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# Dark-acclimation of the Chloroplast in *Koliella antarctica* Exposed to a Simulated Austral Night Condition

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## Abstract

The acclimation response of the chloroplast was studied in the green marine microalga *Koliella antarctica* exposed to a simulated austral night of 90 d. On the basis of the micro- and submicroscopic aspects observed, the photosynthetic pigment patterns monitored spectrophotometrically, and the course of the assembly of the PSII chlorophyll-protein complexes evaluated microspectrofluorimetrically in vivo on single living cells, it was established that the alga tolerates the stress of light absence when cultured in the laboratory. During the treatment, the organism undergoes substantial structural and functional reorganization of the plastid, resulting in the formation of a chlorochromoplast-like structure, suitable for the storage, in different times and in specialized structures, of the products coming from the breakdown of the pre-existing plastid constituents. On the whole, the dark acclimation occurs in two different steps: a first acclimation, quickly realized, during the first 6 d and maintained up to 20 d of darkness, and a second acclimation period, starting from the 21st day, maintained up to the end of the experiment.

## Introduction

Our understanding of how photosynthetic apparatus responds to environmental stress has largely been based on studies using higher plant tissue systems (Basra and Basra, 1997; Pinhero et al., 1999; Hormaetxe et al., 2004). By comparison, there is relatively less information available regarding microalgae (Bidigare et al., 1993; Hoham et al., 1998; Masojidek et al., 2004). This seems to be paradoxical considering that these organisms can be the only photosynthetic colonizers of extreme environments, where the biodiversity can be substantially reduced, even to a single species. The difficulty in finding these organisms and setting up culture conditions may account for the limited number of studies. Since the organisms colonizing extreme environments are mostly unicellular and have rather simple structures, studies on such organisms may also be relevant to the photosynthetic performance of higher plants in extreme environments.

Among extreme environments, polar regions are characterized by low temperatures and extreme variations in irradiance during the summer/winter cycle. Microalgae that inhabit these environments must be adapted to diverse extreme growth-light conditions from very high to low light irradiance, up to prolonged dark condition. Light appears to be the most important factor in the control of the algal communities of high latitudes (Lizotte and Sullivan, 1991; Lizotte and Priscu, 1992). In fact, the light climate of polar regions undergoes such extreme seasonal and spatial changes (Duntun and Dayton, 1995), that several species are obliged both to grow at low irradiance to achieve maximal growth rates, and to possess photoacclimatory mechanisms for protection against short-term photoinhibition (Bidigare et al., 1993; Kirst and Wiencke, 1995).

In polar regions the highest stability for living organisms is surely found in the marine waters. Actually, the antarctic marine environment has a complex nature and includes several subenvironments that differ in their physical, chemical, and biological characteristics (Grossmann et al., 1996; McMin, 1996). The microalgal biodiversity in these subenvironments is an interesting subject for studies. For instance, microalgal communities have been studied in sea-ice brine channels and ice-free sea waters (Scott et al., 1994; McMin, 1996; Andreoli

et al., 2000). Under the pack-ice cover, the “ice platelet” layers also constitute a favorable environment for development of ice biota (Grossmann et al., 1996).

The antarctic marine microalgae that are included in microbial assemblages associated with ice play an important ecological role, because, after the long antarctic night, they are the inocula for the spring algal bloom and form the energy base of the marine food chain (Andreoli et al., 2000, and references therein). It is clear that the continuity of life in the antarctic marine environment is closely dependent on the ability of microalgae to survive the austral night. For instance, some antarctic microalgae can behave as heterotrophic organisms during the polar night (Friedmann, 1993; Bell and Laybourn-Parry, 2003). However, the study of the cell acclimation in microalgae adapted to prolonged darkness remains a seldom treated biological argument. The survival of these microorganisms is surely related to deep structural, physiological, and biochemical changes at the cellular level, which involve the photosynthetic apparatus and are still not understood. In particular, it is not known how adapted microalgae modulate the structure and function of their chloroplast membranes in response to prolonged darkness. Thylakoid function and structure are in turn influenced by the modulation in the relative proportion of light-harvesting pigments, PSII and PSI reaction centers, electron carriers, and ATP synthetase (Anderson, 1986).

A series of studies was undertaken on *Koliella antarctica* (Andreoli et al., 1998), a green marine microalga recently assigned to Trebouxiophyceae (Andreoli et al., 2000), which is adapted to conditions of very low light intensity in the Ross Sea (Antarctica), where polar nights are characterized by a long twilight and a 3-mo period of complete darkness. The first studies on the morphological and biochemical characterization of the alga were published by Andreoli et al. (1998, 2000), Piro et al. (1997, 2000), and Zanetti et al. (2001). Our attention was focused on the morphological and biochemical variations that occur in the photosynthetic apparatus when the alga, grown under low light conditions similar to that of the field, is exposed to 3 mo of complete darkness under a simulated austral night condition. We examined micro- and submicroscopic changes that occur in plastids

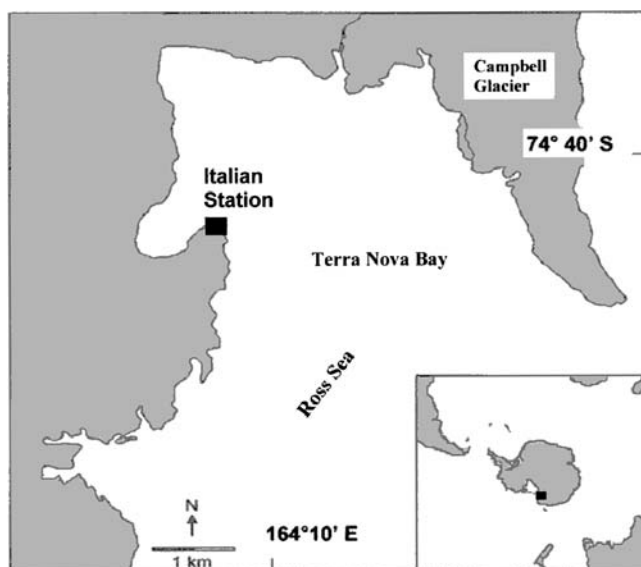


FIGURE 1. Location of the Italian Station at the Terra Nova Bay (Ross Sea, Antarctica).

during dark acclimation, and whether these changes are correlated with the variations in the photosynthetic pigment patterns evaluated spectrophotometrically. We also examined the correlation with the assembly degree of the Chl-protein complexes of PSII, monitored microspectrofluorimetrically in vivo.

## Material and Methods

### PLANT ORGANISM AND CULTURE CONDITIONS

*Koliella antarctica* Andreoli, Lokhorst, Mani, Scarabel, Moro, La Rocca et Tognetto was isolated during the austral summer from samples of ice-free sea water of Terra Nova Bay (Ross sea, Antarctica, 74°41'60"S, 164°07'60"E, Fig. 1) (Andreoli et al., 1998). It was maintained axenically in sea water enriched with F/2 medium (Guillard, 1975) under conditions almost similar to those of origin:  $5 \pm 1^\circ\text{C}$  temperature, 34‰ salinity, continuous light ( $8 \mu\text{mol m}^{-2}\text{s}^{-1}$  PAR, white day-light fluorescent Philips tubes), no shaking (Andreoli et al., 1998; Zanetti et al., 2001).

For the experiments reported in this research, 10 mL of the stock suspension ( $10 \times 10^6$  cells  $\text{mL}^{-1}$ ) were inoculated into culture flasks containing 90 mL of F/2 medium enriched sea water and grown under the above culture conditions in a thermostatically controlled incubator (doubling time of 8.5–10 d). When the culture suspension reached the concentration of about  $5 \times 10^6$  cells  $\text{mL}^{-1}$ , it was placed in the dark and maintained for 3 mo in complete darkness; the other culture conditions remained unchanged. Cell number was controlled throughout the experiment. Micro- and submicroscopic observations, biochemical and microspectrofluorimetric analyses were carried out on vegetative cell samples collected at different time intervals from 0 (light condition) to 90 d of darkness.

