

Cytoarchitecture of the desiccation-tolerant green alga *Zygonium ericetorum*

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Received: 17 February 2009 / Accepted: 30 April 2009 / Published online: 16 May 2009
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Abstract In this study, the filamentous green alga *Zygonium ericetorum* (Zygnematales, Chlorophyta), collected at its natural habitat in the high alps, was investigated by light, scanning, and transmission electron microscopy. The field samples were separated into a moist fraction when wetted by splattering water of a nearby spring or a desiccated one when visually dried out. Light microscopy demonstrated a purple pigmentation of the sun-exposed upper layers, the central position of the nucleus, and the starch content in the pyrenoids. The smooth surface of the cells occasionally covered with fungal hyphae was shown by scanning electron microscopy. The cytoarchitecture of moist cells revealed many vacuoles and only a thin cytoplasmic area surrounding the two chloroplasts. The secondary cell walls of older cells were up to 4 µm thick. Organelle membranes as well as thylakoid membranes occasionally showed an inversion of contrast. In the chloroplasts, distinct areas with granular content surrounding the pyrenoids were detected. Within the cytoplasm, electron-dense particles with electron-translucent crystalloid structures were observed. When desiccated samples were investigated, the vacuoles and cytoplasmatic portions appeared destroyed,

whereas nucleus and chloroplasts generally remained intact. The thylakoid membranes of desiccated samples showed lumen dilatations and numerous plastoglobules. Water-soluble extracts were separated by high-pressure liquid chromatography that revealed two major compounds with UV-absorbing capacities.

Keywords Green algae · Desiccation · Ultrastructure · Electron microscopy

Introduction

When looking for organisms that have the capacity to survive desiccation, bare soil is a good choice (Hoppert et al. 2004; Nienow 1996; Trainor and Gladych 1995). Edaphic algae are at least periodically exposed to desiccation stress, and this may last for prolonged time periods depending on the habitat. Among green algae, some filamentous Zygnematales may form soil crusts together with cyanobacteria and lichens (Büdel 2002, 2005).

For this study, we have selected *Zygonium ericetorum* as this species forms green mats on the soil in a high alpine habitat. The study site in the high alps was chosen because cells are exposed to harsh environmental conditions with short vegetation periods, high irradiation, and a chance of natural desiccation through wind exposure (Körner 2003). *Z. ericetorum* is a cosmopolite, found in a variety of habitats, and all of them have been described as acidic and with low nutrient content (Hoppert et al. 2004; Lynn and Brock 1969; Pope and Pyatt 1984). Upon desiccation, this alga forms conspicuous macroscopic paper-like sheets, and upon rewetting a recovery of physiological processes is possible (Hoppert et al. 2004). Sun-exposed cells turn purple, a fact that caused debate over the nature and role of

Dedicated to Professor Cornelius Lütz on the occasion of his 65th birthday

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this pigment for a long time and is still not yet solved (Alston 1958; Lagerheim 1895; Pope and Pyatt 1984). Similar pigments have been described in some other members of the Zygnematales (e.g. Hoppert et al. 2004; Nishizawa et al. 1985; Remias et al. 2009). Despite the surviving potential of *Z. ericetorum* in harsh environments, no modern cytological studies (besides the classical works by Fritsch (1916), Gau (1934), and Transeau (1933)) have been performed.

In the field, the alga can be relatively easily harvested and, as the mats contain almost exclusively this species, samples can be subjected directly in the field to preparation for the transmission electron microscope (TEM). As it turned out, this is of substantial advantage when attempting to get a reasonable picture of the ultrastructure of naturally exposed *Z. ericetorum* cells. Many phenomena may not be observable when simply using cultured material under standard conditions.

Very limited information on structural changes as a consequence of desiccation is available in green algae (for a recent summary see Holzinger 2009). The situation is different for vascular resurrection plants, where information on structural and ultrastructural changes is available for different species (for a recent review, see Moore et al. (2009)). We are aware that the presently used attempt in simply comparing “moist” versus “desiccated” field samples is a rather simple strategy. This however allowed us to analyze the effects of desiccation in situ, giving valuable information on generated changes. Algae like *Z. ericetorum* are difficult to cultivate. They can survive under standard culture conditions but cannot develop their full potential like producing purple pigmentation in sun-exposed layers in the field. This pigmentation is lost in cultivated samples. In general, many aspects of adaptation strategies may be overseen when investigating only laboratory-grown organisms.

Materials and methods

Sampling site and cultivation methods

Mats consisting of *Z. ericetorum* (Kütz.) from the high alps at the “Schönwieskopf” (GPS data: N 46°50.998', E 11°00.903' reported with Garmin eTrex) near Obergurgl, Ötztal, Austria (2,350 m a.s.l.) were collected during the summer seasons of 2007 and 2008 (June, July) on sunny days in the early afternoon. The field samples were separated in “moist” when wetted by splattering water of a nearby spring or “desiccated” when visibly dried out. The pH of the spring was measured at pH=5.9. Samples were either fixed directly at the growing site (see below) or transferred to liquid Bold's basal medium (Bischoff and

Bold 1963) for cultivation, incubated at ~30 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, 12°C, 12/12 h light/dark regime, and stored at the Culture Collection of the University of Innsbruck (Gärtner 1996).

