig. 3A, where short filamentous structures appear in between corners of the leaves N+1 and N+2 (where N is mother leaf of the area, cf. Fig. 4B).

Neckera menziesii (Fig. 5E–J)

Pellucid area around bud is apparent in younger stems (Fig. 5E, G, I), bluish or pinkish of fuchsine, not brownish of indigocarmin (Fig. 5F, H, J). The border of pellucid area in some young shoots is the place of foliose structures, traditionally referred to pseudoparaphyllia. Having a position identical with the proximal branch leaves in the above discussed species (Figs. 3–4), foliose structures around the buds of *N. menziesii* are arranged more evenly and it is usually impossible to attribute to them numbers of leaves (= numbers of the branch merophytes). Paraphyllia of *Leskea*-type are numerous around the bud and near. Sometimes they occur shortly above the leaf axil, in a position where the branch bud is usually developed (arrowed in Fig. 5F, J). In thick stems, the paraphyllia are arranged partly intermingled with the larger proximal branch leaves that form an imperfect ring around the bud. These outermost large lanceolate proximal branch leaves have at their bases large teeth or sometimes small lobes (Fig. 5I). The abundance of paraphyllia close to branch primordia is obvious. *Neckera menziesii* was circumscribed by Lawton (1971) as a species with paraphyllia; Guerra (2014) mentioned numerous branched pseudoparaphyllia, while paraphyllia were mentioned by him as occurring in the genus, without mentioning them for particular species.

Neckera chilensis Schimp. is similar in paraphyllia presence to *N. humilis*, while *N. himalayana* Mitt. and *N. flexiramea* Cardot to *Exsertotheca crispa*.

The anomalous size and position of the dormant buds inspired us to look for the explanation of their structure, or at least characters that are associated with these unusual features. We undertook anatomical sections of buds of *Exertotheca* (Figs. 7A–D, 8–11) and also compared the bud position in *Neckera* and other groups of pleurocarps (Figs. 7E–H).

Fig. 6. Paraphyllia of *Leskea*-type in between leaves N+1 and N+2 on the stem of *Leptodon smithii*. SEM image (from the series studied and discussed by Ignatov & Hedenäs, 2007). Note that some foliose structures are pointed upwards and some downwards, cf. also with Fig. 5D.

50 µm
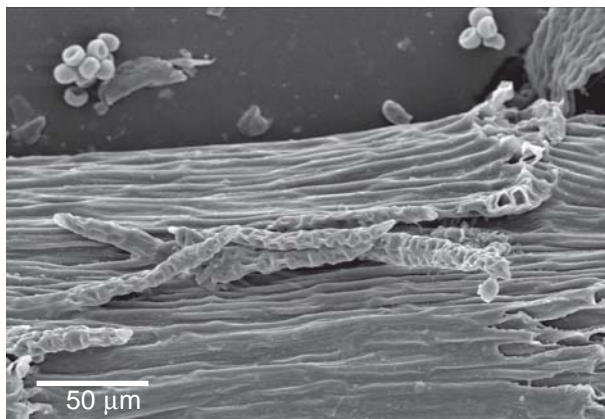
Anatomy of apical cell

Using living material from the experimental studies, a number of shoots from the series with ABA treatment and control were embedded in resin medium and cut by 2 µm sections. Longitudinal sections through the stem apical cell are shown in Figs. 7A–D. Four pictures (A–D) that perform only 8 µm are the only place where the apical cell is seen. Further sections have shown it without upper exposing surface, which width is 11 µm in the widest part (Fig. 7C). Available images of the pleurocarpous moss apical cells (Figs. 7E–H), taken from our previous studies, show that the of apical cell looks very different. <u>The apical cell is discussed here in a traditional sense, and it is usually seen in light microscope where already thickened walls are clearly seen as a large tetrahedral-obovate cell. LCSM (Fig. 7F) and TEM (Fig. 7G) images demonstrate that the apical cell *sensu lato* includes in addition to the apical cell *sensu stricto* just cut-tin off merophytes with imperfectly formed cell walls.</u>

The apical cell of *Exsertotheca* differs from AAC of representatives of other families of pleurocarpous mosses in its shape: is it widest in the middle, contrary to widest well above the middle in other groups (Fig. 7E–H). This shape seems to be associated or even defining the mode of division of the first merophyte, which first division forms the inner cell (II in Frey's (1971) terminology) much larger than outer cell (I in Frey's terminology) (Fig. 7A). In the classical scheme of Frey (1971) the cells I and II (results of the first division of a merophyte cell) are subequal. The same is a rule for most pleurocarpous mosses, or even if the inner cell is larger as in *Brachythecium* (Fig. 7G), the outer cell is not so small as in *Exsertotheca crispa* (Fig. 7A–D). This shape of AAC of *Exsertotheca* seems also to correlate with the leaves near its apex that are already more developed, often 2–3 cells long, whereas in other mosses only a unicellular raisings occur at this distance from the apex.

The cells II are actively divided, forming numerous medullar cells (or mothers of medullar cells), and the stem apical zone has blunt, not acute general outline (Fig. 7A). Branch apical cell (Figs. 8–11) are similar to stem apical cell in rather small size, being broadest well below the surface.