Aseptic conditions were ensured throughout all procedures, and all manipulations of samples maintained in darkness were performed under a dim green safe-light, according to Stürzl and Schiff (1986). The entire experiment was replicated three times.

### CELL NUMBER

Cell number was periodically controlled by hemocytometer counts throughout the experiment (Zanetti et al., 2001).

### OPTICAL AND FLUORESCENCE MICROSCOPY

For in vivo microscopic observations, a Zeiss model Axiophot photomicroscope equipped with conventional or fluorescent attachments was employed. For fluorescence microscopy examinations, the light source was a HBO 100W pressure mercury vapor lamp, with a filter set for UV excitation: BP 436/10, FT 460, LP 470. High speed Fujifilm Superia 400 ASA was used for photography.

### TRANSMISSION ELECTRON MICROSCOPY (TEM)

Cells were harvested by centrifugation (500 g), washed with cacodylate buffer 0.1 M (pH 6.9) and fixed with 6% glutaraldehyde in the same buffer at  $4^\circ\text{C}$  for 24 h. After being rinsed with cacodylate buffer, cells were postfixed with 1%  $\text{OsO}_4$ , prepared in the same buffer, at  $4^\circ\text{C}$  for 2 h. After rinsing, the pellets were embedded in 1% agar and then dehydrated in a graded ethanol series (Pancaldi et al., 2001). Embedding in Araldite-Epon resin and staining procedures for TEM were performed as previously described by Pancaldi et al. (2001). Observations were made with a Hitachi H800 electron microscope (Electron Microscopy Centre, Ferrara University, Italy).

### X-RAY MICROANALYSIS

X-ray microanalysis for iron ( $K_\alpha = 6.4$  keV) was performed on the same samples prepared for TEM. Thin sections were observed with a Cambridge S 360 scanning electron microscope, equipped with a Link Analytical W.D.S. spectrometer (Electron Microscopy Centre, Ferrara University, Italy) (Bonora et al., 2000).

### PIGMENT ANALYSES

#### Pigment Extraction

Cell suspensions of the alga were pelleted by centrifugation at 500 g for 5 min and the supernatant fluid was removed. Cells were mixed with sand quartz and cold acetone and broken by a strong sonication with a Microson Ultrasonic Cell Disruptor (Heat System Inc., New York, USA), then they were extracted with absolute acetone at  $-20^\circ\text{C}$ . The extraction procedure was repeated until the pellets appeared grayish-white. Samples were stored at  $-20^\circ\text{C}$  until analysis. All the operations were carried out on ice under a dim green safe-light to avoid photodegradation.

#### Spectrophotometric Determination

Absorption spectra were recorded at room temperature, 380 to 750 nm range, with a Perkin-Elmer model 554 UV-VIS double beam spectrophotometer at band pass 1 nm. The concentration of the total photosynthetic pigments (chlorophylls *a* and *b*, and carotenoids) was determined using the Lichtenthaler's equations (1987).

### MICROSPECTROFLUORIMETRY

This method constitutes a useful nondestructive tool for studying the assembly kinetics of photosystem II (PSII) under physiological conditions (Pancaldi et al., 2002).

#### Acquisition of Spectra

In accordance with Pancaldi et al. (2002), fluorescence emission spectra were recorded using a microspectrofluorimeter (RCS, Florence, Italy), associated with a Zeiss model Axiophot photomicroscope. The spectra were recorded in vivo, at room temperature, on single living cells that were selected using the microscope under fluorescent light (1000 times magnification).

The excitation light (436 nm) was focused on a single cell at a time, using a 1.6-mm diaphragm. The emission light was collected by the objective lens and deviated to the detector system which included a monochromator reticle (band pass 0.25 nm), endowed with a computer-assisted stepper-motor, and a photomultiplier tube (PMT), coupled with an analogic/digital converter (ADC) for data transfer to the “Autolab” software (RCS, Florence, Italy). The Autolab software was also employed to set the recording range and optimize the photomultiplier response. Fluorescence levels, which were measured in arbitrary units directly established by the setting system, were visualized as emission spectra by the same program. For each cell sample at least four spectra were recorded.

#### Elaboration of Spectra

Elaboration of the spectra was performed with the “Origin 6.0” program (Microcal Software Inc.). For each elaboration, experimental spectra were normalized and averaged. The resulting graph was smoothed by averaging adjacent data points, by means of the “Adjacent averaging” smoothing function of “Origin 6.0”. The degree of smoothing was controlled by specifying the number of points (10) to be used for calculating each averaged result. The smoothed graph was resolved into Gaussian components (Pancaldi et al., 2002). Peak positions were determined on the 4th derivative of the curve in order to obtain the best deconvolution. In this way, the Gaussian multipeak fitting was performed by fixing the following starting parameters: (1) the number of components and their position, resulting from the 4th derivative; (2) the halfband-width, directly estimated by the computer program. The halfband-width was modified to yield the best fit, as indicated by the comparison of the experimental spectrum with the sum of Gaussian curves. Only the final result is reported.

Although the present method provides a precise definition of the components responsible for experimental curves, it does not allow quantitative evaluation. Nevertheless, a single experimental curve yields much information from the reciprocal relations between Gaussian components, whose areas were provided by the computing system.

## Results and Discussion

#### LIGHT CONDITION

When viewed with a light microscope, the light-grown vegetative cells of *K. antarctica* showed the same morphological characteristics described by Andreoli et al. (1998) and Zanetti et al. (2001). In particular, the alga formed short unbranched filaments that were made up of rows of coupled cells of about 10  $\mu\text{m}$  in length and 3 to 4  $\mu\text{m}$  in diameter without a gelatinous sheath (Fig. 2A). Moreover, when observed under UV light, the cells appeared normally red-fluorescent (Fig. 2B). Observations with TEM revealed that the cells were uninucleate and had a parietal agranal chloroplast, which partially surrounded the cell lumen (Fig. 3A). The chloroplast, which was devoid of pyrenoid, contained small starch grains and was filled with several lamellae, formed by two or three apposed thylakoids, regularly arranged along the longitudinal axis of the organelle (Fig. 3B and its insert). Spectrophotometric analyses of the acetone extracts revealed the presence of chlorophyll *a* (Chl*a*) and chlorophyll *b* (Chl*b*) and of a large amount of carotenoids (Car) (Table 1). Chl*a*/Chl*b* molar ratio was 1.43, i.e., a very low value due to the high content of Chl*b*. This value confirms that *K. antarctica* is certainly a shade-type alga. In fact, the ratio, which is an index of the available light in the green algae as in higher plant photosynthetic tissues, varies from 2.8 to 3.5 in the sun-type forms, from 2.3 to 2.8 in the shade-type forms, and up to 1.5 and minor levels in the extremely shade-type forms (Morgan et al., 1998; Ensminger et al., 2001). The high contents of Chl*b* and Car indicate the

