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Sub-cellular distribution of nitrogen compounds in *Azolla* and *Anabaena* by ESI and EELS analysis

Patrizia Albertano*, Antonella Canini, and **Maria Grilli Caiola**

Department of Biology, University of Rome "Tor Vergata", Rome

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Summary. The sub-cellular localization of some nitrogen compounds within the leaf cavities of *Azolla filiculoides* Lam. was obtained by means of electron spectroscopic imaging (ESI) and electron energy loss spectroscopy (EELS). The analyses were performed on ultrathin unstained sections of different *Azolla* leaf cavities which contain epidermal hairs, *Anabaena azollae* Strasb. and bacteria. Net nitrogen distributions were visualized by image analysis, and nitrogen peaks were evidenced in spectra recorded in the same areas. Different distributions of nitrogen compounds were observed within the leaf cavities along the stem, in particular inside the epidermal hairs of *Azolla* and the vegetative cells and heterocysts of *A. azollae*.

Keywords: *Azolla-Anabaena* symbiosis; Ultrastructure; Nitrogen distribution; Electron spectroscopic imaging; Electron energy loss spectroscopy.

Introduction

The leaf cavities of *Azolla* Lam. contain the nitrogen fixing cyanobacterium *Anabaena azollae* Strasb., bacteria and hair cells (Peters and Meeks 1989, Peters 1991, Grilli Caiola 1992). The ability of *A. azollae* to fix nitrogen allows the fern to live in waters with low combined nitrogen. Nitrogen fixed as ammonium (Meeks et al. 1987) by the heterocysts of the cyanobacterium is released in the leaf cavities of *Azolla*. Furthermore, fixed nitrogen is transported to the apex of *Azolla* which does not contain active heterocysts (Kaplan and Peters 1981). The fern assimilates ammonium and organic products as glutamine or glutamate through the glutamate synthase cycle (Meeks et al. 1987). Since epidermal hair cells of *Azolla* contain a very high amount of ammonia-assimilating enzymes (Uheda 1986) and exhibit transfer cell ultrastructure (Calvert et al. 1985), it has been hypothesized that these cells are the site for carbon and nitrogen exchanges. In particular, epidermal branched hairs could be functional in nitrogen exchange between *Azolla* and *Anabaena*, whereas the simple hairs could be involved in carbon exchange (Calvert et al. 1985). Nevertheless, the hypothesis of nitrogen transport from mature leaf cavities to the apex was based on the measurement of nitrogen content in various segments of the fern (Kaplan and Peters 1981). Moreover, the molecular interactions between surface molecules of lectins and receptors expected to be involved in the exchanges of fixed nitrogen compounds among the symbiotic partners are still not resolved (Tel-Or et al. 1991). Therefore, the role of epidermal hairs in the nitrogen exchange remained to be demonstrated.

Most of the knowledge on the biochemical, physiological, and ultrastructural features of *Anabaena azollae* arose from studies carried out on the symbiont of *Azolla pinnata* Presl., *A. mexicana* R. Brown, and *A. caroliniana* Willd. (Braun-Howland and Nierzwicki-Bauer 1990), while few recent data were available for the symbiont of *Azolla filiculoides* (Braun-Howland et al. 1988). The first detailed ultrastructural study of *Anabaena azollae* within *Azolla filiculoides* was made by Lang (1965). In the same *Azolla* species, Duckett et al. (1975) revealed the presence of multicellular hairs with the transfer cell ultrastructure within the leaf cavities. Hill (1975) evidenced a variation in the number of heterocysts with a maximum at about 12 leaves from

* Correspondence and reprints: Department of Biology, University of Rome "Tor Vergata", via della Ricerca Scientifica, I-00133 Rome, Italy.

the apex, and an increase in vegetative cell size according to the increasing leaf age. The presence of two different classes of epidermal leaf hairs was firstly reported by Calvert and Peters (1981) through light microscopy. These authors showed that only primary branched hairs are present at the apex, while two secondary branched hairs and up to 25 simple hairs are in each mature cavity. The ultrastructural characteristics of vegetative cells of *A. azollae*, symbiont of *Azolla filiculoides*, and the ultrastructural changes which occur during the differentiation of vegetative cells into heterocysts were examined by Braun-Howland et al. (1988) who found the highest nitrogenase labeling in mature heterocysts.

By using microelectrodes, low ammonium concentrations were found within the *Azolla filiculoides* leaf cavities of apex to group III which has the maximum heterocyst frequency and nitrogen fixation activity, while high ammonium concentrations were found in basal groups (Canini et al. 1990). These data supported the presence of an active utilization of ammonium from the cavities which contained the highest number of nitrogen-fixing heterocysts. Moreover, cytochemistry in combination with transmission electron microscopy demonstrated the presence of peptidic material, positive to tannic acid fixation, in the mucilaginous matrix of the leaf cavity and within the epidermal hair cells (Albertano and Grilli Caiola 1988, Grilli Caiola and Albertano 1988). Recently, by using the energy-filtering transmission electron microscopy (EFEM) which allows the subcellular localization of elements with low atomic number by ESI and EELS analysis (Reimer et al. 1988), it was possible to visualize and identify nitrogen containing structures. Preliminary results were obtained with these methods which suggested the presence of nitrogen amounts in primary branched hairs, in vegetative cells and in heterocysts (Albertano and Grilli Caiola 1991 a).

Therefore, the aim of the present work was to investigate the sub-cellular localization of nitrogen compounds in the cyanobacteria and the leaf hair cells of *Azolla* by ESI and EELS analyses in the attempt to clarify the contribution of each partner to the nitrogen metabolism of the symbiosis.

Materials and methods

Plants

Azolla filiculoides Lam., from Botanical Garden of Milan, were grown in Hoagland nitrogen-free medium at "Tor Vergata" University. For this study, the plants were exposed at 140 $\mu E/m^2/s$ light irradiance, at 25 °C. The leaves located on the main stem axis were pooled as follows: apex (meristems and leaf primordia), group I (1–4 leaf cavities from the apex), group II (5–8 leaf cavities), group III (9–12 leaf cavities), group IV (13–16 leaf cavities).