Branch primordia that originated in the apical part of the stem are inconspicuous because of rather small size, which is shown in Fig. 8 by selected sections of a complete series. Apical cell is 16–20 µm long, and, similarly to the stem apical cell, is narrower at apex, 13 µm (Fig. 8-0), and broadest in its middle, to 22 µm (Fig. 8-8 and 8-10). At 20 µm and even at 16 µm below the branch primordium apex (Figs. 8-16, 8-20), the apical cell is guessed without confidence.

Another series (Fig. 9-2–9-18) performs sections transverse and somewhat oblique to the branch primordium axis. This bud was situated farer from the stem apex than that shown in Fig. 9, at 0.2–0.3 mm. It is more developed,
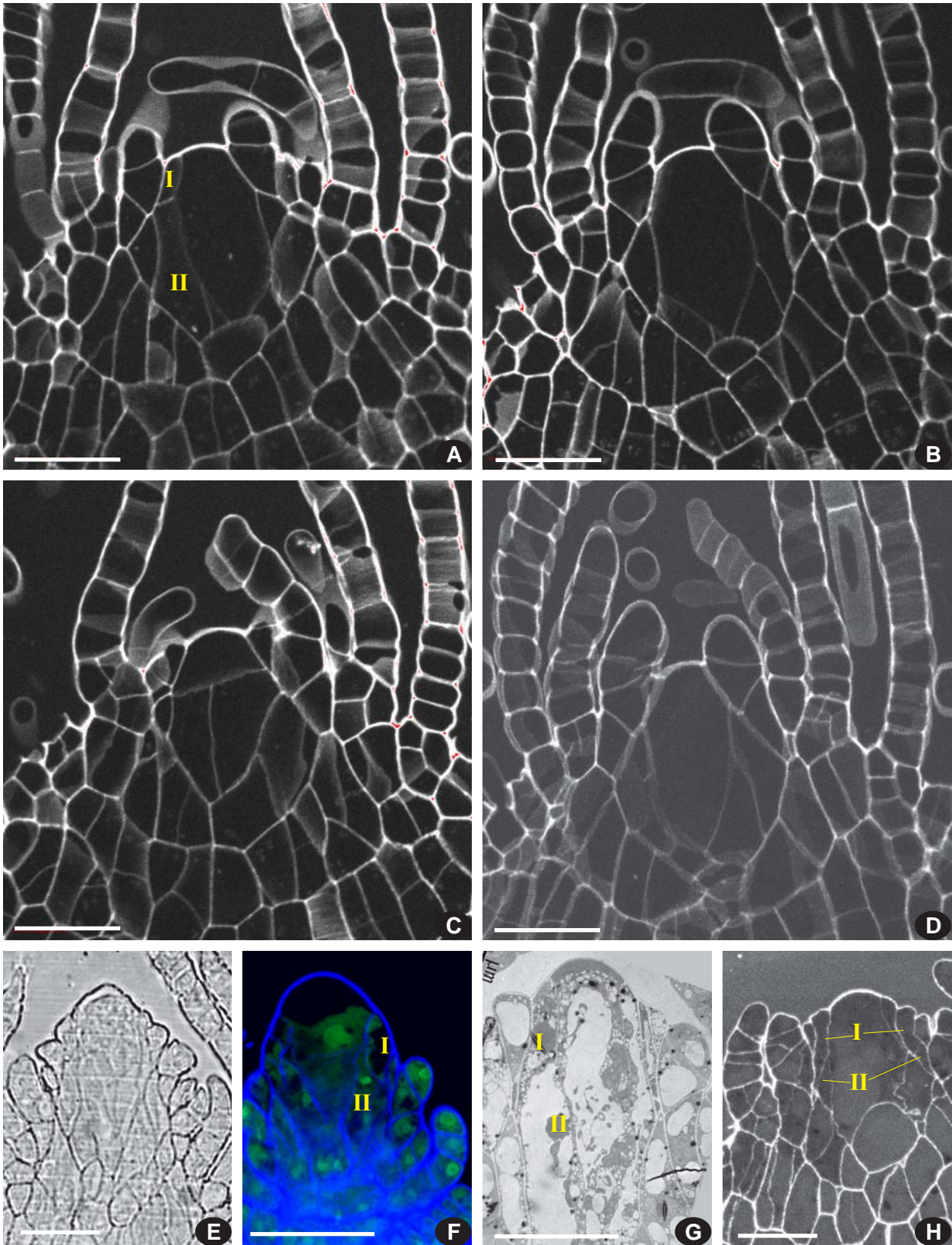
Fig. 7. Apical cells of *Exsertotheca crispa* (A–D), and their comparison with representatives of other families of Hypnales: *Hypnum cupressiforme* (E), *Fontinalis antipyretica* Hedw. (F), *Brachythecium rutabulum* (Hedw.) Schimp. (G), and *Weymouthia cochlearifolia* (Schwägr.) Dixon (H). Four sections of *Exsertotheca* comprise complete series of 2 μm sections where the stem apical cell is presented. Note that the apical cell of *Exsertotheca* is more deeply immersed in the stem tissue (a somewhat similar aspect has only *Weymouthia*, though less obvious). Scale bars are 20 μm for all. (A–D, H: LCSM, berberine staining; E: light microscopy of longitudinal section, osmius tetroxide fixed; F: LCSM, FITC and fluorescent brightner staining; G: TEM.
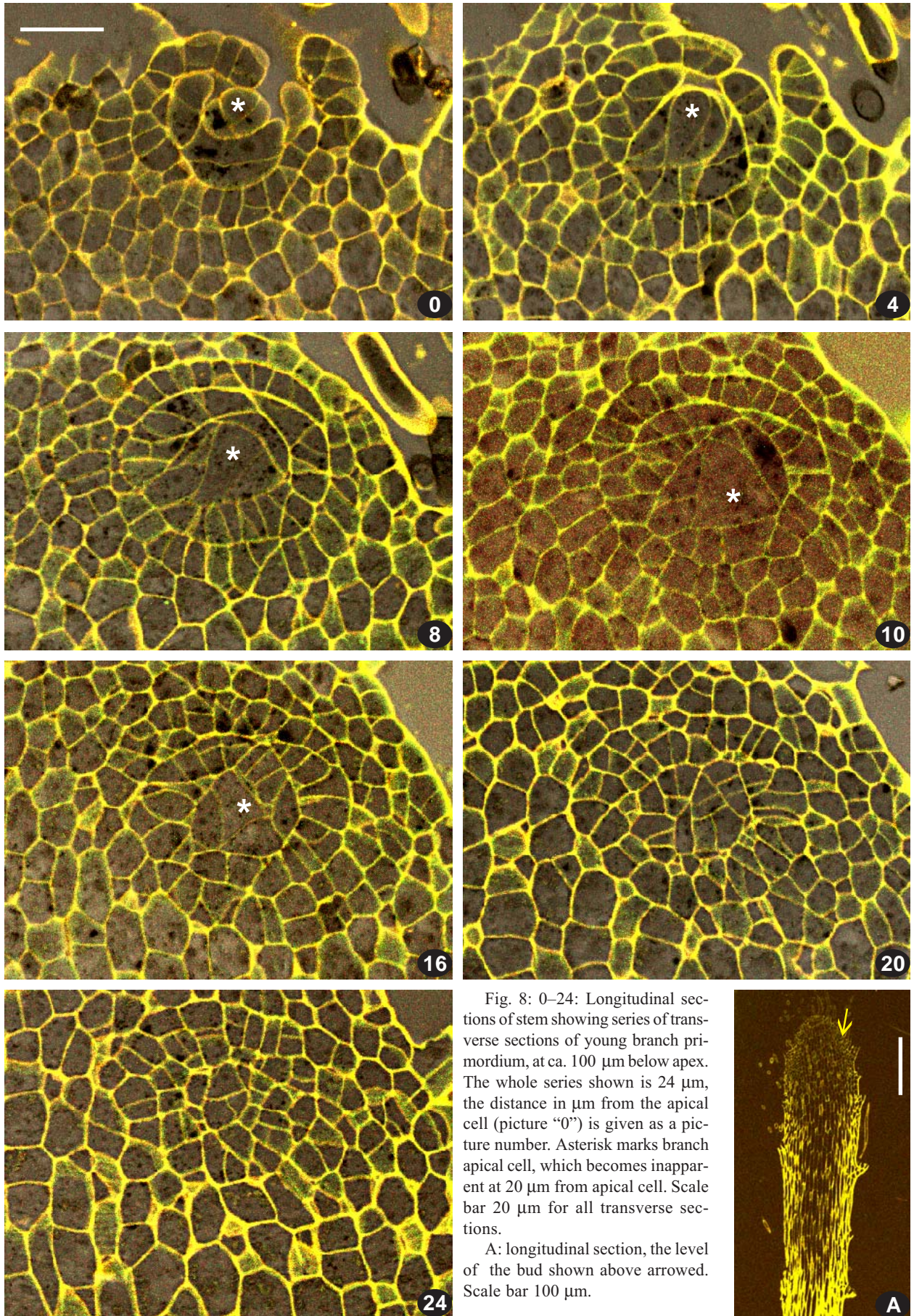
Fig. 8: 0–24: Longitudinal sections of stem showing series of transverse sections of young branch primordium, at ca. 100 μm below apex. The whole series shown is 24 μm, the distance in μm from the apical cell (picture "0") is given as a picture number. Asterisk marks branch apical cell, which becomes inapparent at 20 μm from apical cell. Scale bar 20 μm for all transverse sections.