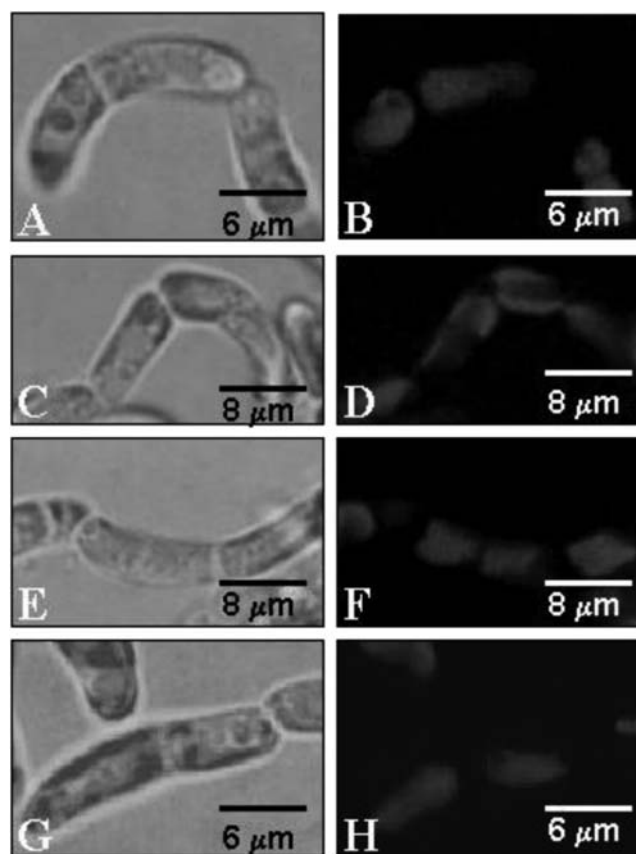


FIGURE 2. Light and fluorescence microscope aspects of *K. antarctica* cells cultured under continuous light (A, B) or in complete darkness for 3 (C, D), 21 (E, F), and 90 (G, H) days. The images show filaments of coupled cells in which the fluorescence of the chloroplasts under UV light persists, although with decreasing intensity, throughout the experiment.

presence of wide antennae, mainly of PSII (LHCII) (Anderson, 1986). This pigment pattern makes chloroplasts more effective in capturing the low light intensities that are especially enriched in green-blue and poor in red radiations, typical of the site of origin of *K. antarctica* (Lizotte and Priscu, 1992). Indeed, far-red and red light are attenuated and transmission is restricted to the blue-green region of the spectrum as the water depth increases (Larkum and Barret, 1983). Some green marine algae just contain special Car which increase the capacity for harvesting blue light (Hiller et al., 1991; Green and Durnford, 1996).

Microspectrofluorimetric analyses were problematic due to the rapid fluorescence decay in chloroplasts during observations. Therefore, the spectrum reported in Figure 4A was a collage of six records: short spectra of 20 nm, recorded from six cells, were joined together to cover the whole range (640–760 nm). The resulting spectrum was then smoothed and mediated in order to obtain a continuous profile. The rapid fluorescence decay can be due to the dissipation of the excess energy deriving from the light of the microscope mainly as heat, instead of fluorescence. The nonphotochemical quenching of the energy seems to be common in antarctic marine microalgae, which are very sensitive even to low light intensities and in which the excess of light absorbed is dissipated greatly as heat. This could also favor metabolic activities at the low temperatures that characterize their habitats (Giacometti et al., 2001). This process is mediated by Car, whose function is not only light harvesting in the blue region of the spectrum, but also the protection of the photosynthetic apparatus from photodestruction under strong light (Bidigare et al., 1993; Niyogi, 1999; Masojíddek et al., 2004). However, despite the practical difficulties in carrying out analyses, the spectrum

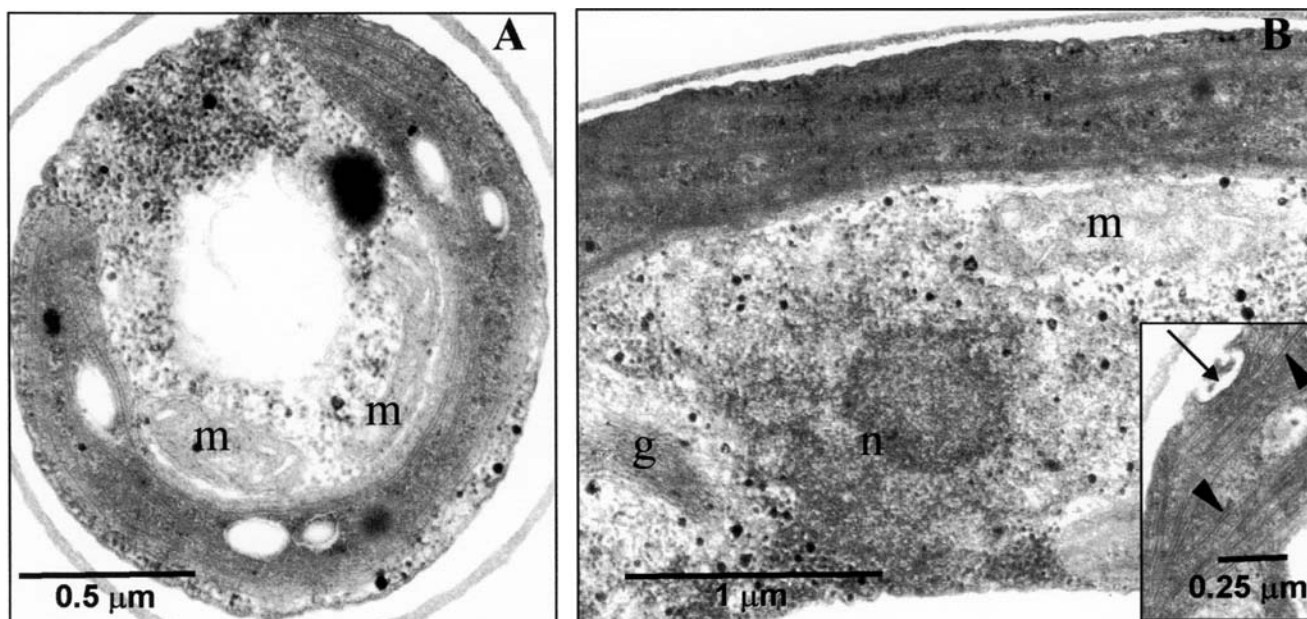


FIGURE 3. Electron microscope aspects of *K. antarctica* cells grown under continuous light. (A) Section through a cell showing a parietal chloroplast surrounding the cell lumen. Some mitochondria (m) are also visible in the cytoplasm. Note the absence of pyrenoid in plastid. (B) Section through a cell showing a conspicuous nucleus (n), a Golgi apparatus (g) and a chloroplast with the stroma filled by lengthened lamellae that, at higher magnification (insert), appear to be formed by two or three apposed thylakoids (arrow heads). A small starch granule is also visible in the stroma (insert, arrow).

was resolved into Gaussian components, analogous to those found also in other green algae and higher plants. In detail, the emission at 677 to 680 nm was ascribable to phaeophytin, the primary electron acceptor in the reaction center of the PSII (RC), as identified also in *Euglena* and higher plants (Ignatov and Litvin, 1994; Pancaldi et al., 2002; Baldisserotto et al., 2004). The emissions at 683 to 698 nm are characteristic of the PSII inner antennae (IA), i.e., the Chl-proteins of 43 kDa and 47 kDa (CP43–47) (van Dorssen et al., 1987; Vassiliev et al., 1995). Attribution of the emissions in the 702- to 715-nm spectral region to LHCII is supported by several evidences in higher plants (Zucchelli et al., 1992) and in algae (Vassiliev et al., 1995; Pancaldi et al., 2002; Ferroni et al., 2004). Other emissions were not relevant for this work.

The Gaussian deconvolution allowed for the evaluation of the RC/IA and LHCII/PSII ratios, where IA is the sum of the emissions of CP43 and CP47, and PSII is the sum of the emissions of the inner antennae and the reaction center. RC/IA and LHCII/PSII ratios were 0.34 and 0.45, respectively. These low ratios (i.e., below 0.5) show the normal PSII functionality, as inferred by comparison with ratios estimated in *Euglena gracilis* (Pancaldi et al., 2002), *Trapa natans* (Baldisserotto et al., 2004), *Pinus brutia*, *Phaseolus vulgaris*, and *Guzmania* sp. (unpublished).