TEM preparations

The different groups of *Azolla filiculoides* leaves were fixed in 2.5% glutaraldehyde in phosphate buffer 0.2 M pH 7.2, washed three times with phosphate buffer, post-fixed in 1% osmium tetroxide in the same buffer, dehydrated in ethanol series, and embedded in Agar Aids resin Agar Kit 100. Ultrathin (< 40 nm) unstained sections were collected on uncoated 600 mesh copper grids and observed with a CEM 902 Zeiss energy filtering electron microscope at 80 kV.

ESI and EELS analyses

Inelastically scattered electrons of defined energy losses were imaged using a 20 eV energy window to obtain high contrast darkfield images at energy losses between 150 and 250 eV. At such values most of the important biological elements except carbon contribute to the signal (Bauer 1988). The electron spectroscopic images (ESI) for the localization of nitrogen in the specimens were taken at 410 eV just beyond the ionization edge (IE) of the nitrogen (N_K edge at 401 eV) and at the pre-ionization edge (PIE) at 370 eV as reference carrying out information on the background. The net nitrogen distribution images were obtained by using the digital image analyser with an IBAS image processing system. The elemental mapping resulted by the subtraction of the IE and PIE images recorded with a highly sensitive camera. Electron energy loss spectra (EELS) of N_K were acquired by an integrated photomultiplier with a 90 µm objective lens diaphragm at a scan rate of 1 eV/channel and 30,000× magnification on 200 or 300 nm diameter areas of the ultrathin sections where nitrogen was localized.

Results

Apex of *Azolla* with vegetative cells of *Anabaena azollae* around meristematic cells and leaf primordia is shown in Fig. 1. The image has been obtained at $\Delta E = 150$ eV, the value at which the contrast is due to the light element content of cell structures. Two body cells and two terminal cells of a primary branched hair (PBH) are visible. The terminal cells show variations in cell diameter from 25–35 µm and in cell length from 55 to 90 µm, and appear in different stages of differentiation of the transfer cell ultrastructure (TCU). Two vacuoles are generally present in each terminal cell at the mature stage, and very thin granular particles are visible inside them. The cross and longitudinal sections of the terminal cells (Figs. 2 and 3) also show many plastids with unusually elongate shape, no differentiated thylakoid system and starch granules. Numerous mitochondria with cristae are visible within the dense cytoplasm. *Anabaena* cells and other bacteria lie near the outer side of the hair cell walls. The image taken at $\Delta E = 250$ eV (Fig. 4) shows a mature terminal cell of a PBH without visible TCU of the cell wall but with a thick fibrillar wall structure (up to 1.5 µm wide) and a thin outer

layer of different composition and irregular distribution. The cytoplasm appears extremely reduced and with few organelles. Vegetative cells of *A. azollae* are rich in carboxysomes and adhere to the cell wall of the leaf hair. After image analysis of the images taken at 370 eV and 410 eV, nitrogen has clearly been shown to be localized in the outer layer of the cell wall and in great amount in the cytoplasm of the terminal cells and in the membrane system as well as in carboxysomes of the vegetative cell of *Anabaena* (Fig. 5).

In the leaves of group I and II, the terminal cells of branched and simple hairs (SH) show the same differentiation pattern observed in the former at the apex. The formation of two large vacuoles occurs by confluence of small vacuoles during elongation of the young cell, while the nucleus occupies the central part of the cell (Fig. 6). The first stages of SH differentiation show a thin cell wall, cytoplasm rich in mitochondria and plastids with starch granules. The SH terminal cells show a more elaborated TCU at the mature stage, until the senescence (Fig. 7); the vacuoles, which fill almost entirely the body cell volume, appear full of electron-dense material. The senescent terminal hair cells collapse and probably loose some parts of the additional outer layer of cell wall to which many bacteria adhere (Fig. 7). In leaf cavities of these groups amounts of nitrogen compounds were detectable in the outer layer of the cell wall and inside the cytoplasm only in mature stage of secondary branched hairs (SBH) (data not shown). In contrast, nitrogen has not been appreciably detected in senescent stage.

In leaves of group III, secondary branched hairs appear as observed in the previous leaf cavities (data not shown), while mature simple hairs (Figs. 8–11) appear always surrounded by bacteria which adhere to their cell walls. Cytoplasm is extremely reduced, and some granular material is visible inside the vacuoles. No nitrogen spots appear to be localized in the cell wall and few in the underlying cytoplasm (Fig. 9), but a certain amount is visible inside the bacteria. In contrast to this situation, much more nitrogen could be localized inside both vegetative cells and heterocysts of *Anabaena azol-*

Figs. 1–3. ESI images of the apex of *A. filiculoides*. Bars: 5 μm

Fig. 1. Low magnification image taken at ΔE = 150 eV. The filaments of *Anabaena azollae* are entrapped among the apical meristem, leaf primordia, and primary branched hairs. Two terminal cells (*tc*) of the hair and part of body cells (*bc*) are visible

Fig. 2. ΔE = 250 eV. Part of cross section of the terminal cell of the hair during differentiation of the transfer cell ultrastructure. Plastid (arrowhead) possesses few thylakoids, starch granule, and plastoglobules; cytoplasm is dense of ribosomes, endoplasmic membranes, and many vesicles. Vegetative cells of *Anabaena azollae* show many carboxysomes and cyanophycin granules

Fig. 3. ΔE = 250 eV. Longitudinal section of terminal cell of primary branched hair in mature stage. The TCU elaborations of the cell wall (arrowhead) are more extended and contain bright material. The vacuole (*v*) is filled by granular material

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Figs. 4 and **5.** Detail of the end part of a terminal cell of primary branched hair in the apex of *A. filiculoides* and vegetative cells of *A. azollae* which adhere to its surface