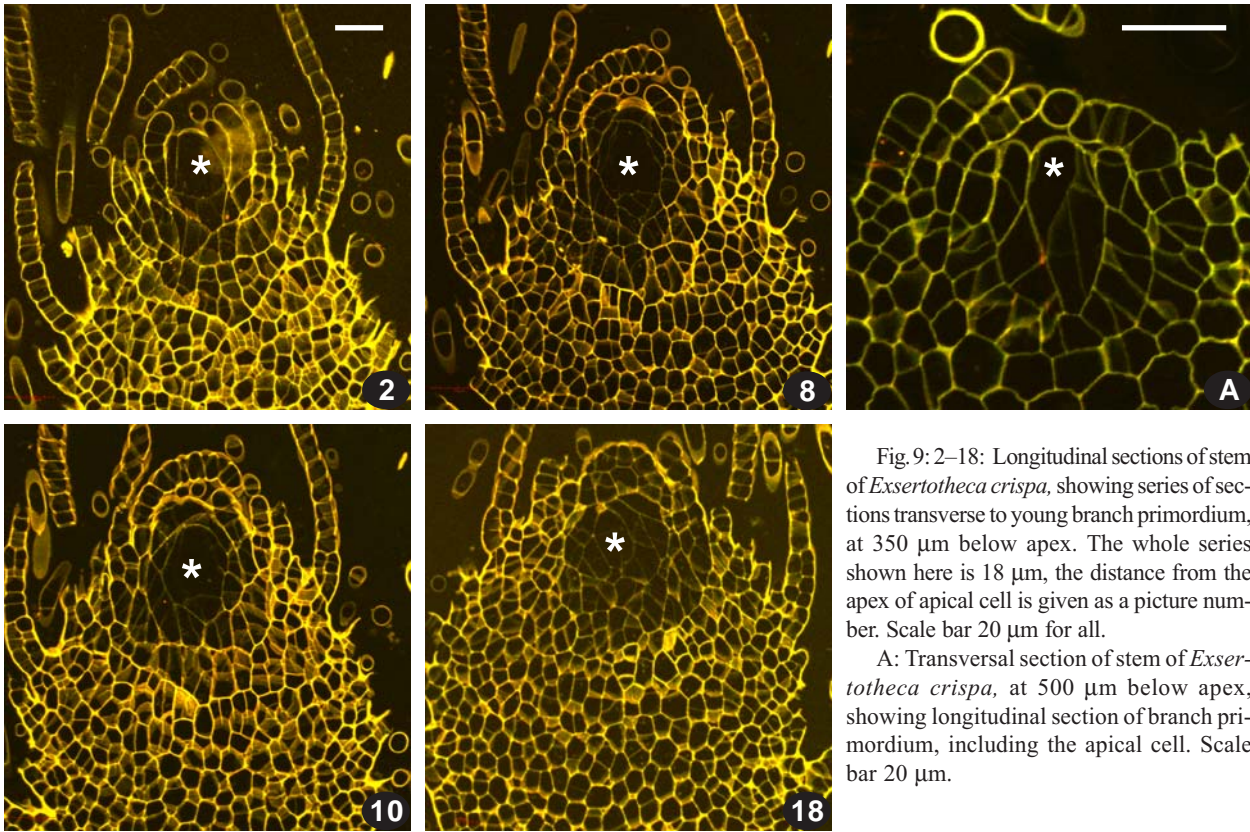A: longitudinal section, the level of the bud shown above arrowed. Scale bar 100 μm.