Light microscopy and staining procedures

For light microscopy, either an Olympus BH-2 light microscope (Olympus, Tokyo, Japan) with a ProgRes C10 plus Jenoptic digital camera and PICEd Cora image analysis software (Jomesa Meßsysteme) or a Zeiss Axiovert 200 M microscope (Carl Zeiss AG, Oberkochen, Germany), using a Zeiss Axiocam MRc5 camera with Zeiss Axiovision Software for image generation, was used. Algal filaments were stained with one of the following solutions: methylene blue for cell walls and general staining, Lugol's iodine solution for starch, Sudan IV (pre-dissolved in isopropanol) solution for lipids. For nuclear visualization, cells were stained with 2 μM syto 16 dye (Molecular Probes, dissolved in water from 1 mM stock in DMSO). After the latter staining, fluorescence images were captured with a Zeiss filter set 09 (excitation BP 450–490 nm, emission LP 515 nm long pass). Additionally, autofluorescence was recorded using the Zeiss filter set 01 (excitation 365/12 nm, emission LP 397 nm).

Scanning electron microscopy

For scanning electron microscopy (SEM) investigation, moist field samples of *Z. ericetorum* were first transferred into distilled water and dehydrated in a series of ethanol with increasing concentration (30 min each). For chemical dehydration, the material was transferred to formaldehyde-dimethyl-acetal (FDA, dimethoxymethane) for 24 h, followed by FDA for 2 h (for methods, see Gerstberger and Leins (1978) and Tschaike et al. (2008)). The sample was critical-point-dried with liquid CO₂ using FDA as intermediate, sputter-coated with gold-palladium, and examined with a Philips XL20 SEM microscope at 10 kV.

Transmission electron microscopy

Moist and desiccated field samples and cultivated algae were fixed either in 10 mM cacodylate buffer, pH=6.8, containing 1.25% glutaraldehyde, or in 50 mM cacodylate buffer, pH=6.8, containing 2.5% glutaraldehyde for 1.5 h. After extensive rinsing steps, samples were postfixed, respectively, in 10 or 50 mM cacodylate buffer containing 1% OsO₄ for 14–17 h at 4°C. Alternatively, samples were fixed by a potassium hexacyanoferrate fixation protocol or high-pressure freeze fixation followed by freeze substitution with 1% OsO₄, 0.2% uranyl acetate in acetone (for a summary of the methods and their original citations, see

Holzinger et al. (2009)). All samples were dehydrated in increasing ethanol concentrations, embedded in Spurr's resin or low-viscosity embedding resin (Agar Scientific, England). Ultrathin sections (60 nm) prepared with a Leica Ultracut were counterstained with uranyl acetate and Reynold's lead citrate and investigated at a LIBRA 120 transmission electron microscope at 80 kV. Images were captured with a ProScan 2k SSCCD camera, camera controller: "sharp:eye" controlled with OSIS iTEM software.

High-pressure liquid chromatography

Water-soluble algal constituents were analyzed by diode array detector (DAD) high-pressure liquid chromatography (HPLC), using an Agilent 1100 ChemStation with a Phenomenex Rezex RCM resin column, thermostated at 80°C. The solvent was ultrapure water with a flow rate of 0.6 ml/min and each run lasted for 30 min. Sample preparation: algal filaments were freeze-dried on glass fiber filters for 24 h and disintegrated with a grinding mill according to Remias and Lütz (2007). Afterwards, the material was suspended in 2 ml ultrapure water. Prior to injection, the extract was centrifuged (10,000×g, 10 min) and filtered through a 0.45-µm syringe filter.

Results

Light microscopy

When viewed under the light microscope, moist field samples from the sun-exposed upper layers of the *Z. ericetorum* mats had a purple appearance, both in younger (Fig. 1a) and older (Fig. 1b) developmental stages. The cylindrical cells were typically 18–20 µm wide and two to four times longer than broad, depending on the number of divisions performed. The two chloroplasts were pillow-shaped, surrounded by small unpigmented particles. The nucleus is found in the center of the cells (Fig. 1a, g). Due to additional duplications, older filaments contained shorter cells, and the cell walls reached a width of up to 4 µm. Upon cultivation, the cells lost their purple coloration, and the axial chloroplasts appeared light green (Fig. 1c). When stained with methylene blue, the cell walls of young cells became visible, and especially in vicinity to the chloroplasts blue dots were obvious (Fig. 1d). After staining with Lugol's iodine solution, two pyrenoids were visible in the chloroplasts of each cell (Fig. 1e), whereas staining with Sudan IV did not yield any particular coloration of a distinct structure (Fig. 1f). Fluorescence microscopical observation of syto 16-stained cells gave a clear visualization of the central nucleus combined with a little background fluorescence in the vacuoles and the marked red

autofluorescence of the chloroplasts (Fig. 1g). When exciting unstained cells with a wavelength of 365 nm, a marked blue fluorescence in distinct parts of the cells was visible (Fig. 1h).

Scanning electron microscopy

Z. ericetorum filaments had a smooth surface without any particular ornamentation (Fig. 2a, b). The algal mat contained virtually only this species; occasionally, smaller diatoms were observed (Fig. 2b). The ends of the cells were also smooth; when broken apart, a small cell wall ring remained at the top (Fig. 2c). Most interestingly, several cells were covered with filament-like structures, presumably fungal hyphae (Fig. 2d). These structures grew centrifugally away from the cellular surface. Minute spore-like objects, presumably of fungal origin, covered the whole surface of older cells (Fig. 2e).