Fig. 4. ΔE = 250 eV. Cell wall is thick and fibrillar, with an outer layer of different element composition (arrowhead) extending on it. Cytoplasm is dense and restricted to the cell periphery. Square boxed is the area where the EELS spectrum was acquired. *PBH* Primary branched hair. Bar: 2 μm

Fig. 5. Net nitrogen distribution obtained from the image analysis of ΔE = 410 eV and ΔE = 370 eV images of the region boxed in white in Fig. 4. The amounts of nitrogen compounds are shown in black dots and appear conspicuous in the outer layer of the cell wall and in the cytoplasm of the primary branched hair. Nitrogen is also present in the thylakoids and carboxysomes of vegetative cells of *A. azollae*

Figs. 6 and **7.** Longitudinal sections of simple hairs in the cavities of leaves of group I–II. Bars: 2 μm

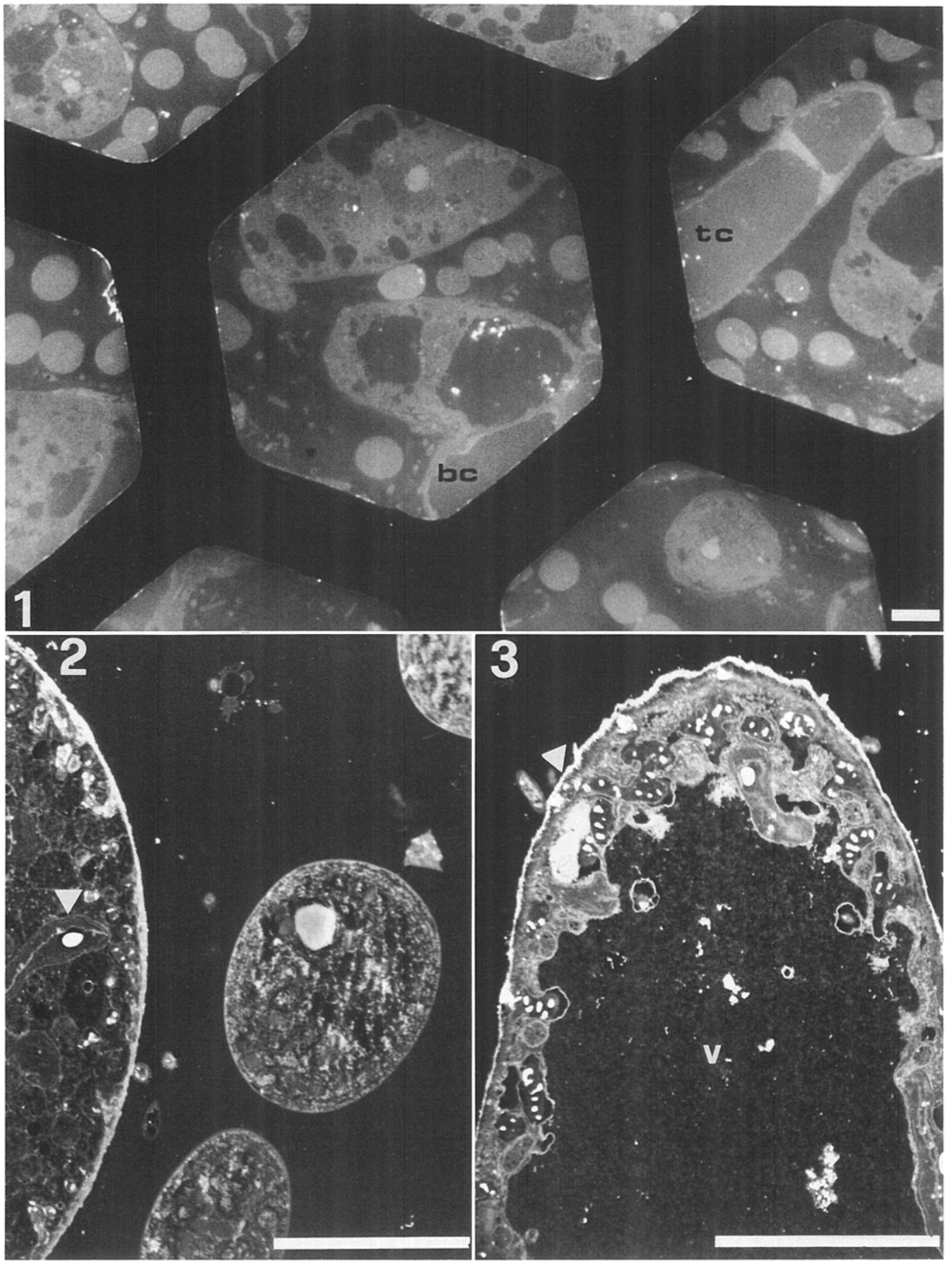
Fig. 6. ΔE = 200 eV. Stage in maturation of the terminal cell: the small vacuoles in the apical region are coalescing to form a large vacuole, the nucleus is in median position, many vesicles and endoplasmic membranes are in the cytoplasm

Fig. 7. Image taken in front of the nitrogen edge at ΔE = 410 eV. Terminal cell at the beginning of senescence. The cell wall is starting to collapse, the outer layer is thicker than in previous stages and irregularly disrupted and one bacterium adheres to its surface. The whole vacuole is filled by dense granular material

Figs. 8–11. ESI images of the same cross section of a terminal cell of a simple hair in the leaves of group III. Bars in Figs. 8, 10, and 11: 2 μm

Fig. 8. ΔE = 250 eV. Bacteria adhere to the cell wall which shows an elaborated transfer cell ultrastructure. Boxed is the area shown in Fig. 9