Fig. 9: 2–18: Longitudinal sections of stem of *Exsertotheca crispa,* showing series of sections transverse to young branch primordium, at 350 μm below apex. The whole series shown here is 18 μm, the distance from the apex of apical cell is given as a picture number. Scale bar 20 μm for all.

A: Transversal section of stem of *Exsertotheca crispa,* at 500 μm below apex, showing longitudinal section of branch primordium, including the apical cell. Scale bar 20 μm.

with apical cell surrounded by proximal branch leaves, some having axillary hairs in their axils. These apical cell is 26 μm wide in the middle, at depth of 10 μm from cell apex.

Bud in Fig. 9A was situated still slightly farer from the stem apex than that in Fig. 9: 2–18. Its apical cell is 32 μm long, 11 μm wide at apex, 14 μm wide in the middle.

The bud has already 3–4 leaves flanked by parts of stem destroyed in the course of the leaf removal. This means that at this stage all cells of the stem surface formed leaves.

Further down on the stem, at 0.6 mm from the stem apex, the dormant buds (Fig. 10) are strongly flattened, with the apical cell ca. 15 μm wide, but less than 15 μm in length, measuring by number of sections. No less than

Fig. 10: Longitudinal and somewhat oblique section of the stem of *Exsertotheca crispa,* showing series of sections transverse to young branch primordium. The whole series shows apical cell only slightly larger than surrounding cells. Scale bar 50 μm for all.
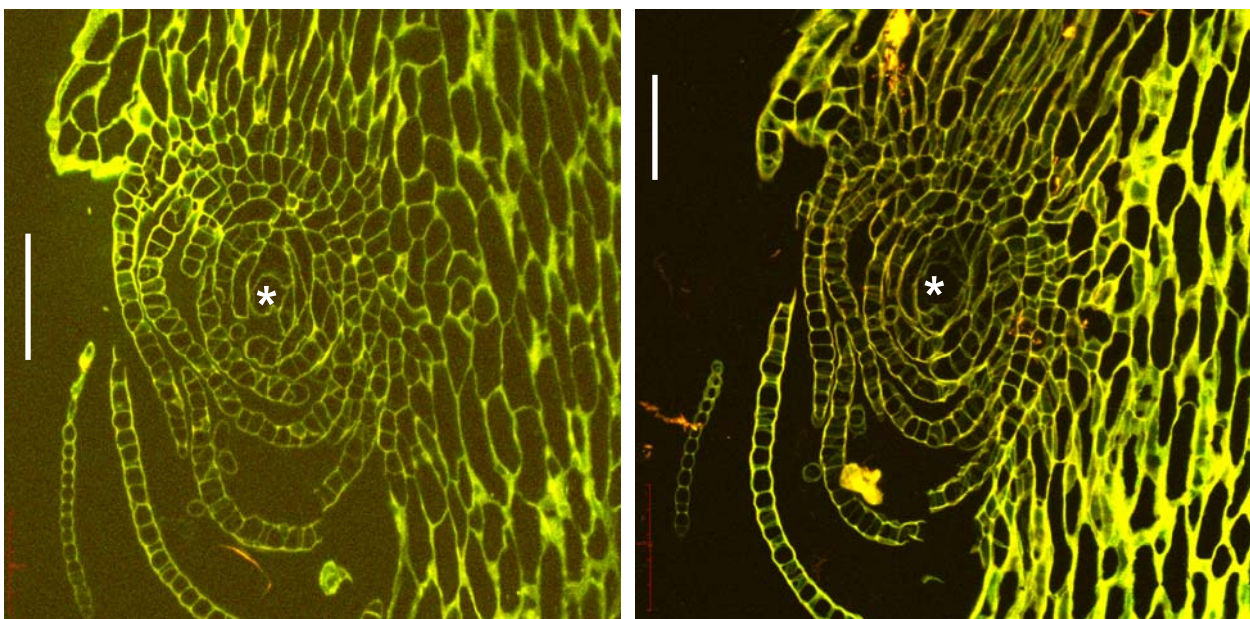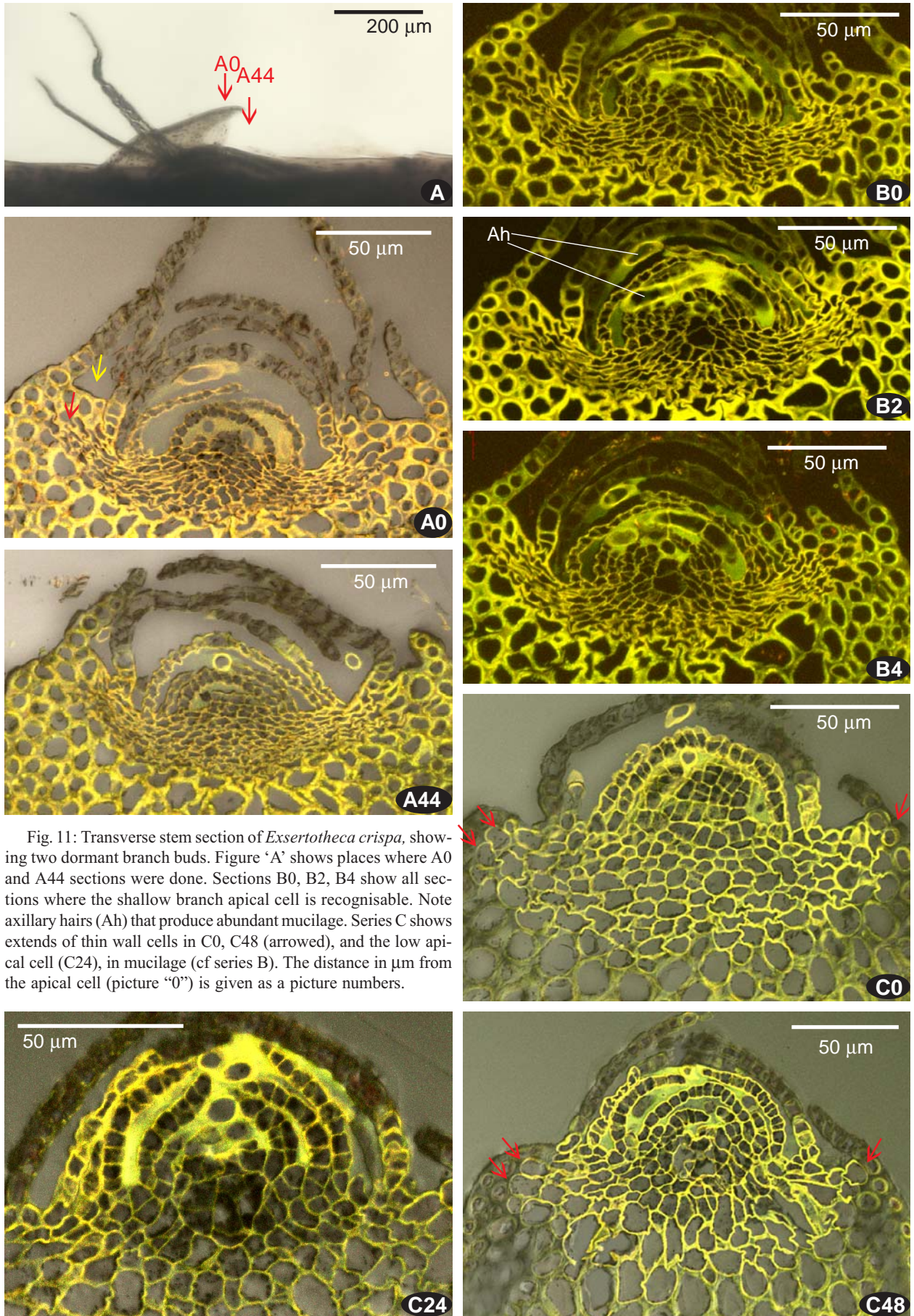
Fig. 11: Transverse stem section of *Exsertotheca crispa,* showing two dormant branch buds. Figure 'A' shows places where A0 and A44 sections were done. Sections B0, B2, B4 show all sections where the shallow branch apical cell is recognisable. Note axillary hairs (Ah) that produce abundant mucilage. Series C shows extends of thin wall cells in C0, C48 (arrowed), and the low apical cell (C24), in mucilage (cf series B). The distance in μm from the apical cell (picture "0") is given as a picture numbers.
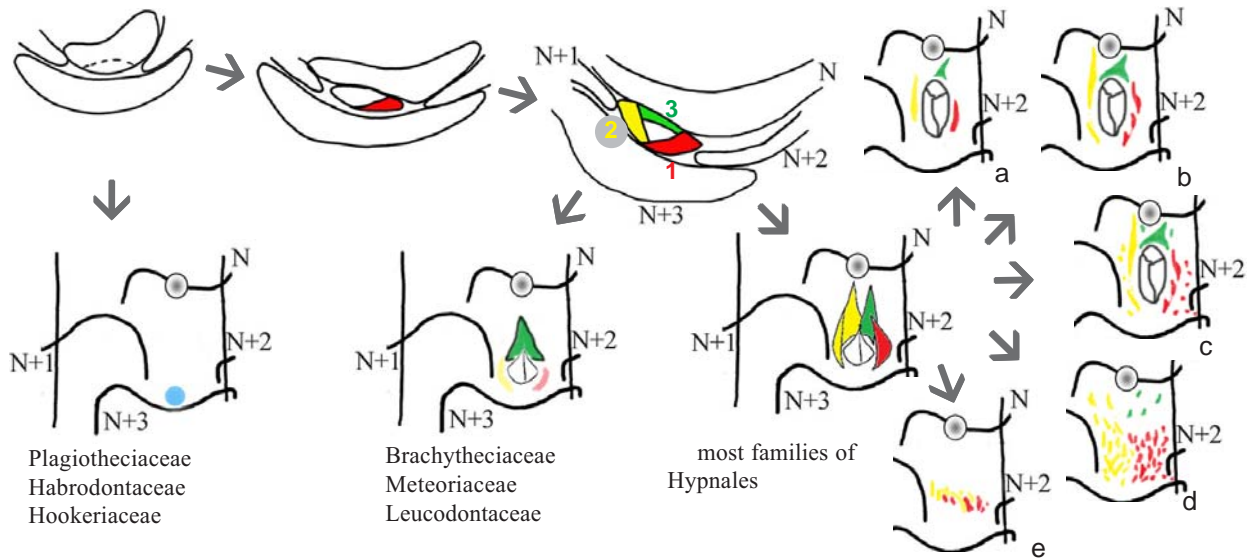
Fig. 12. Early stages (A to C) of branch development in pleurocarpous mosses: a schematic summary of Berthier (1971). N to N+3 – order of stem leaves; 1–3 – order of branch leaves. The case 'most common of Hypnales' is modified in Neckeraceae through series a–b–c–d–e. Variants 'a' and 'b' occur in *Neckera douglasii* and *Exsertotheca crispa* (cf. Fig. 3, 4A–D), variants 'b–e' in *Neckera andina* (cf. Fig. 5A–D); 'c–d–e' are common in *Neckera menziesii* (Fig. 5E–J). In addition, 'e' is shown for *Leptodon smithii* in Fig. 6, and 'b' for *Alleniella complanata* in Fig. 13.