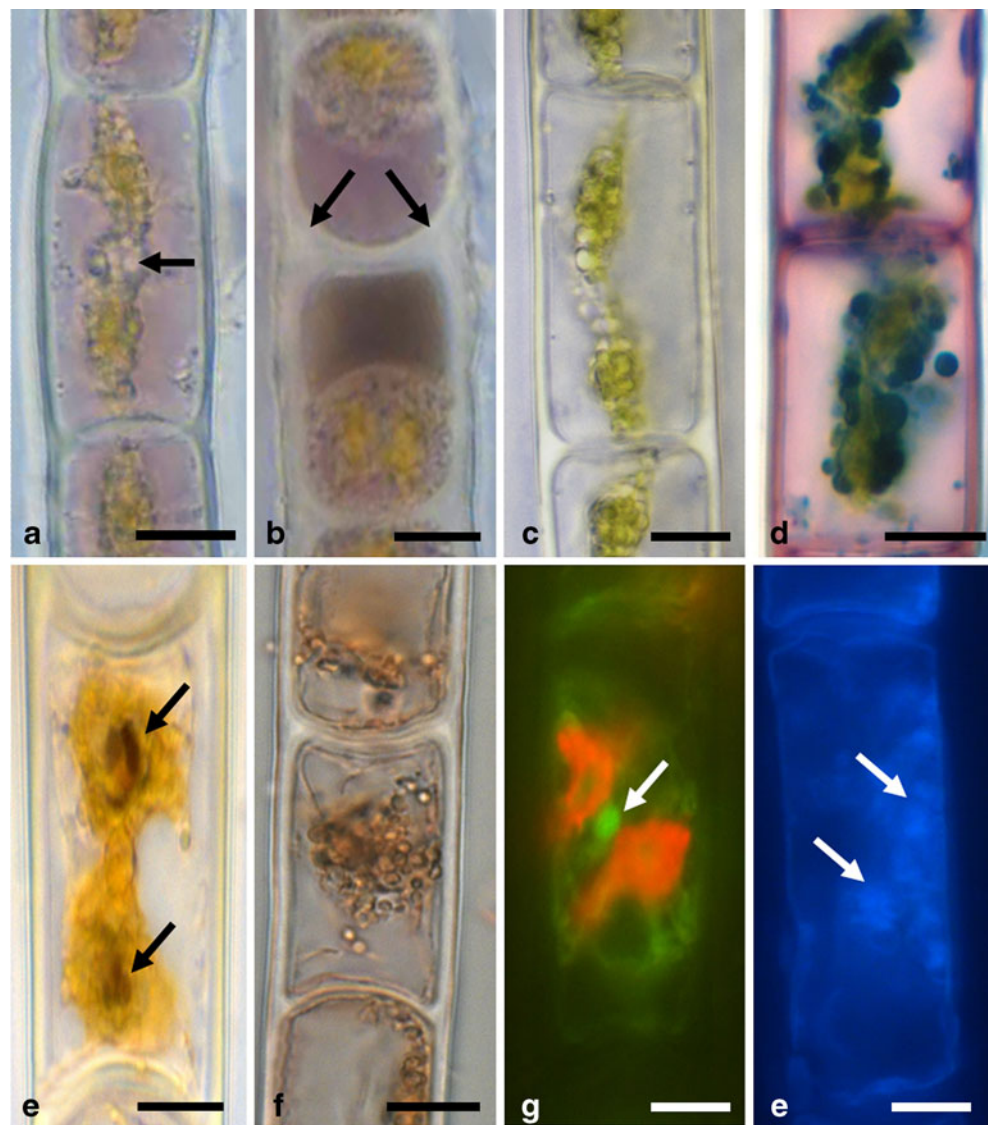
Transmission electron microscopy

Z. ericetorum was extremely difficult to fix for transmission electron microscopy. Most of the initial attempts using 50 mM cacodylate buffer and 2.5% glutaraldehyde, a potassium hexacyanoferrate fixation protocol, or attempts using high-pressure freeze fixation followed by freeze substitution failed to give a reasonable preservation of the ultrastructure (data not shown). When reducing the buffer concentration to 10 mM and the glutaraldehyde content to 1.25%, a reasonable proportion of the highly vacuolated cells showed good preservation of the ultrastructure (Figs. 3, 4, 5, and 6). Moist (Figs. 3, 4, and 5) and desiccated (Fig. 6) field samples were fixed separately. During the observation at the TEM, younger and older cells could be distinguished. It is important to state that all samples shown here have been directly fixed in the field at the collecting site.

The cells are dominated by two chloroplasts, each with a pillow-shaped central part (Fig. 3a–d) holding the pyrenoid (Fig. 3a–d) and irregular radiating processes (Fig. 3a, b). The pyrenoid was surrounded by large starch grains, filling up most of the space (Fig. 3a–d). The central part of the pyrenoid appeared dense, electron-opaque, and invaginated by tubular structures (Fig. 3c). Most interestingly, the central pyrenoid was occasionally surrounded by round structures with a diameter of about 1 µm (Fig. 4a, b), filled in part with dense granular material (Fig. 4b).