Figs. 9–11. Net nitrogen distribution (Fig. 9) obtained from the computerized analysis of Fig. 10 at ΔE = 410 eV and Fig. 11 at ΔE = 370 eV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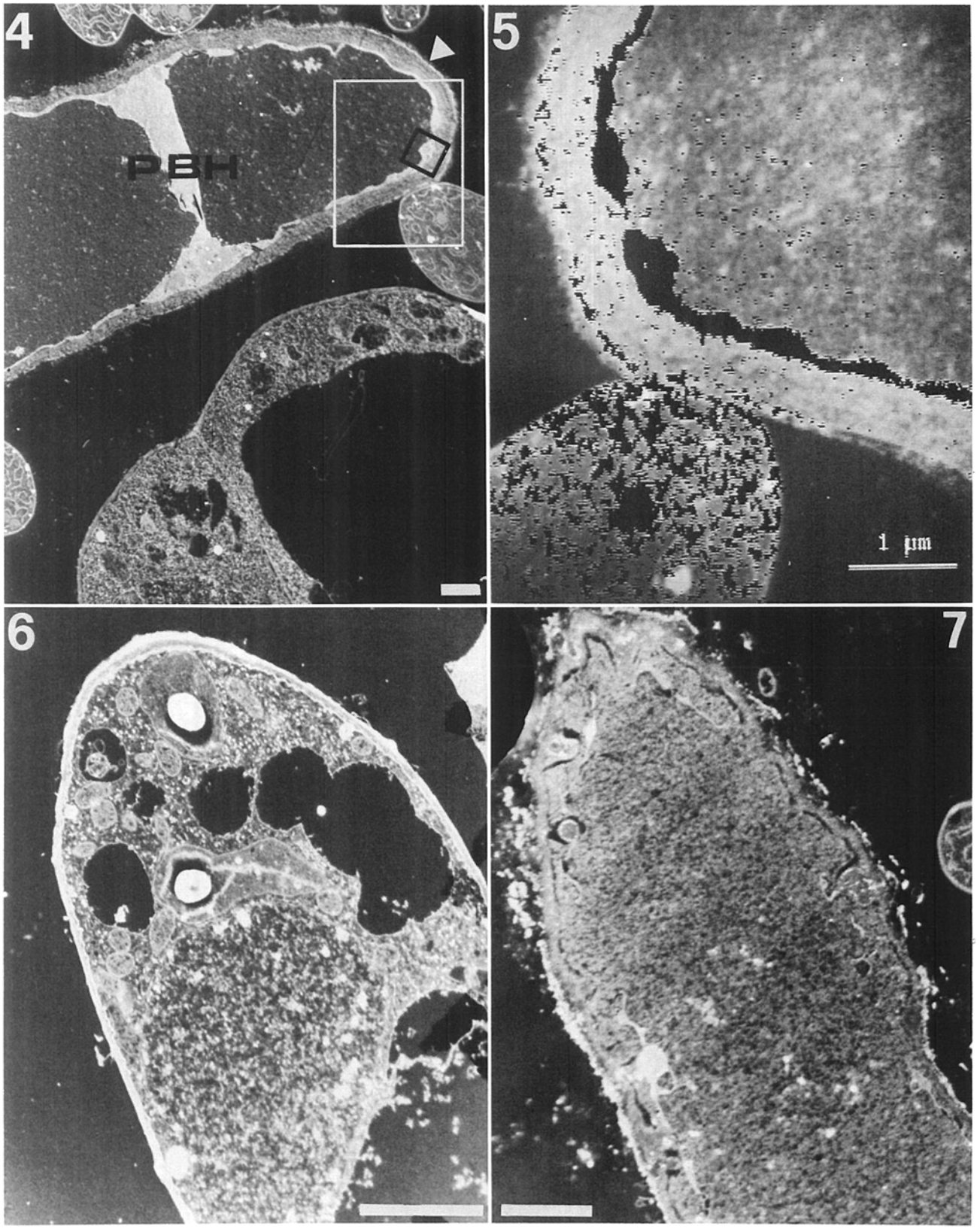
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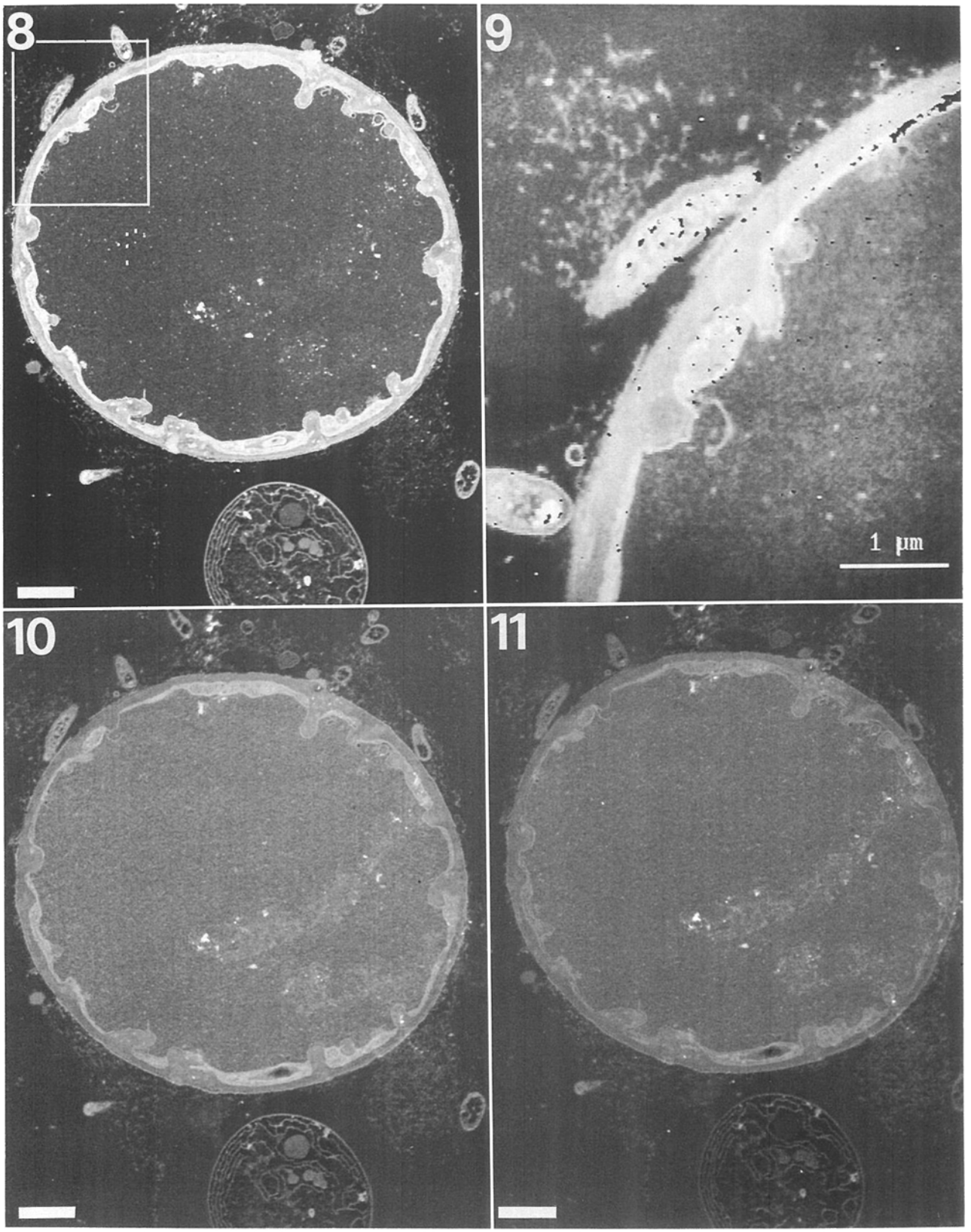