#### DARK CONDITIONS

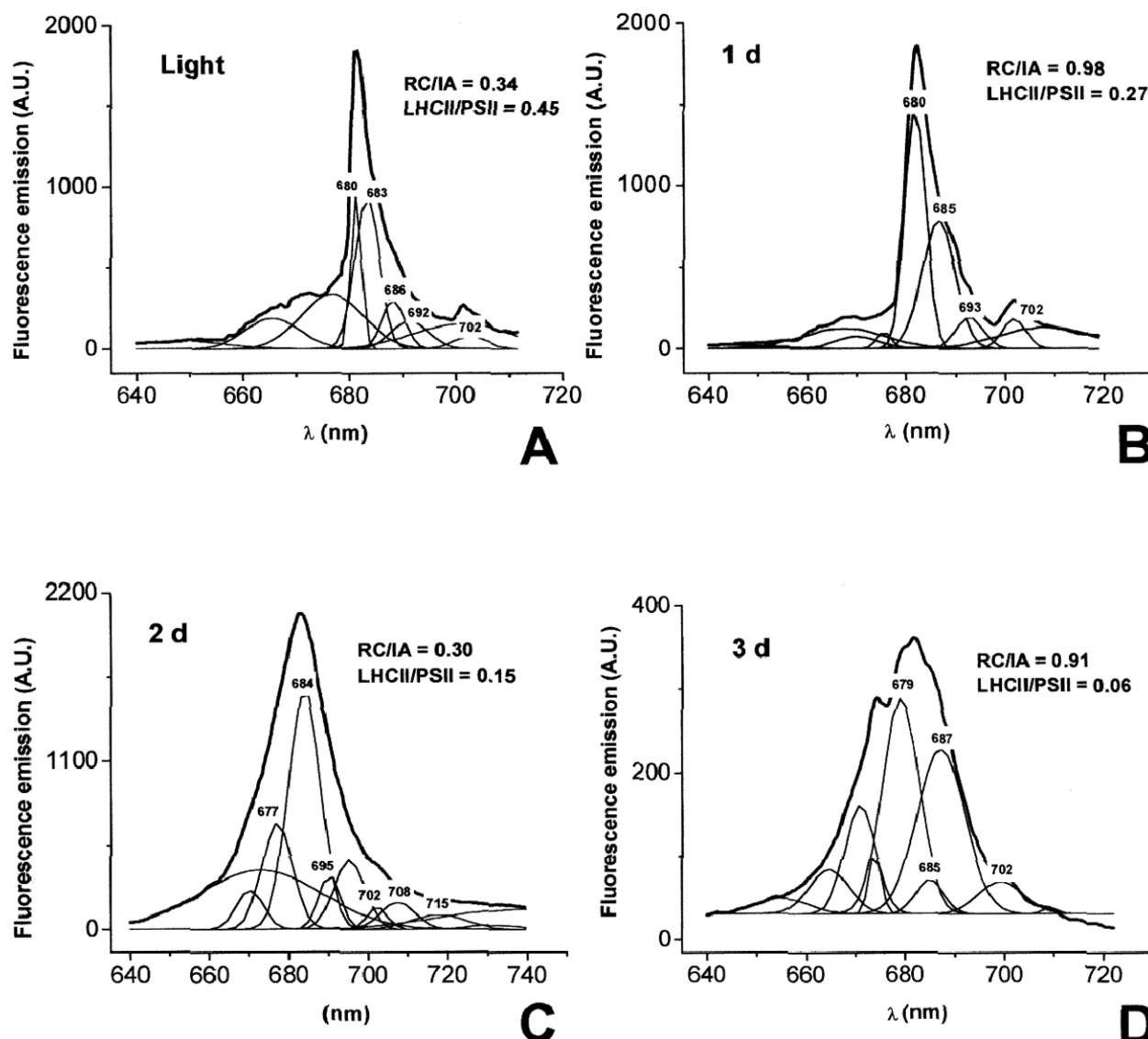
During 3 mo of darkness, only vegetative cells were present in the culture (Fig. 2). These were viable, as revealed by the persisting red fluorescence of the chloroplasts under UV light (Fig. 2), but did not multiply as if they were in resting conditions. The organisms maintained substantially the same structural characteristics described in light conditions, except for the chloroplast compartment, which underwent several dark-acclimative changes. The absence of cell multiplication favored the study of these changes because structural and biochemical modifications frequently associated with cell division were absent in chloroplasts.

The first adaptive modifications in chloroplasts were already visible after 1 d of darkness. In the central portion of the stroma, thylakoids were less numerous and some pale globules appeared in their place (Fig. 5A). These globules could accumulate Car, typical components of the external antennae LHCs, but not galacto- or phospholipids that are typical constituents of thylakoid membranes. Indeed, Car do not color up with osmium tetroxide, while the membrane lipids do (Gahan, 1984). Small starch granules were still present in the stroma, while the remaining thylakoid system appeared normally featured (Fig. 5A). Therefore, the pale globules could be composed

TABLE 1

Photosynthetic pigment contents and their ratios in *K. antarctica* maintained in continuous light or in complete darkness for 90 d (mean  $\pm$  standard deviations).

Culture conditions	Chla (nmol mL <sup>-1</sup> )	Chlb (nmol mL <sup>-1</sup> )	Car (nmol mL <sup>-1</sup> )	Chla/b	Car/Chla
Light	4.47 $\pm$ 0.39	3.13 $\pm$ 0.30	3.17 $\pm$ 0.30	1.43 $\pm$ 0.06	0.71 $\pm$ 0.06
1 d darkness	4.18 $\pm$ 0.38	3.43 $\pm$ 0.35	3.13 $\pm$ 0.31	1.22 $\pm$ 0.06	0.75 $\pm$ 0.07
2 d darkness	4.27 $\pm$ 0.40	3.28 $\pm$ 0.27	2.99 $\pm$ 0.32	1.30 $\pm$ 0.07	0.70 $\pm$ 0.05
3 d darkness	4.01 $\pm$ 0.37	3.28 $\pm$ 0.32	2.56 $\pm$ 0.27	1.22 $\pm$ 0.08	0.64 $\pm$ 0.09
6 d darkness	3.81 $\pm$ 0.31	3.12 $\pm$ 0.21	2.51 $\pm$ 0.28	1.22 $\pm$ 0.07	0.66 $\pm$ 0.08
21 d darkness	3.87 $\pm$ 0.30	2.25 $\pm$ 0.19	2.57 $\pm$ 0.25	1.72 $\pm$ 0.13	0.66 $\pm$ 0.06
90 d darkness	3.68 $\pm$ 0.29	3.06 $\pm$ 0.28	2.39 $\pm$ 0.21	1.20 $\pm$ 0.10	0.65 $\pm$ 0.09



**FIGURE 4.** Microspectrofluorimetric responses of *K. antarctica* maintained in continuous light (A), or in complete darkness for 1 (B), 2 (C), 3 (D), 6 (E) 21 (F), and 90 (G) days. The analyses were carried out at room temperature, on single cells, in vivo. Fluorescence emission spectra = thick line. Gaussian deconvolutions = thin lines. Excitation wavelength: 436 nm. RC/IA and LHCII/PSII ratios, calculated on the basis of the Gaussian components, are reported on the top right of each picture. PSII = photosystem II; RC = reaction centre of PSII; IA = inner antennae of PSII; LHCII = light harvesting complex of PSII. The curves are the mean of 12 determinations made on 4 cells for each of 3 separate experiments. SDs, evaluated at fixed  $\lambda$ , were  $\leq 5\%$ . The difference between the Gaussian sum and the original data (not reported) was always negligible ( $r^2 \geq 0.999$ ).

of Car reserves, which were deriving from LHCs under incipient degradation. However, the presence of a still high content of total Car in plastids was shown by spectrophotometric analysis (Table 1). As in light conditions, the microspectrofluorimetric spectrum came from the collage of six records (Fig. 4B), due to the persisting rapid fluorescence decay of the chloroplasts. The RC/IA ratio increased to 1.2. This was an indication of the beginning of the PSII disassembly. In fact, the higher value of this ratio indicates a failure of aggregation of the reaction centers with the inner antennae (Pancaldi et al., 2002). On the other hand, LHCII/PSII ratio decreased to 0.27, suggesting good association of the remaining LHCII with the PSII (Pancaldi et al., 2002).