The cells had several closely arranged larger vacuoles (Fig. 3a, b). While in younger cells the vacuoles had a homogenous electron-opaque appearance (Fig. 3a), in older cells the vacuoles had granulated contents (Fig. 3b). Toward the chloroplast, these vacuoles became fragmented. A "bubble-like" appearance of membranes was seen in the vicinity of the chloroplasts (Fig. 3a, b). The cytoplasmic

Fig. 1 Light and fluorescence microscopic images of *Z. ericetorum*. **a** Moist field samples of younger cells with purple pigmentation, nucleus (*arrow*) in the center; **b** moist field samples of older cells with rigid cell walls (*arrows*) and purple pigmentation; **c** cultivated cells, no purple pigmentation; **d** cultivated cells stained with methylene blue; **e** cultivated cells stained with Lugol's iodine solution, starch grains in the pyrenoid are stained dark (*arrows*); **f** cultivated cells stained with Sudan IV; **g** cultivated cell stained with syto 16 dye, nucleus in the center of the cell (*arrow*), red autofluorescence of the chloroplasts; **h** autofluorescence (excitation 365/12 nm, emission LP 397 nm filter) of cell from moist field sample; areas with bright blue fluorescence are marked with *arrows*. Bars 10 μ m



portion was hardly visible and appeared mostly granulated and electron-dense, including organelles like mitochondria (Fig. 4c, d) and Golgi bodies (Fig. 4d). Golgi bodies often showed an “inverted” contrast with electron-translucent membranes. In older cells of *Z. ericetorum*, the cell walls were up to 4 μ m thick and rigid (Fig. 5a–c). These cell walls contained a clearly distinguishable outer layer (Fig. 5b). In the regions where two cells of a filament meet, an electron-dense line was visible between the individual cells (Fig. 5c) and additional layers could be distinguished (Fig. 5c). Fungal hyphae on the outer surface of the cell wall were occasionally observed (Fig. 5d).

Within the cytoplasm, electron-dense structures with an electron-translucent crystalloid content were observed in moist and desiccated samples (Figs. 5b and 6a).

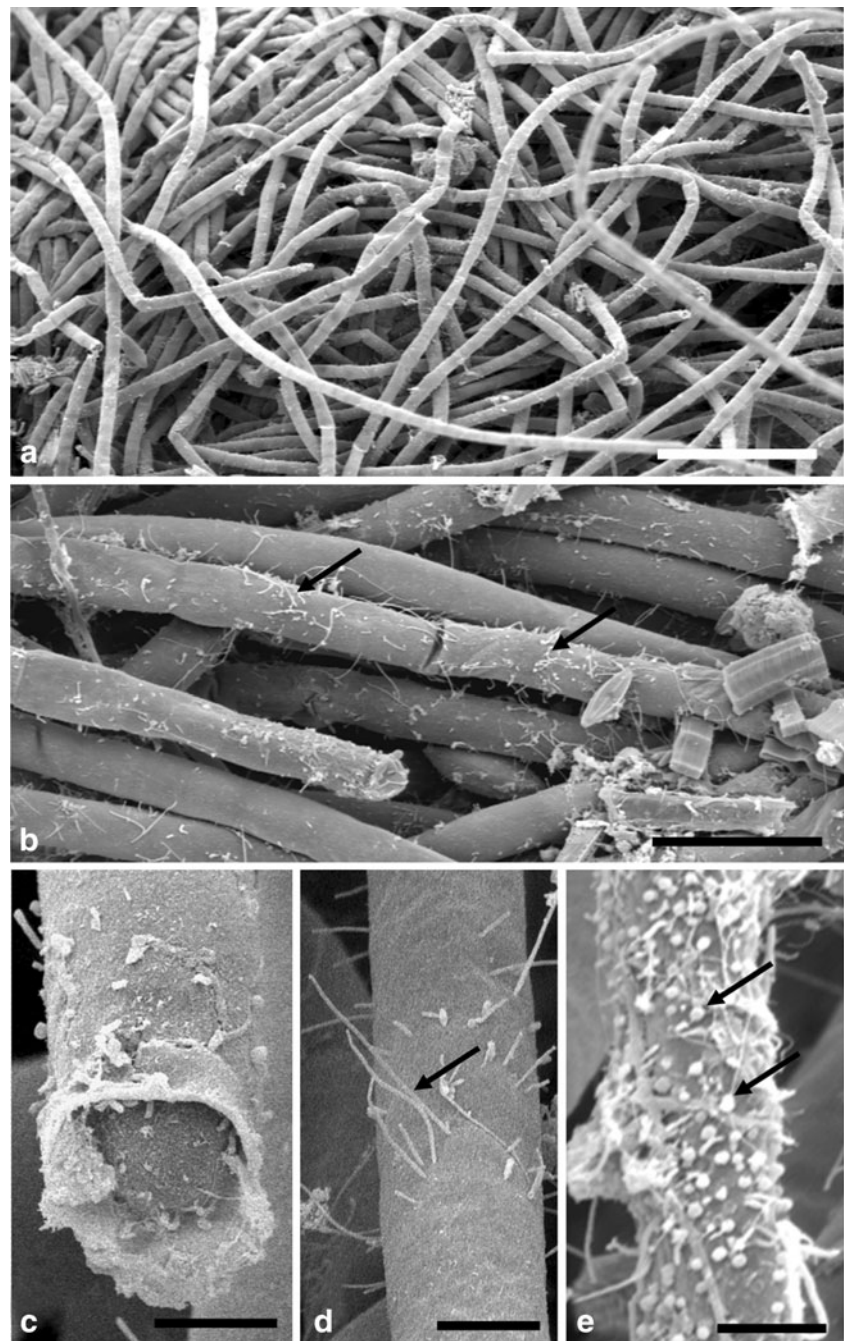
When desiccated filaments were fixed, a severely damaged appearance of the cytoplasmic and vacuolar portion of the cells was visible (Fig. 6a–d). Vacuoles

appeared lacerated and the cytoplasmic portion was in most parts electron-dense and accumulated (Fig. 6a). Numerous electron-dense particles were observed throughout the cytoplasm, in part with an electron-translucent crystalloid content (Fig. 6a). In contrast, the nucleus (Fig. 6a) and chloroplasts of the desiccated samples did not appear severely damaged, the latter structure showing thylakoid membranes with lumen dilatations (Fig. 6b–d). These chloroplasts contained a substantial amount of plastoglobules (Fig. 6a, d). The starch grains appeared to be reorganized, with an electron-translucent appearance and electron-dense surrounding (Fig. 6c, d).