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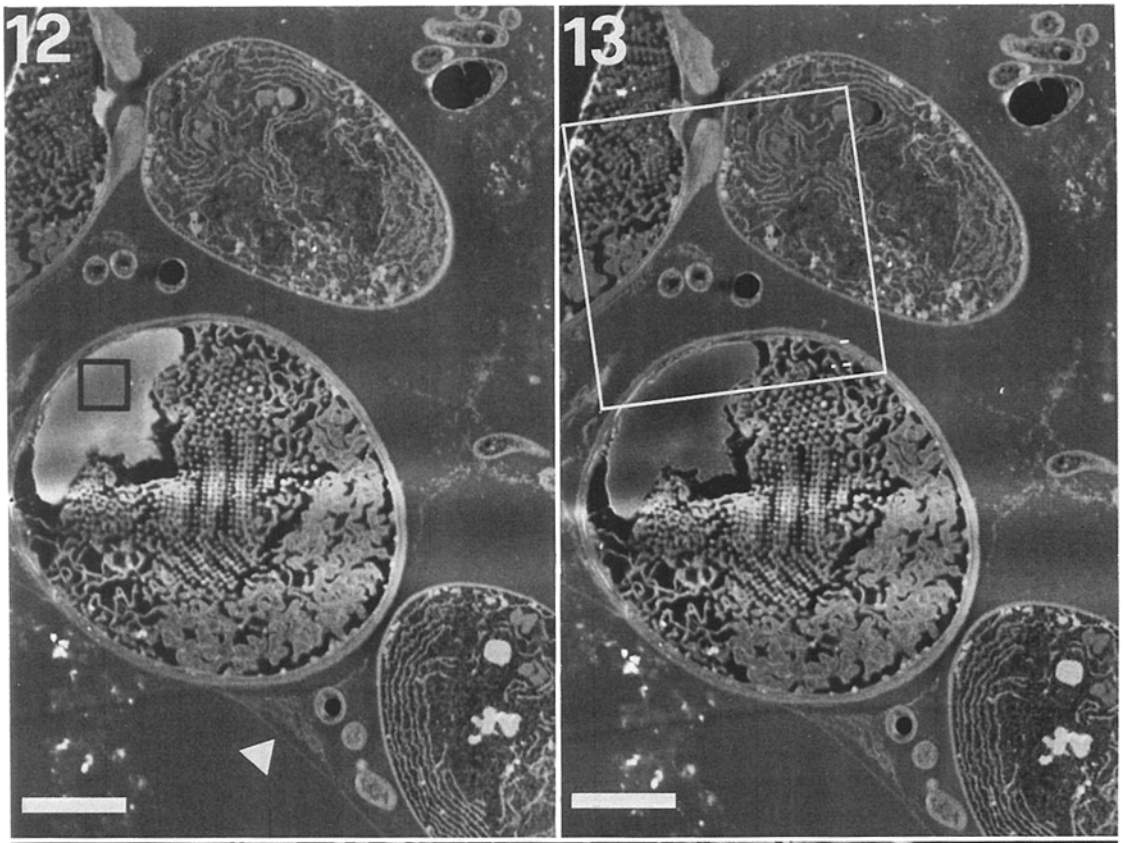
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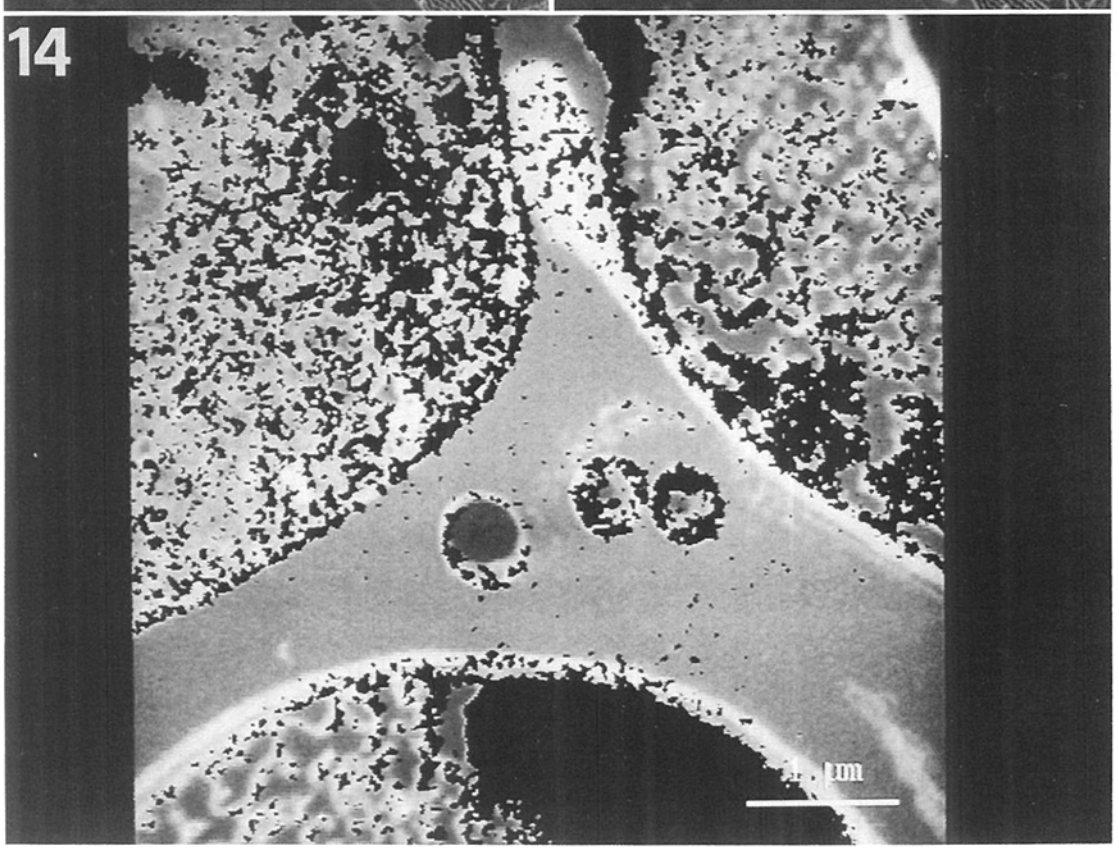
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1 µm