On the whole, the first structural and biochemical modifications in the photosynthetic apparatus of *K. antarctica* were triggered by only 24 h of darkness, since Car began to be removed from LHCII, where their role was less important. In all probability, LHCI also underwent the same process of degradation, but the microspectrofluorimetric method does not give complete information about this.

After 2 d of darkness, the thylakoid system was more reduced and confined chiefly to the periphery of the chloroplast, where the first signs of membrane stacking occurred (Fig. 5B, C). The portion of the stroma lacking thylakoids was still occupied by pale globules that were more numerous and conspicuous than in the previous step, while starch granules were no longer visible (Fig. 5B). Moreover, osmiophilic globules and tubules, which had never been observed during the previous 24 h, appeared in the stroma (Fig. 5D). The tubules were found either by themselves or connected to the globules, and sometimes grew out the globules assuming the shape of tadpoles (Fig. 5D). These structures have been observed in chromoplasts of pepper fruit, cultivar Sweet Chocolate (Deruère et al., 1994) and of *Arum italicum* fruit during maturation and ripening (Bonora et al., 2000), where they have been interpreted as substructures bearing cyclic Car. It is likely that, in *K. antarctica*, osmiophilic tubules and globules store the Car arising from degraded external antennae (LHCs), together with the lipids released from the degraded thylakoid membranes. The presence of

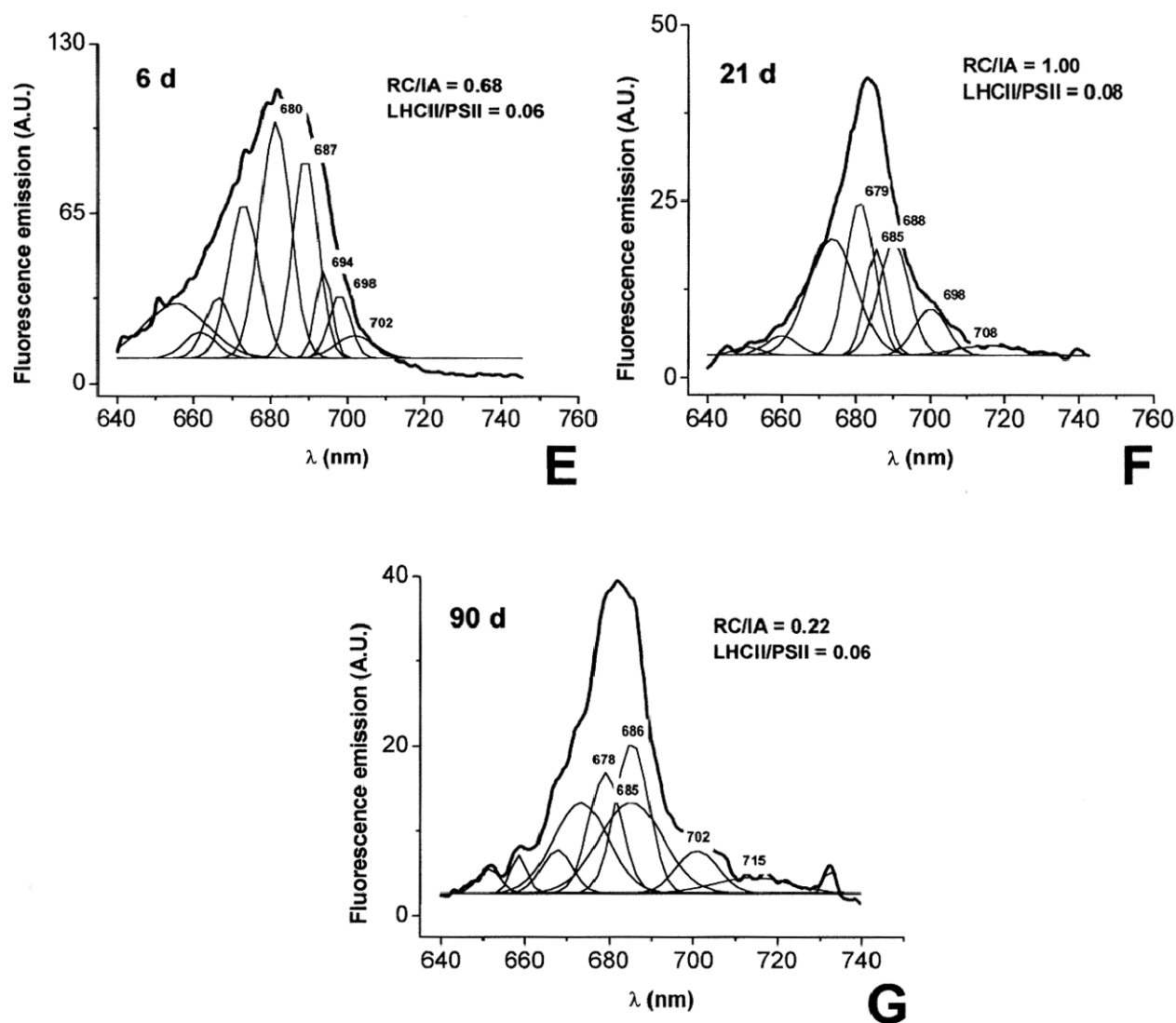
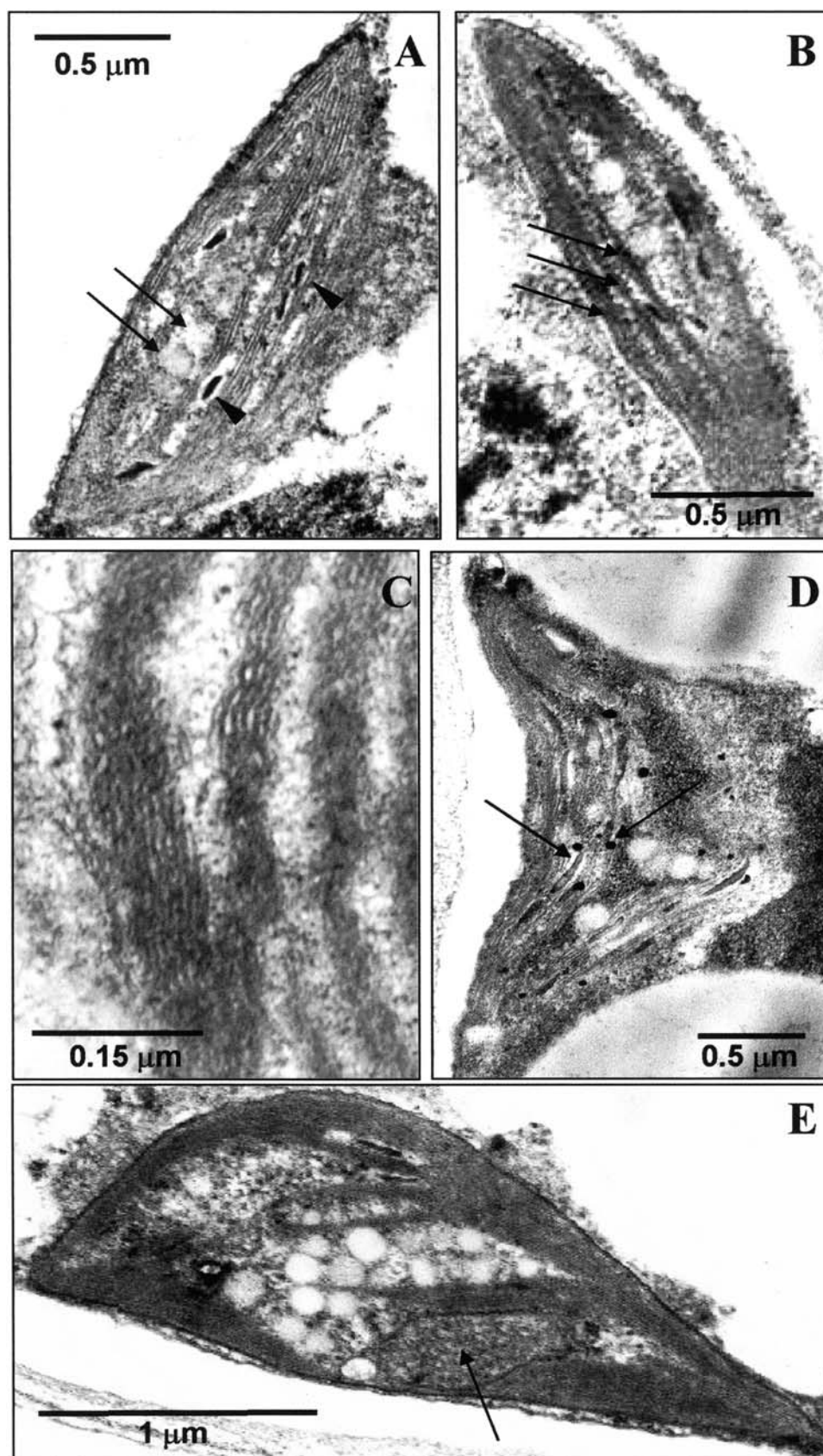