four leaves from each side surround this bud. Shortly behind the outermost ring of them, the stem cells are long and thick-walled.

Transverse sections at about this distance from the stem apex are shown in Fig. 11. Apical cell is small and well packed under surrounding leaves, where axillary hairs are already well developed. Axillary hairs show bright fluorescence of their inner contents, and also the same fluorescence, likely of mucilage, is seen in the space in between leaves surrounding apical cell.

Cells below the apical cell seem to be especially thin-walled (Fig. 11C). This group of cells occupies a broad area that extends at places beyond outermost parts of the bud (Fig. 11C, arrowed). The plasmolisis occured in majority of our samples and seems the ability of large cells for strong shrinking (and perhaps vice versa?) hide the apical cell zone in a well protected pit, covered by many layers of leaves.

DISCUSSION

The exogenous ABA treatment of the plants of *Exertotheca crispa* leads to a number of morphogenetic responses. ABA increases stem increments, number of buds, length of the proximal branch leaves and number of cases of their subdivision into independent units. In one case the paraphyllia of *Leskea*-type were recorded (Fig. 3A). They appeared in the place where the branch bud is typically appearing. Otherwise, no accelerating of the paraphyllia appearing was noticed, which differs *Exertotheca crispa* from mosses where paraphyllia of *Leskea*-type became more numerous after ABA application (*Leskea, Cratoneuron, Leptodon*, cf. Spirina *et al.*, 2020). This may be considered as an evidence of the absence of real paraphyllia in *Exsertotheca*. At the same time, the proximal branch leaves of *Exsertotheca* are often subdivided into narrowly lanceolate or even filamentous parts (Fig. 3) and therefore can be very easily misunderstood as para-

Fig. 13. Dormant brunch buds of *Alleniella complanata,* SEM images, showing filamentous structures, which arrangement allows us to interpret them as a compound proximal branch leaves, with respective numbers for them or their parts. Scale bar 50 μm for all.
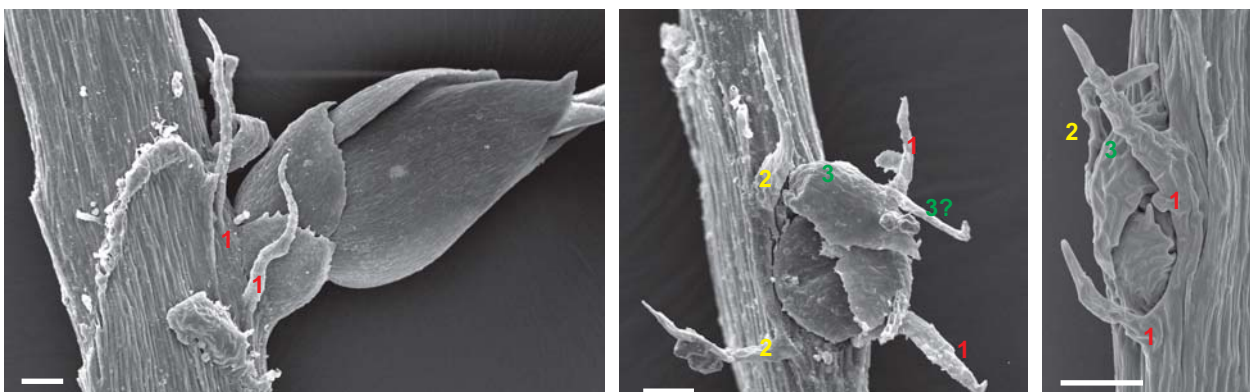
phyllia. This is especially likely because the dormant branch buds are situated in the lateral position on the dorsiventrally flattened stems, which strongly restricts the possibility to notice phyllotaxis as only one half of a bud is visible.