lae (Figs. 12–14). The large polar cyanophycin granules of heterocysts are surrounded by the highly regular honey-comb structure of membranes. The multi-layered structure of heterocyst envelope appears more clearly visible at the cell poles by the thickening of both the glycolipidic and polysaccharide layers. A certain degree of "vacuolization" is visible among the thylakoid membranes. Vegetative cells do not present relevant differences in their ultrastructure from those observed in the previous cavities. The number of bacteria within this group of leaf cavities starts to increase. By image analysis of the images taken at 370 and 410 eV, the net nitrogen distribution in the *Anabaena* and bacteria cells (Fig. 14) shows that nitrogen compounds are mainly localized on the cell walls, cell membranes, cyanophycin granules, and carboxysomes. A certain amount of nitrogen is also visible in the envelope of the heterocyst in both glycolipidic and polysaccharide layers that surround the cell wall.

In leaves of group IV, the development of *Azolla* hairs within the leaf cavities seems generally to proceed as in the other leaf groups. Only few plastids are visible inside the cytoplasm of terminal cells during their differentiation (Figs. 15 and 16), and transfer cell ultrastructure is less evident. Several vegetative cells of *Anabaena azollae* present extensive "vacuolization" of thylakoids and increase in the number of carboxysomes (Figs. 17 and 18). While the cyanophycin granules are still present, their dimensions seem generally smaller than those found in vegetative cells of *Anabaena* in the other leaf cavities. Accumulation of nitrogen compounds is not detectable inside the cells of leaf hairs, but is present in a reduced amount on membranes and on both carboxysomes and cyanophycin granules of *Anabaena* (data not shown).

The EELS spectra were acquired in all the areas where the presence of nitrogen compounds was observed by ESI (Figs. 19 and 20). Nitrogen peaks at 401 eV confirmed the presence of the element in the cell structures. The EELS spectrum reported in Fig. 19 refers to the cytoplasmic area of *Azolla* leaf hairs shown in Figs. 4 and 5. It shows a carbon peak at 281 eV, calcium at 346, and nitrogen peaks at 406 eV. The EELS spectrum in Fig. 20 was acquired on a small area of the cyanophycin granule shown in Figs. 12–14 and confirms the presence of large amounts of nitrogen compounds in the cell inclusion. In addition, the nitrogen edge outline of this spectrum differs from that above reported for the nitrogen compounds localized in the hair cells of *Azolla*. Besides the presence of nitrogen, the spectrum also recorded carbon and calcium peaks at energy loss of 281 and 346 eV, respectively.

Discussion

The sub-cellular distribution of nitrogen compounds in the cells of the *Azolla* leaf hairs, *Anabaena azollae* and bacteria was reported for the first time. Moreover, differences in the localization of nitrogen compounds among the different leaf groups of *Azolla* were also obtained.

Generally, nitrogen can be expected to contribute to almost all the structures of cell components and mainly proteinaceous compounds of *Azolla* and *Anabaena*, but accumulation sites of this element within the cells of the leaf cavities suggest a relationship with the nitrogen metabolism of the symbiotic partners.

Terminal cells of the branched hairs in the apex and in all leaf cavities of *Azolla* have shown to possess great amounts of nitrogen compounds which are localized in the outer layer of the cell wall and widely in the cytoplasm. Opposite, little nitrogen has been detected in body or stalk cells at the mature stage of branched hairs. Therefore, the reduced quantities of nitrogen compounds inside these latter cells could be due to a very efficient synthesis and translocation of glutamate by the fern. Moreover, no evident differences have been detected in the sub-cellular distribution of nitrogen between primary branched hairs in the apex and secondary branched hairs in the mature leaf cavities.