FIGURE 4. (Cont.)

thylakoid galactolipids within similar plastid substructures has also been reported (Deruère et al., 1994). Chl and Car contents remained nearly unchanged, as did the Car/Chl *a* ratio (Table 1). In contrast with the previous steps, continuous fluorimetric spectra were recorded at this stage, as fluorescence emission was high and stable (Fig. 4C). This stability suggested that fluorescence was the path for the dissipation of the surplus energy. These responses indicated that the physiological role of nonphotochemical fluorescence quenching in removing excess absorbed energy was lacking, because it was no longer required in dark conditions (Horton et al., 1996). After 2 d of darkness, remaining LHCII narrowed in comparison with the previous interval. This may have been due to the progressive removal of no longer necessary components. Therefore, TEM observations and fluorimetric data seemed to highlight a reduction in the outer antennae due to the removal of Car in surplus; probably the more peripheral in the LHCII complex. A close relationship existed between energy dissipation as heat and the Car of the xanthophyll cycle, which are known to bind at the periphery in the LHCII complexes (Horton et al., 1996). In fact, the organization of the complexes in the membrane may control Car availability (Horton et al., 1996). On the other hand, the low ratio of RC/IA (0.30) denoted that the remaining PSII were still well associated and well functioning (Pancaldi et al., 2002). On the contrary, LHCII/PSII ratio (0.15) decreased by about 50%, reinforcing the hypothesis that some LHCII were degraded. However, the incipient stacking of

thylakoids confirmed that the remaining LHCII were still tightly associated with PSII (Fig. 5C) (Pancaldi et al., 2002). The broad emission band at about 675 nm, not associated with stable complexes, showed the presence of Chl molecules uncoupled from the antenna pigment matrix (Santabarbara et al., 2001) and, consequently, the partial degradation of the pigment-protein complexes that appeared evident in this step (Fig. 4C).

After 3 d of darkness, plastids exhibited characteristics of both chloro- and chromoplasts. The stromal area, deprived of thylakoids and filled with nonosmiophilic globules, continued to be larger in plastids. The remaining membrane system was organized in bundles of tightly stuck thylakoids located in the peripheral region of the organelles without regular orientation (Fig. 5E). These structures have been described in young chromoplasts of ripening pumpkin fruits (Ljubesic, 1977). They are attributed to degraded thylakoids which adhere after losing their lumina and seem to be related to the accumulation of acyclic carotenes. However, these structures could also contain chlorophylls, whose presence was still evident in spectrophotometric analysis (Table 1). The persistence of these pigments was also inferred by red fluorescence of the cells observed under UV light (Fig. 2D). In some chloroplasts, an osmiophilic body was observed in the stroma (Fig. 6A). This body was identified as phytoferritin, a complex of protein and iron that has been found in the stroma of etioplasts (Kirk and Tilney-Basset, 1978) and some immature chromoplasts (Ljubesic, 1976; Bonora et al.,



**FIGURE 5.** Plastids in *K. antarctica* maintained in darkness for 1 (A), 2 (B, C, D) and 3 (E) days. (A) Thylakoids are reduced in number while some pale globules (arrows) lie in the inner portion of the stroma. Starch granules are still present (arrow heads). (B) The thylakoid system is confined in the periphery of the organelle, where the first signs of membrane stacking occur (arrows). Numerous pale globules lie in the stroma. (C) Detail of a plastid as in (B) showing tightly stacked thylakoids. (D) Minute electrondense globules and tubules are spread in the stroma. The tubules are found either by themselves or connected to the globules and sometimes protrude from the globules, assuming the shape of tadpoles (arrows). (E) Pale globules largely occupy the central portion of the stroma. Tightly stuck thylakoids are irregularly deposited mainly at the periphery of the organelle. A conspicuous membrane-bound body is also visible tightly associated with the stacked thylakoids (arrow).

2000). In fact, the X-ray spectrum analysis of these aggregates showed a peak at 6.4 keV, specific for the  $K_{\alpha}$  line of iron (Fig. 6B). Phytoferritin deposits in the chromoplasts derive from the cytochromes and the ferredoxin of degraded thylakoids (Bonora et al., 2000), while in the

etioplasts they serve as a storage of iron to be used during chloroplast differentiation (Kirk and Tilney-Bassett, 1978, and references therein). For *K. antarctica* cells maintained in darkness, phytoferritin could have derived from the degraded thylakoids, to be used when light conditions

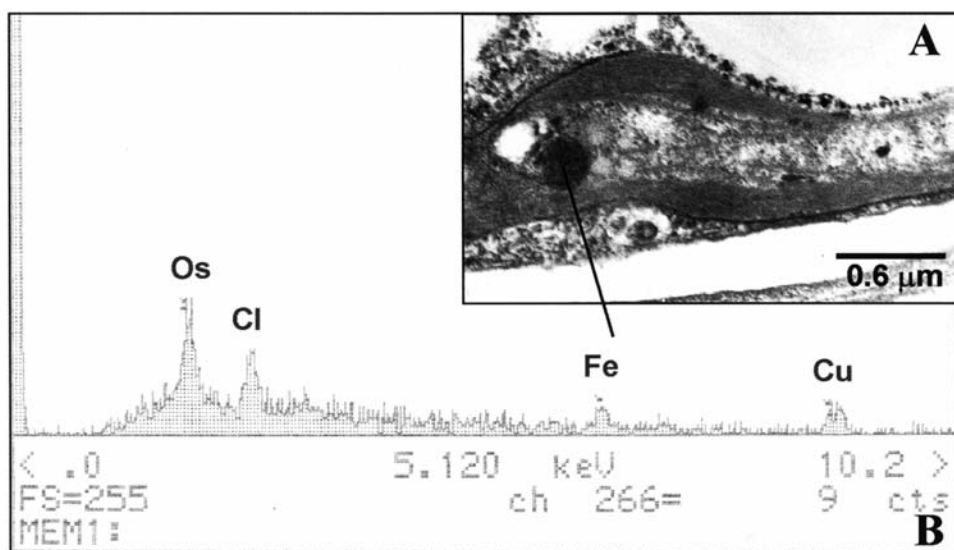


FIGURE 6. (A) Detail of a plastid in *K. antarctica* cultured for 3 d in darkness showing a granular osmiophilic body identified as a phytoferritin deposit, as confirmed by X-ray microanalysis which includes a peak at 6.4 keV, specific for the  $K_{\alpha}$  line of iron (B).