Anatomical studies of Akiyama & Nishimura (1993a) showed that there is no principal difference between outermost proximal branch leaves (scaly leaves in terminology of these authors) between Neckeraceae (represented in their studies by *Homaliodendron flabellatum* (Sm.) M. Fleisch.) and Brachytheciaceae (represented by *Brachythecium wichurae* (Broth.) Paris). In *Homaliodendron*, the outer branch leaves are somewhat spaced from the next inwards, however, their development is identical and they obviously originate from the branch apical cell. Our observations of *Exsertotheca* also demonstrated that the outermost leaves of branch primordia belong to bud (Fig. 11).

The arrangement of foliose structures along the border of pellucid area around branch buds (Figs. 1, 3–5) is another evidence that they are homologous of the proximal branch leaves. However, in many species of the Neckeraceae shape of the proximal branch leaves and of further (4th–6s) branch leaves is so contrastingly different that it is difficult to believe in their homology. A conspicuous case occurs in *Alleniella* (*Neckera*) *complanata* (Fig. 13), where proximal branch leaves are subdivided into two subequal lanceolate structures. Cubero *et al.* (2006) found that the mean number of 'pseudoparaphyllia' (as they named such lanceolate structures) in *Exsertotheca crispa* was 3.63±0.18, while *Alleniella complanata* differs from it (and from four other species of the genus involved in that study) in significantly higher number, 5.42±0.19. As it can be traced in Fig. 13, the young branches/buds with ovate leaves are surrounded with pairs of lanceolate structures, which actually represent compound proximal branch leaves.

The descriptive terminology of the above mentioned facts remains controversial. The case shown in Fig. 3A comprises the filamentous structures that are situated at the place usual for branch bud and in position of about 4 and 11 o'clock. Therefore, their relation to branch is obvious. However, they are spaced one from another and therefore their origin from one cell seems unlikely. A similar case was discussed by Spirina *et al*. (2020, Fig. 4B): filamentous structures with obvious phyllotaxis were even more spaced, no obvious candidate for apical cell was found among epidermal cells in between them.

Thus, the question is how to call structures that are:

(1) not of common origin from a single cell and therefore cannot be called proximal branch leaves, but (2) are obviously upregulated in their development by a 'vestigial primordium', so these structures are arranged with phyllotaxis. Their examples are shown in Fig. 3A, Fig.5D, 5J (arrow), and Fig. 6 (cf. Fig. 12e). Spirina *et al.* (2020) suggested to refer them to paraphyllia of *Leskea*-type.

The term 'pseudoparaphyllia' often applies to such structures; however, we avoid to use it, as a source of confusion. Warnstorf (1904–1906) introduced it for foliose structures around the branch primordia in *Rhynchostegium* (Brachytheciaceae) and *Rhytidiadelphus* (Hylocomiaceae). Ireland (1971) in his global overview of pseudoparaphyllia annotated these two genera as lacking pseudoparaphyllia. Another problem was that this term was applied both to whole proximal branch leaves and, e.g. in *Hypnum*, for narrowly lanceolate parts of compound leaves (Spirina & Ignatov, 2008; Ignatov & Spirina, 2012). And finally, a gradual transition between proximal branch leaves and paraphyllia, illustrated in the present paper (cf. Fig. 12a–e), and especially apparent in *Neckera andina* (Fig. 5A–D), makes the term pseudoparaphyllia still more controversial. The difference between pseudoparaphyllia and proximal branch leaves, from one side, and from pseudoparaphyllia and paraphyllia, from another side, will require additional criteria for delimitation.

Therefore we refer to branch leaves (including proximal ones) descendants of the branch apical cell (Fig. 12 a,b), leaving the rest to paraphyllia (shown outwards proximal branch leaves in Figs. 12 c,d,e). If the latter are more abundant near branch initial, then they are paraphyllia of *Leskea*-type; if they are evenly spreading throughout the stem surface, then they are of *Climacium*-type (Ignatov & Hedenäs, 2007; Spirina *et al.*, 2020)