HPLC analysis of constituents

At a wavelength of 230 nm, the DAD-chromatogram of *Z. ericetorum* showed two main peaks of water-soluble compounds. The spectrum of the first peak at retention time

Fig. 2 Scanning electron micrographs of moist field samples of *Z. ericetorum*. **a** Overview of the filaments; **b** detail, several algal cells are covered with filament-like structures, presumably fungal hyphae (*arrows*); **c** terminal region of a filament; **d** surface of an algal cell covered with filament-like structures (*arrow*); **e** individual filament covered with spore-like objects (*arrows*). Bars **a** 200 μm , **b** 50 μm , **c–d** 10 μm



7.9 min had an absorption peak at 270 nm and a shoulder at 380 nm as well as a slight absorption in the visible region. The spectrum of the second peak with a retention time 18.7 min had different characteristics with absorbance only at less than 330 nm and a maximum at 280 nm.

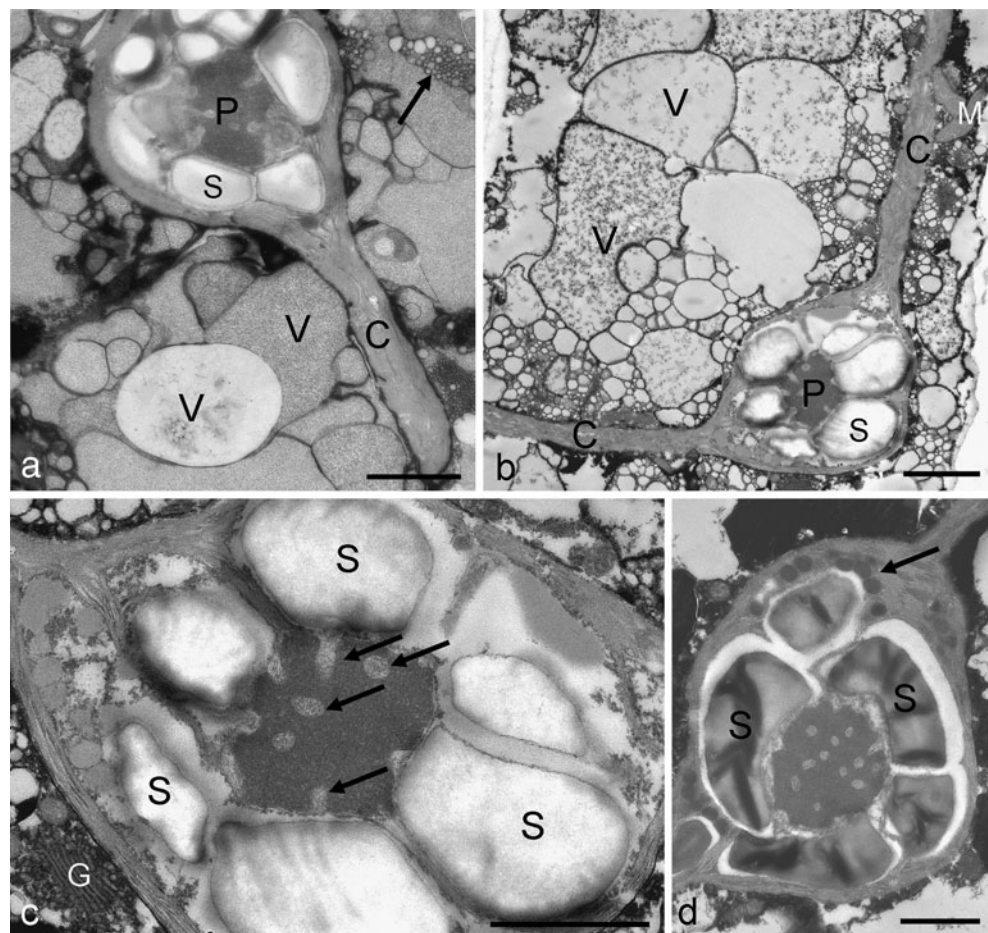
Discussion

This study described in detail the structural and ultrastructural aspects of *Z. ericetorum*, collected in a high-mountain

soil crust, its habitat known for harsh environmental conditions (Körner 2003). To our knowledge, this was the first study investigating the ultrastructure of this species in detail. Most interestingly, in desiccated samples, the nucleus and chloroplasts remain generally intact. Moreover, the occurrence of two UV-absorbing compounds could be of great significance for the species' adaptation to the environmentally stressful habitat. The chemical nature of these water-soluble compounds, however, remains to be identified.