are restored. Concomitantly, a membrane-bound intraplastid body often appeared, tightly associated with the stacked thylakoids (Fig. 5E). These inclusions have been frequently observed in higher plants and interpreted as proteinaceous bodies, storing materials utilized for thylakoid formation in developing chloroplasts (Kirk and Tilney-Bassett, 1978). Actually, in *K. antarctica* the inclusion could have derived from the progressive degradation of the photosynthetic machinery. This may have constituted a protein reserve employed for the successive reconstitution of normally structured chloroplasts when the alga is exposed to light conditions again. Microspectrofluorimetric analysis permitted the calculation of very interesting ratios: RC/IA, around 1, indicated the beginning of a second phase in the disassembly of the Chl-protein complexes of PSII, and in the degradation of the IA (Fig. 4D). The LHCII/PSII ratio decreased to about 0. This event was probably not due to a complete loss of LHCII, since Chla/Chlb and Car/Chla ratios remained substantially unchanged with respect to the previous steps (Table 1). This value indicated that a very tight association of peripheral antennae to PSII was still maintained, as the very stacked feature of the remaining thylakoid membranes showed (Fig. 5E). All these aspects remained nearly unchanged until 5 d of darkness.

After 6 d of darkness, the new rearranged thylakoid system was more and more organized in spindle-shaped bundles of stacked membranes occupying the outer portion of the organelle (Fig. 7A). Bundles were strongly osmiophilic and resembled the stuck thylakoids observed in chromoplasts of pepper cultivar Sweet Chocolate, whose unusual pigmentation derives from Car that are developed in the presence of Chl (Deruère et al., 1994). The spectrophotometric analysis attested to the persistence of chlorophylls, even if they were slightly diminished (Table 1). The remaining internal structure in chloroplasts appeared more complex with respect to the previous days of darkness. The pale globules in the stroma were also accompanied by osmiophilic globules (Fig. 7B). These osmium-reactive globules could well be related to polar lipids (Deruère et al., 1994), i.e., galactolipids and phospholipids, in association with proteins. Other accumulation sites of degradation materials in plastids were slack bundles of wavy osmiophilic tubules and clusters of minute globules, which were contained inside small electron-transparent areas outlined by concentrically arranged osmiophilic lamellae (Fig. 7C). These substructures, which have been frequently described in chloroplasts during transition into chromoplasts, are considered as deposits of carotene pigments (Harris and Spurr, 1969; Bonora et al., 2000). Finally, microspectrofluorimetric and pigment analyses indicated that the Chl-protein

complexes continued to be stable, since RC/IA and LHCII/PSII ratios remained low (0.68 and 0.06, respectively) (Fig. 4E, 8), and Chla/Chlb and Car/Chla were substantially unchanged with respect to the previous phase of darkness (Table 1). Ultrastructural, microspectrofluorimetric, and pigment characteristics remained essentially unchanged for 20 d of darkness.

After 21 d of darkness, cells were less red-fluorescent under UV light (Fig. 2F), in accordance with the minor content of chlorophyllous pigments (Table 1) and with the reduced thylakoid system (Fig. 9A). Moreover, many structural changes that had previously been observed in plastids still persisted and/or were enhanced. Electron-dense globules and bundles of tightly stacked thylakoids were increasingly evident in the stroma, a sign of the continuing dark-acclimation (Fig. 9A). The membrane bound area, bearing a dense amorphous material and interpreted as a proteinaceous body, was still evident in the stroma (Fig. 9A). Concomitantly, new membranous systems appeared in plastids. These consisted of many concentrically arranged layers lying usually at the periphery of the organelles (Fig. 9B) and resembling the "chromoplast internal membranes" (CIMs) found in many chromoplasts during fruit maturation (Ljubesic et al., 1991; Bonora et al., 2000). The presence of CIMs represented a further step in dark acclimation. In chromoplasts they are achlorophyllous membrane systems bearing lipids (mostly galactolipids), special proteins, and Car, among which lutein is a main pigment component (Wrischer and Ljubesic, 1984; Ljubesic et al., 1991). This pigment is one of the most internal carotenoid in LHCII (Kühlbrandt et al., 1994; Pineau et al., 2001). The presence of CIM-like structures, which were probably rich in lutein, would confirm the further degradation process of peripheral antennae that occurred during the previous steps. Pigment analyses indicated a diminished adsorption by the Chlb (Table 1). Consequently, Chla/Chlb ratio was higher and was consistent with the antennae degradation process. Microspectrofluorimetry also showed another phase of degradation of the PSII. This was inferred mainly by the RC/IA ratio that increased to 1.0 (Figs. 4F, 8), an aspect related to the uncoupled state of complexes, which preceded a new degradation step of PSII. Nevertheless, this event was not evident from the LHCII/PSII ratio, which still yielded a very low value (Fig. 8), in accordance with the behavior already observed after 72 h in darkness.

From 22 to 90 d of darkness, *K. antarctica* continued to be fluorescent under UV light (Fig. 2H), and contained plastids with very similar structural features and pigment contents (Table 1) observed after 21 d of darkness. Microspectrofluorimetric analysis also indicated the achievement of a stable state; RC/IA and LHCII/PSII ratios maintaining