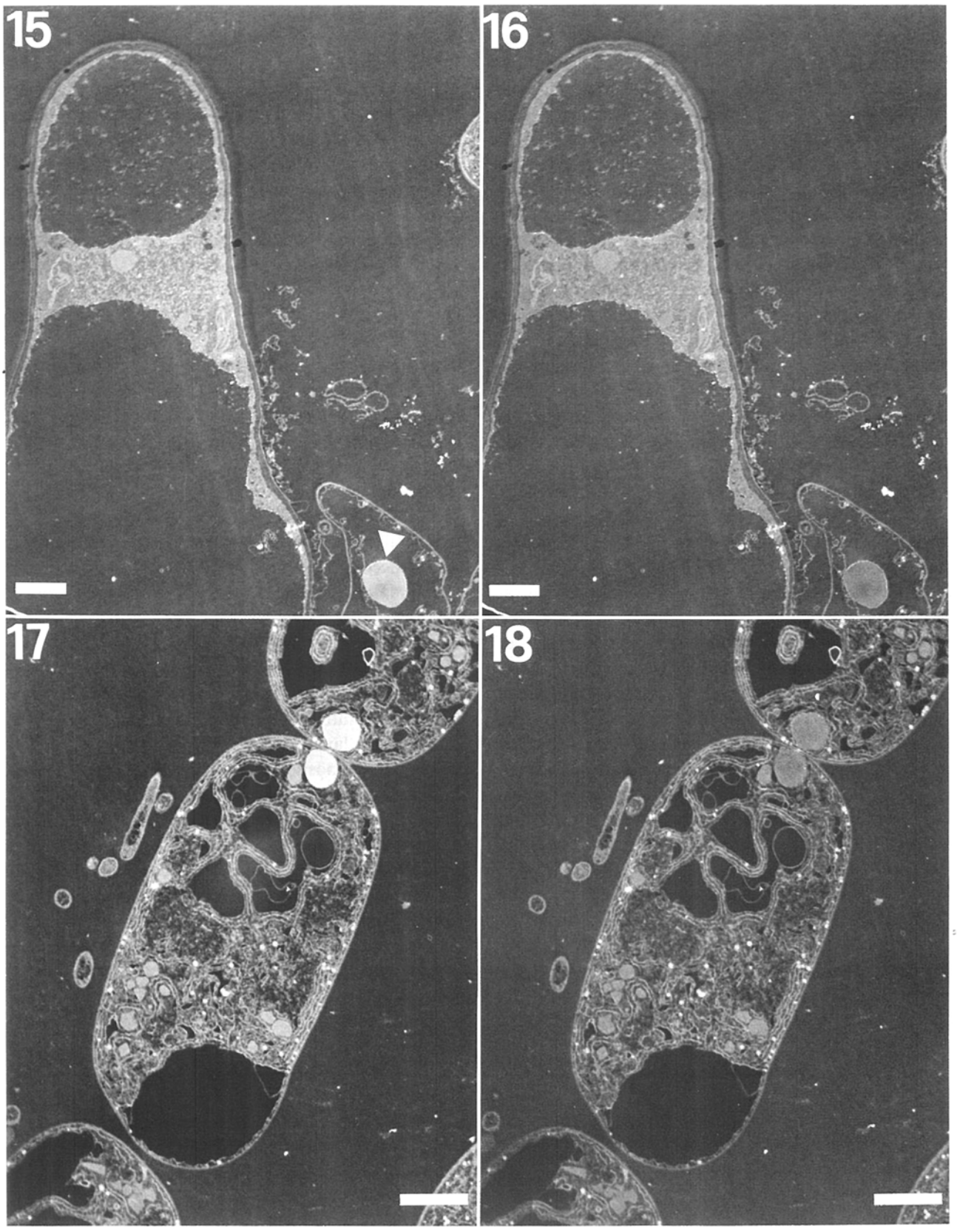
The nitrogen fixed as ammonium by *Anabaena azollae* and released in the leaf cavities is incorporated as glu-

Figs. 12–14. Mature heterocysts and vegetative cells of *Anabaena* and bacteria within a leaf cavity of group III. At the pole region of heterocysts the envelope thickening, large cyanophycin granules, and honey-comb structure of membranes are visible. Bars in Figs. 12 and 13: 2 μm

Fig. 12. $\Delta E = 410$ eV. The outer envelope (arrowhead) delimits the mucilaginous matrix of the leaf cavity. Square boxed is the area where the EELS spectrum was acquired

Fig. 13. Same section as in Fig. 12 at $\Delta E = 370$ eV. The square indicates the area shown in Fig. 14

Fig. 14. Net nitrogen distribution obtained by image analysis of Figs. 12 and 13. In *Anabaena* cells the element appears mainly localized inside cell wall, membranes, and cell inclusions, while in bacteria it is present in the cytoplasm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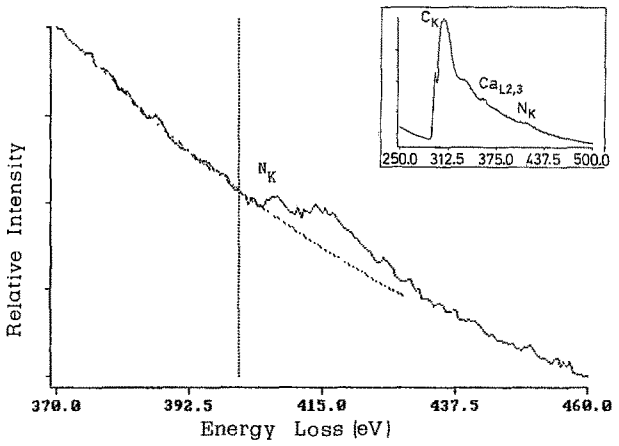