The observed cell architecture was well in accordance with earlier observations (Fritsch 1916; Pope and Pyatt

Fig. 3 Transmission electron micrographs of moist field samples of *Z. ericetorum*. **a** Overview of younger cell showing vacuoles (*V*) with electron-opaque content, chloroplast (*C*) with pyrenoid (*P*), and starch grains (*S*), “bubble-like” membranous structures (*arrow*); **b** overview of older cell showing vacuoles (*V*) with granulated content, chloroplast (*C*) processes and a pyrenoid (*P*), mitochondria (*M*); **c** detail of the center of the same chloroplast as in **b**, central part of the pyrenoid with tubular invaginations (*arrows*), surrounded by massive starch grains (*S*), Golgi body (*G*) in vicinity of the chloroplast; **d** chloroplast of an older cell with pyrenoid, starch grains (*S*), plastoglobules (*arrow*); the cytoplasmic area appears electron dense. Bars **a** and **b** 1 μm , **c** and **d** 0.5 μm



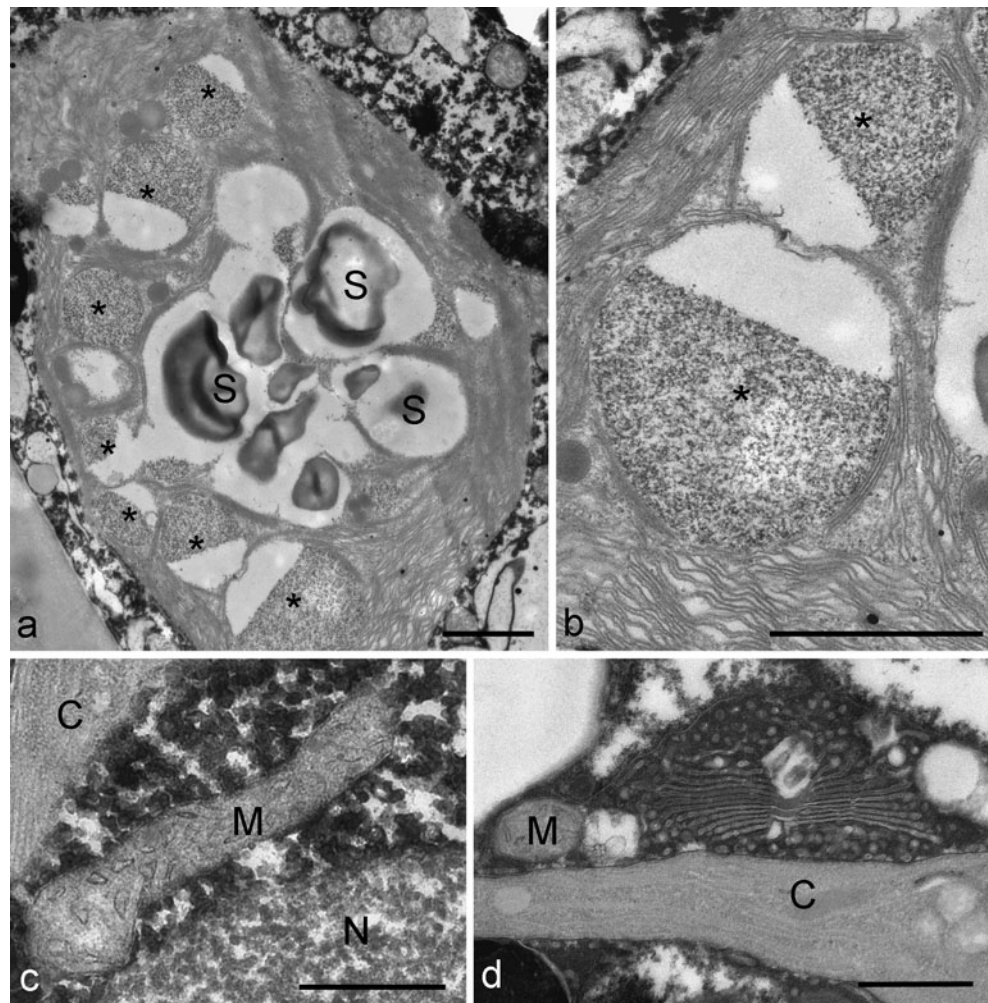
1984; Transeau 1933). The cells were cylindrical and contained two pillow-shaped chloroplasts with processes. The description of the chloroplast shape in the literature is rather vague, ranging from ovoid, weakly star-shaped, pillow-shaped, or more indefinite with irregular radiating processes (Brook and Johnson 2002). It appeared interesting that besides one not very-well-preserved overview image (Hoppert et al. 2004) that did not allow to depict much detail, no further images are available that visualize the ultrastructure of *Z. ericetorum* cells. From our own experiences, this might be due to the observation that standard procedures failed in preserving the ultrastructure well enough. Initially, we were surprised that chemical fixation protocols suitable for a wide range of chlorophycean green algae (e.g. Holzinger 2000; Holzinger et al. 2006; Kiermayer 1968; Meindl 1987; Pickett-Heaps 1972; Remias et al. 2009; Tschalkner et al. 2007) lead to a poor preservation of the ultrastructure in *Z. ericetorum*. This is likely related to a high osmotic value in the *Z. ericetorum* cells. Fritsch (1916) described that 15% sodium chloride (equivalent to ~ 2.5 M) was necessary to generate plasmolysis in the cells. Negative effects of high osmotic values that may lead to inverted contrast of membranes were described earlier, especially after high-pressure freeze

fixation and freeze substitution (Wesley-Smith 2001). However, it remained contradictory that a reduction of the buffer concentration may yield better results.

An interesting observation in *Z. ericetorum* chloroplasts was the round structures with granular content around the pyrenoids. These structures could contain polysaccharides. It could also be possible that these structures are dispersed masses of rubisco, as seen in a similar appearance in the hornwort *Anthoceros punctatus* (Vaughn et al. 1992). If this points toward a phylogenetic relationship (for details of Zygnemophycean phylogeny, see McCourt et al. (2000)), remains unclear. Occasionally observed cytoplasmic electron-dense compartments with a crystalloid content have also been reported in other green algae, but the biological function of these organelles still remains unknown (Holzinger and Lütz 2006; Remias et al. 2005).