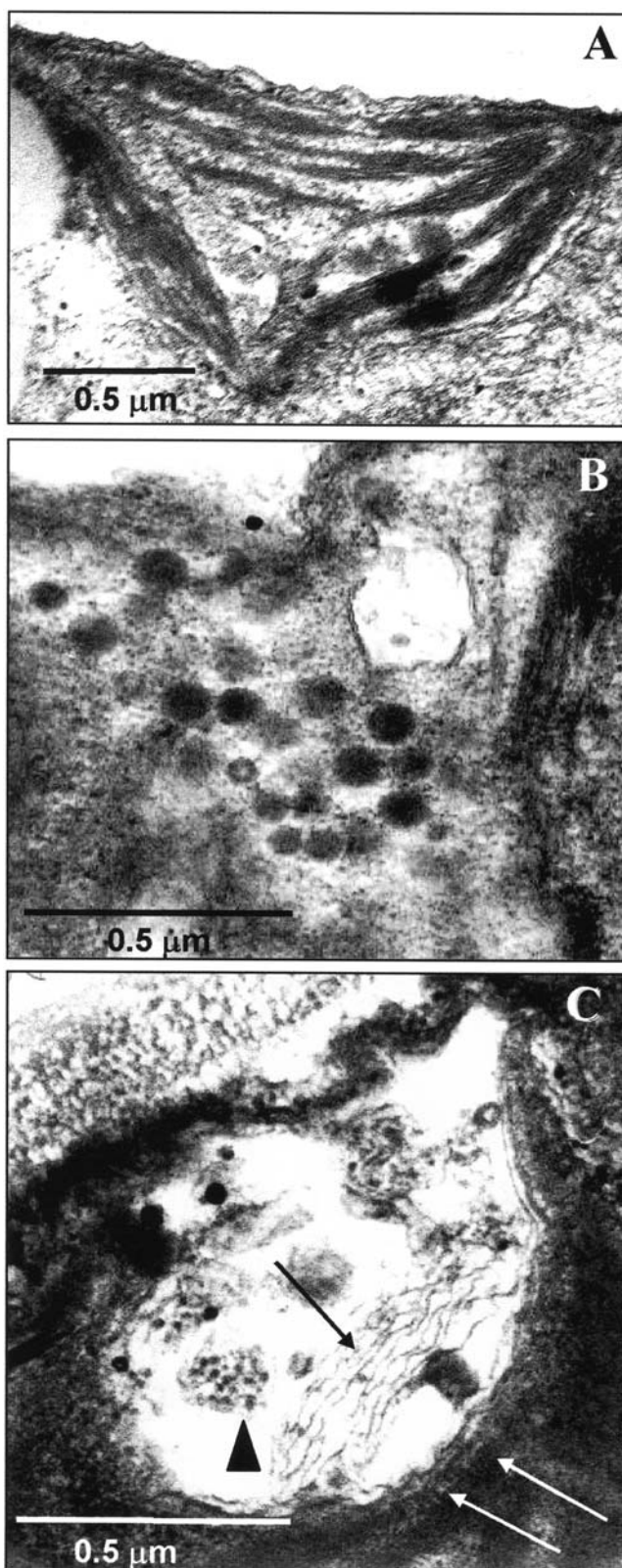


FIGURE 7. Details of plastids in *K. antarctica* maintained in darkness for 6 d. (A) The thylakoid system is mostly organized in spindle-shaped bundles of stacked membranes occupying the outer portion of the organelle. (B) Osmiophilic globules are present in the stroma in addition to pale globules. (C) Bundles of slack wavy tubules (arrow) and minute globules (arrow head) contained inside a small electron-transparent area, outlined by osmiophilic lamellae (double arrow).

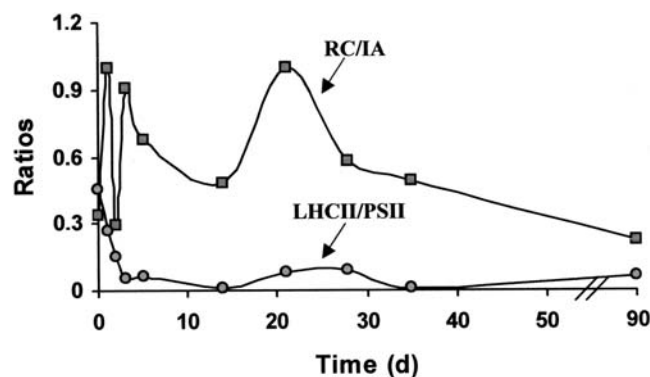


FIGURE 8. Time-course variations of the RC/IA and LHCII/PSII ratios calculated on the basis of microspectrofluorimetric analysis (see Fig. 4) during the entire experiment.

values of 0.22 and 0.06, respectively (Figs. 4G, 8). This was an indication of a condition of balance in the structural and biochemical changes of the plastid, reached during dark acclimation.

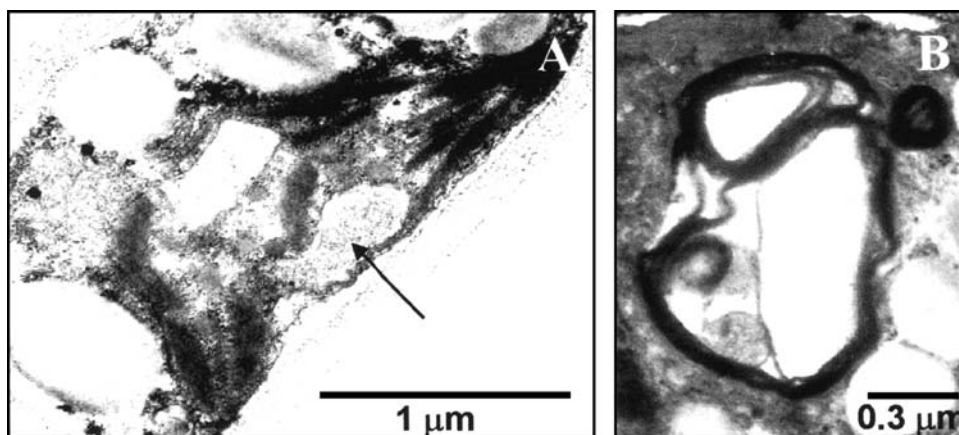
## Conclusions

The study of the adaptive characteristics of microorganisms to their extreme environmental conditions is often difficult because of the lack of cell samples at the site of origin. In our case this is primarily due to the inaccessibility of the habitat in antarctic winter. Simulation of the extreme environmental conditions in the laboratory may be required to study the acclimation strategies carried out by the cells.

The extremophilic microalga *K. antarctica* is a very sciaphilous marine microorganism, adapted to very low light intensities. The  $8 \mu\text{mol m}^{-2} \text{s}^{-1}$  PAR value is that recorded in 1988 at a depth of 3 m in the Ross Sea by the meteorological station of Terra Nova Bay (Antarctica) (Andreoli et al., 2000). The alga has been maintained at this intensity under continuous light for laboratory culturing for over 10 yr (Andreoli et al., 2000). Previous studies had shown the high adaptive ability of *K. antarctica* to grow in a variety of experimental conditions (Andreoli et al., 2000; Zanetti et al., 2001). For instance, the morphological changes following different salinities (from 5 to 70‰) were linked to the plasticity of the cell wall, made up of more than 90% matrix polysaccharides and less than 10% cellulose (Piro et al., 1997, 2000). This surprising acclimatory ability has surely evolved for the selective pressure in the antarctic marine environment. The question that is addressed in our work is: has this environmental pressure also selected efficient mechanisms to tolerate complete darkness in the antarctic winter?

Our study shows that *K. antarctica* does tolerate the stress of long dark periods. Under prolonged darkness, the alga exhibits structural and functional reorganization of the plastid. This results in the formation of a chlorochromoplast-like structure suitable for the storage of the products resulting from the breakdown of pre-existing plastid constituents. Dark acclimation occurs in two different steps: a first acclimation phase, quickly realized during the first 6 d and maintained up to 20 d of darkness, and a second acclimation period, starting from the 21st day and maintained up to the end of the 90 d experiment (Fig. 8).

When light is limiting in antarctic environments, heterotrophy has been suggested to be important for the acquisition of carbon (Jones, 2000; Bell and Laybourn-Parry, 2003). Mixotrophy constitutes a strategy by which microalgae sustain vegetative winter populations offering them a competitive advantage and promoting their survival in polar marine and lacustrine environments (Bell and Laybourn-Parry, 2003). Linked to mixotrophy could be the transition of the chloroplast to the chlorochromoplast observed in darkened *K. antarctica*.



**FIGURE 9.** Details of plastids in *K. antarctica* maintained in darkness for 21 d. (A) Note very electrondense bundles of tightly stacked thylakoids and a membrane bound proteinaceous body (arrow). (B) Osmiophilic membranes arranged as in CIMs (Chromoplast Internal Membranes).

Nonetheless, the relevance of the plastid to mixotrophy remains an open question. Actually, the presence in the same cell of a plastid structure combining characteristics of the chloroplast and chromoplast is surely distinctive among algae.

Finally, it is unclear whether the plastid modifications observed for *K. antarctica* in our study occur in the alga in its natural environment during the winter season. Nonetheless, *K. antarctica* appears to be an organism that is adapted for long dark periods and maintains this acclimatory capacity also when it is cultured for long periods away from Antarctica.

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