The question if a given structure has originated from the branch apical cell or not, can usually be answered based of phyllotaxis and the position in the branch bud area. Usually the delimitation of the branch bud area provides no problem (as the proximal branch leaves are usually appressed to the branch leaves further inwards). However, the Neckeraceae are exceptional, as their proximal branch leaves may be well-spaced from the 'inner part' of bud, which was already noticed by Akiyama & Nishimura (1993). The arrangement along the pellucid zone (Fig. 1A,B, 3D, 4B,F,H) around the inner part of the branch bud may be revealing for the referring to proximal branch leaves in this case, as was suggested by New-
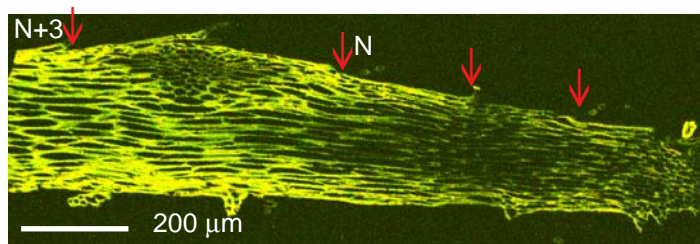

Fig. 14. Longitudinal section of the stem, almost from apex (<100 μm not shown on the right), showing the average size of bud at 30–50 μm below the stem surface. Arrows mark the position of detached leaves (mainly by remained fragments of axillary hairs). Note that the bud fills a considerable part of space between leaves N and N+3.

ton & De Luna (1999). And especially accurate would be conclusions based on the anatomy studies, similar to those done by Akiyama & Nishimura (1993). The latter however are time-consuming, and cannot be applied to every specimen. An increase of the number of paraphyllia scatterd over 'internode' in such species as *Leskea polycarpa*, *Cratoneuron filicinum* and *Leptodon smithii* (Spirina *et al.*, 2020) in response of the exogenous ABA treatment may serve as another characteristic of paraphyllia. Unlike them, the proximal branch leaves remain in their position albeit may be divided into more parts (Table 3).

The specifics of branch bud structure in *Neckera* and its outstanding variation likely relate to the shape of their apical cell (or AAC, as specified above), as the latter may have a morphogenetic value, forming a very broad bud. Such bud has numerous cells under the cortical layer, where a rather extensive "foot of a branch" occurs (Fig. 14), spreading almost throughout the whole 'internode', i.e. the areas between leaves N, N+1, N+2 and N+3 (Fig. 4B). This may explain why the border of the bud is somewhat indefinite and a bud fills almost the whole 'internode', so the proximal branch leaves occur unbelievably far from the compact inner part of the bud.

The present report intends to attract attention to the plasticity of leaf-like structures around branch primordia as a potentially useful model object for the morphogenetic studies. Potentially they may disclose the dependence of morphogenesis from the architecture of the apical cells, which studies remain at the same level as it was pointed by Schuster (1988).

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Appedix 1. Specimens used for study:

Species	Country	Collector	Herbarium barcode	N buds studied
Exsertotheca crispa	Russia	A.Kartasheva	MHA 9132139	43
Exsertotheca crispa	Russia	A.Kartasheva	MHA 9132140	32
Exsertotheca crispa	Russia	A.Kartasheva	MHA 9132141	32
Exsertotheca crispa	Russia	A.Kartasheva	MHA 9132142	42
Neckera andina	Peru	P. and E.Hegevald	MHA 9065787	4
Neckera chilensis	Chile	M.Maxu	MHA 9065789	7
Neckera chilensis	Columbia	J.Muñoz	MHA 9065788	4
Neckera douglasii	U.S.A.	D.H.Norris	MHA 9065800	2
Neckera douglasii	U.S.A.	K.Korotkov	MHA 9065790	10
Neckera douglasii	U.S.A.	K.Korotkov	MHA 9065791	8
Neckera douglasii	U.S.A.	M.S.Ignatov	MHA 9065792	4
Neckera douglasii	U.S.A.	M.S.Ignatov	MHA 9065793	3
Neckera douglasii	U.S.A.	M.S.Ignatov	MHA 9065794	2
Neckera douglasii	U.S.A.	M.S.Ignatov	MHA 9065795	7
Neckera douglasii	U.S.A.	M.S.Ignatov	MHA 9065796	9
Neckera douglasii	U.S.A.	M.S.Ignatov	MHA 9065797	5
Neckera douglasii	U.S.A.	M.S.Ignatov	MHA 9065798	7
Neckera douglasii	U.S.A.	M.S.Ignatov	MHA 9065799	5
Neckera douglasii	U.S.A.	M.S.Ignatov	MHA 9065801	4
Neckera douglasii	U.S.A.	M.S.Ignatov	MHA 9065802	8
Neckera douglasii	U.S.A.	M.S.Ignatov	MHA 9065803	8
Neckera ehrenbergii	Mexico	E. de Luna	MHA 9065806	2
Neckera ehrenbergii	Mexico	T.B.Croat	MHA 9065805	3
Neckera flexiramea	China	T.Koponen et all	MHA 9065807	10
Neckera himalayana	Thailand	H.Akiyama	MHA 9065808	9
Neckera humilis	Japan	A.Noguchi	MHA 9065810	5
Neckera humilis	Japan	M.Deguchi	MHA 9065811	6
Neckera humilis	Japan	M.Deguchi	MHA 9065813	8
Neckera humilis	Japan	M.S.Ignatov, E.A.Ignatova	MHA 9065812	7
Neckera menzensii	Canada	D.H.Vitt	MHA 9065815	4
Neckera menzensii	France	G.Gardet	MHA 9065814	8
Neckera menzensii	U.S.A.	M.S.Ignatov	MHA 9065364	1
Neckera menzensii	U.S.A.	M.S.Ignatov	MHA 9065366	4
Neckera menzensii	U.S.A.	M.S.Ignatov	MHA 9065816	2