Gray et al. (2007) gave a comprehensive comparison of the physiological effects when treating desiccation-tolerant desert algae and closely related aquatic relatives. They reported on the survival and recovery rates after a desiccation period in several Chlorophycean genera. The chlorophyll fluorescence F_v/F_m value fully recovered after rehydration in desiccation-tolerant species. However, no structural basis for this physiological observation was

Fig. 4 Transmission electron micrographs of moist field samples of *Z. ericetorum* (older cells). **a** Central part of the chloroplast with starch grains (S) surrounded by a ring of round compartments, partially filled with a granulated content, each marked with an asterisk; **b** detail with two round compartments (marked with asterisks) from the same cell; thylakoid membranes surround these structures; **c** detail in the vicinity of the nucleus (N) showing a mitochondrion (M) and part of the chloroplast (C); the plasma appears electron-dense; **d** detail with Golgi body and mitochondrion (M) in vicinity of a chloroplast (C). Bars **a** and **b** 1 μm , **c** and **d** 0.5 μm



given. The reason for a fast recovery of the photosystem could lay in the ability of certain organisms to keep key structural components intact during periods of desiccation (Holzinger 2009; Viceré et al. 2004). In *Z. ericetorum*, the chloroplasts, with occasionally occurring dilated thylakoid membranes, and the nucleus remain intact in desiccated samples. Hoppert et al. (2004) measured that *Zygonium*-dominated desiccated soil crusts did not show photosynthetic activity, which is corroborated by our TEM data, showing that starch grains have been modified or were virtually absent in desiccated samples. Instead, a larger proportion of plastoglobules (Austin et al. 2006; Ytterberg et al. 2006) was observed in desiccated samples, pointing toward an adaptation mechanism, also observed in higher plants (Moore et al. 2008).

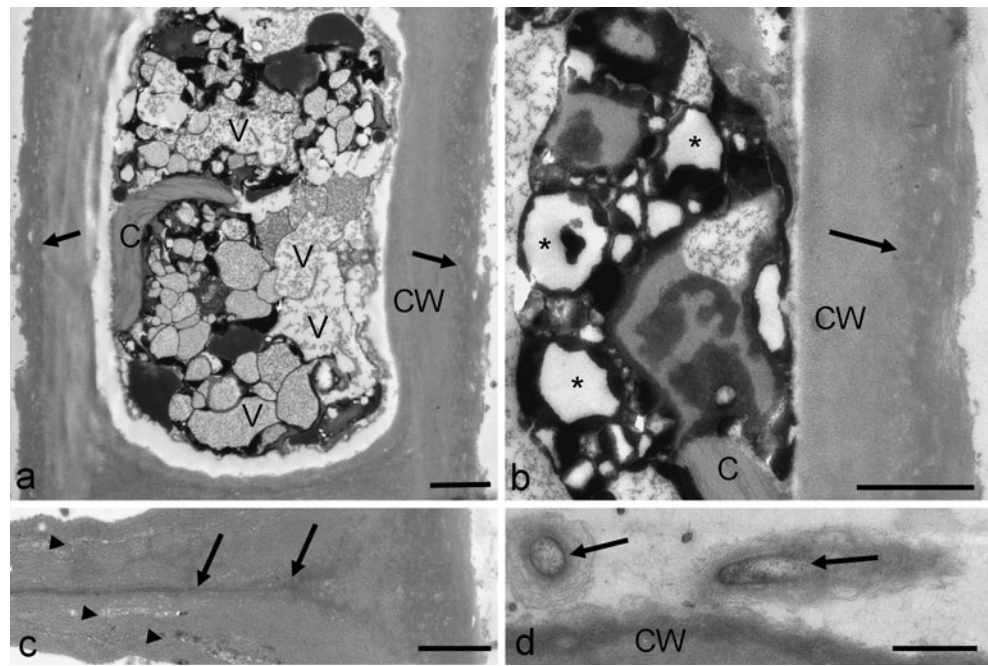
A thick and rigid cell wall of older cells of *Z. ericetorum* may have additional protecting effects (Hoppert et al. 2004). The same authors state that only a small amount of extractable polymers could be isolated from *Zygonium*-containing soil crusts. The fungus *Fusarium* sp. was described as parasitic in *Z. ericetorum* (Hoppert et al. 2004). We have observed fungal hyphae on the surface of

the cell walls, but we have never seen any sign of penetration of the fungus into the algal cells.

Reports of several authors (Lynn and Brock 1969; Pope and Pyatt 1984) that lipids would make the chloroplast difficult to be visualized have not been encountered in our study. When applying Sudan IV, a selective stain for lipids, no particular coloration was found. Moreover, the TEM data did not give any signs on the occurrence of lipid bodies as observed in other green algae from extreme habitats (Holzinger and Lütz 2006; Remias et al. 2005, 2009).