Fig. 19

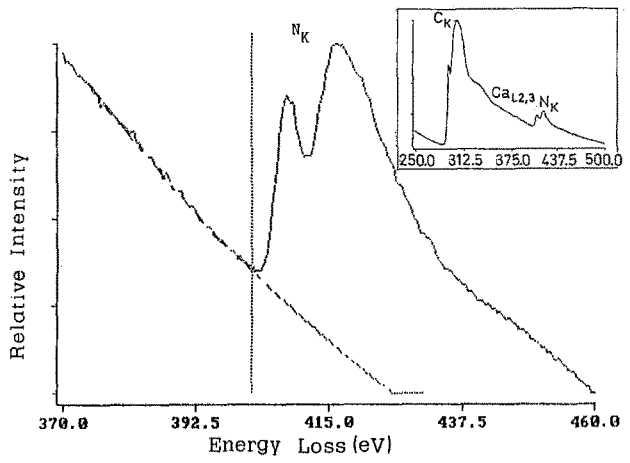

Fig. 20

Figs. 19 and **20.** Expanded range out of EELS spectra shown in the insets. The dotted lines below the spectra represent the calculated background curve for the N_K range. The vertical lines are markers. The EELS spectra were respectively recorded in the cytoplasm of a hair cell (Fig. 4) and on a cyanophycin granule (Fig. 12). The nitrogen edge outline of the two spectra differs. **Insets** Presence of a C_K edge at 281 eV and of a $Ca_{L2,3}$ at 346 eV. The core-loss integral of the edges contributed by nitrogen between 401 and 425 eV (the areas above the dotted lines) are 94.159 and 707.496, respectively

tamate by the fern, and then translocated apo- or symplastically to the other parts of *Azolla* and probably from the apexes to the vegetative cells of *Anabaena* (Calvert et al. 1985). Despite the primary role attributed to primary branched hairs which would function in the partitioning of *A. azollae* filaments into the leaf cavities (Peters and Meeks 1989), the transfer cell ultrastructure of their terminal cells could be functional in supplying nitrogen to the apical colonies. Consequently, it has been hypothesized that the ammonium assimilation could be due to the body cells of the secondary branched hairs (Calvert et al. 1985).

Our observations agree only in part with this hypothesis. However, the presence in the apexes of nitrogen compounds bound to the cell structure of the primary branched hairs and inside the vegetative cells of *Anabaena* would support the hypothesis of a nitrogen transport through the hairs to the apical colonies of the phycobiont (Calvert et al. 1985). Moreover, the supply of nitrogen compounds to the trichomes of *Anabaena* could act as one of the mechanisms by which the cyanobiont is partitioned into the developing leaf cavities. The nitrogen rich layer on the outer surface of the cell wall of all epidermal hairs, which do not disappear in senescent stages, could also be related to the presence of surface cell antigens produced by *Azolla* and interacting with *Anabaena* filaments. This type of cell-cell interaction was recently supposed to trigger the translocation of fixed nitrogen products from the cyanobiont to the host (Tel-Or et al. 1991).

In comparison to the branched hairs, simple hairs showed very little amounts of nitrogen in the cytoplasm, thus supporting the hypothesis that they do not contribute to nitrogen exchanges, but mainly to carbon supply (Calvert et al. 1985).

A. azollae and bacteria appeared more rich in nitrogen compounds at the group III of leaf cavities, where there is the highest nitrogen fixation activity and heterocyst frequency (Canini et al. 1990), thus indicating that the great availability of fixed nitrogen compounds can be utilized by all the symbionts.

No or very few aggregates containing nitrogen have been found inside the mucilaginous matrix in which *A. azollae* and bacteria are embedded. No nitrogen was detected inside the mucilage of the basal leaf cavities of the same strain of *A. filiculoides*, where it was shown a ten-fold higher concentration of soluble ammonium

Figs. 15–18. Leaf cavity of group IV. Bars: 2 μm

Figs. 15 and **16.** ΔE = 410 eV (Fig. 15), ΔE = 370 eV (Fig. 16). Longitudinal section of a mature simple hair of *Azolla* and part of a lysed vegetative cell of *Anabaena*. The hair cell does not show relevant presence of nitrogen compounds. The only cyanophycin granule (arrowhead) inside the *Anabaena* cell indicates its nitrogen composition

Figs. 17 and **18.** ΔE = 410 eV (Fig. 17), ΔE = 370 eV (Fig. 18). Vegetative cells of *Anabaena* with the progressive vacuolization of thylakoid membranes and accumulation of carboxysomes in the cytoplasm. Cyanophycin granules appear near the cross walls of adjacent cells

if compared to the apexes and to the leaves of group III (Canini et al. 1990). The impossibility to localize nitrogen in soluble compounds arose from the washing out of this fraction during conventional fixation or from the usual retention of only water-insoluble elements bound to a proteinaceous matrix in the Epon embedded material (Sorber et al. 1991). Moreover, any noticeable amount of ammonia could be detected after extraction of soluble or detergent treated fraction of *A. filiculoides* (Tel-Or et al. 1991). However, peptidic material was confirmed by tannic acid fixation to be a component of the mucilaginous matrix in different *Azolla* species (Grilli Caiola and Albertano 1988).

The EELS spectra taken at the N_K edge of cyanophycin granules inside heterocysts of *A. azollae* appeared characterized by a double peak that differed from the N-peaks obtained in the "honey-comb" cytoplasmic area of the heterocysts of *Anabaena* as well as from those recorded in the cytoplasmic areas under the cell wall of the leaf hairs of *Azolla*. This latter difference is possibly related to the enzymatic pathways which are metabolizing the ammonium through glutamine synthetase and glutamate synthase in *Azolla* and *Anabaena*, but by glutamate dehydrogenase in epidermal hairs (Peters and Meeks 1989).

The observation of a calcium peak in the cyanophycin agrees with the reports of Albertano and Grilli Caiola (1991 b) on the vegetative cells of *A. azollae* and with those of Golecki and Heinrich (1991) on *Nostoc* and *Gloeothece*. Further comparison of EELS spectra of standard nitrogen compounds with those hereby reported should probably indicate the exact atomic composition of the nitrogen compounds found inside the cells.

In conclusions, the possibility to localize nitrogen in ultrathin sections of plant cells has given a new insight for the understanding of the *Azolla-Anabaena* relationships. On the basis of the results obtained, the knowledge of the subcellular localization of nitrogen could be the basis of further researches on the localization of other elements which could be essential for the functioning of the symbiosis.

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