At the moment, little is known about the vacuolar purple pigmentation in *Z. ericetorum*. Despite the purple coloration has been mentioned already by Lagerheim (1895), no detailed analysis of these compounds is available. The assumption that the compounds are water-soluble anthocyanins (Alston 1958) has never been proven. There are also speculations about the occurrence of a pigment composed of ferric ions and gallo-tannins (Allen and Alston 1959). Gallo-tannins have been confirmed by NMR in the related genus *Spirogyra* sp. (Nishizawa et al. 1985). Recently, Remias et al. (2009) reported on similar

Fig. 5 Transmission electron micrographs of moist field samples of *Z. ericetorum* (older cells). **a** Cell overview with a massive cell wall (CW); two different layers are visible (arrows), vacuoles (V), and chloroplast (C); **b** massive cell wall (CW) with an outer cell wall layer clearly separated (arrow), electron-dense structures with electron-translucent crystals in the cytoplasm (marked with an asterisk), chloroplast (C); **c** cell wall area between two cells in a filament, separation marked with arrows; distinct layers of the cell wall separated by looser material are marked with arrowheads; **d** fungal hyphae (arrows) outside of the cell wall (CW). Bars **a** 2 μm , **b–d** 1 μm

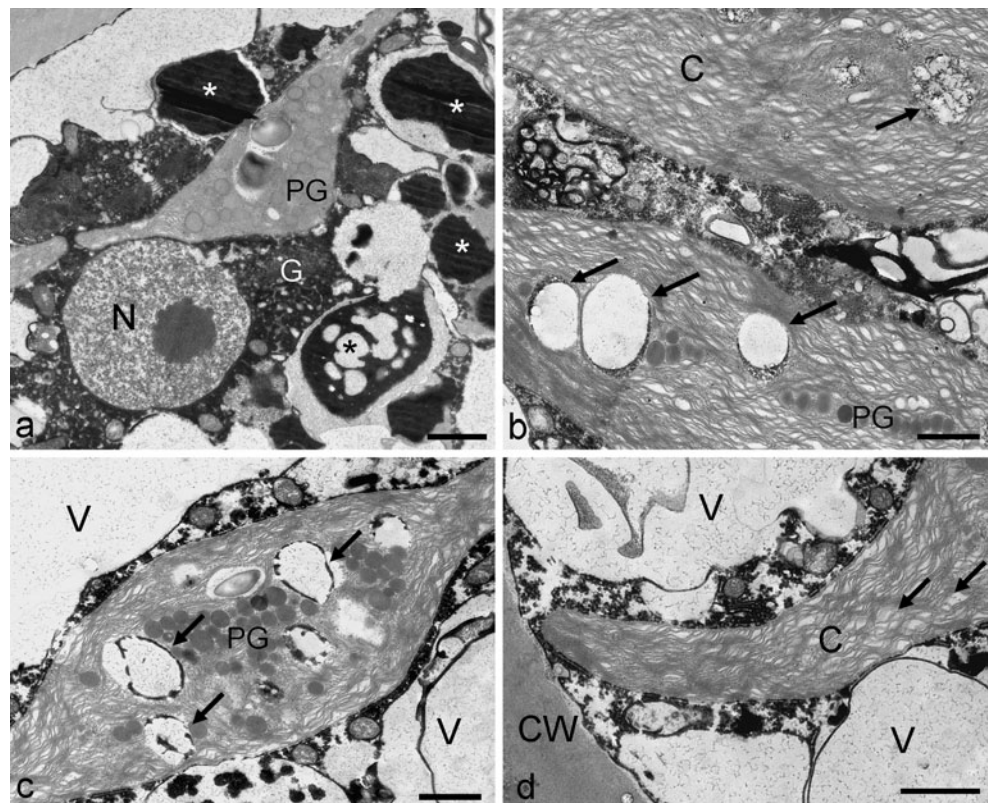


substances in *Mesotaenium berggrenii*, ice algae from alpine glaciers. Probably, these pigments could play a role in protecting the chloroplasts against excessive irradiation. In *Z. ericetorum*, the first UV-absorbing compound (retention time 7.9 min) absorbed in the UV-A and UV-B range and the second (retention time 18.7 min) in the UV-B

range. The first compound with slight absorption also into the visible range up to ~ 460 nm might be related to the purple pigmentation, whereas the second cannot be related due to the spectral characteristics.

Taken together, our observations demonstrated, besides the general cytoarchitecture, that desiccated samples of *Z.*

Fig. 6 Transmission electron micrographs of desiccated field samples of *Z. ericetorum*. **a** Overview with nucleus (N), chloroplast filled with plastoglobules (PG); cytoplasmic portion appears extremely electron-dense with Golgi body (G), numerous electron-dense particles (marked with white asterisks) or with electron-translucent content (marked with black asterisk); **b** chloroplast (C) architecture with modified starch grains (arrows) and plastoglobules (PG); **c** central part of the chloroplast with numerous plastoglobules (PG) and modified starch grains (arrows), lacerated vacuoles (V); **d** chloroplast (C) with dilated thylakoid membranes (arrows), cytoplasmic portion destroyed and electron-dense remnants, lacerated vacuoles (V), cell wall (CW). Bars 1 μm



ericetorum were able to maintain a chloroplast with thylakoid membranes, and a nucleus. The detailed mechanisms are not yet elucidated; however, similar strategies like in other green algae (Holzinger 2009) or other desiccation-tolerant organisms (Alpert 2006) are to be expected. Further investigations, especially concerning the soluble carbohydrate contents, as well as detailed characterization of the desiccation status would enhance our understanding of the adaptation strategies of this desiccation-tolerant organism.

Acknowledgement We would like to thank Werner Kofler from the Department of Systematics, Palynology and Geobotany, Institute of Botany for the expert technical help in performing the SEM studies. Access to the TEM of the Zoology Department, University of Innsbruck is kindly acknowledged. We thank Prof. Cornelius Lütz for critically proofreading the manuscript. This study was supported by a research grant from the “Universitätszentrum Obergurgl” to AH. The authors declare that they have no conflict of interest.

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