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The fine structure of *Dunaliella acidophila* (Kalina) Massyuk grown under chronical salt stresses

ANTONINO POLLIO*, GENNARO CAFIERO**, GABRIELE PINTO*, SALVATORE SANTISI*

*Dipartimento di Biologia vegetale e **Centro Interdipartimentale di Ricerca sulle Ultrastrutture Biologiche, Fac. di Scienze, Università di Napoli Federico II, Via Foria, 223 - I-80139 Napoli, Italy.

Riassunto

E' stata studiata l'ultrastruttura dell'alga verde acidofila *Dunaliella acidophila* cresciuta in condizioni di stress salino. La microanalisi a raggi-X del campione di suolo dove è stata isolata l'alga in natura ha evidenziato un'abbondanza di zolfo ed alluminio mentre sodio e cloruro non sono stati trovati in quantità apprezzabili. La concentrazione di sodio, zolfo e cloruro all'interno delle cellule non varia dopo sette giorni di esposizione a 0.5 M di NaCl e 0.6 M di Na_2SO_4.

Sotto stress salino, *D. acidophila* presenta significative modificazioni a livello del complesso del Golgi. Vescicole trasparenti agli elettroni divengono più abbondanti nell'area del Golgi ed esse tendono a fondersi formando ampi vacuoli contenenti porzioni di membrane e materiale granuloso. Tale incremento di vescicole potrebbe essere coinvolto nell'equilibrio osmotico dell'alga.

INTRODUCTION

The genus *Dunaliella* Teodoresco (Chlorophyceae, Chlorophyta) comprises unicellular biflagellate algae, lacking a rigid cell wall and provided by a cup-shaped chloroplast with pyrenoid and eyespot. Most species belonging to this genus occurr in saline and brackish waters and are capable of growing over a wide range of salinity (BEN AMOTZ, 1975; GIMMLER *et al.*, 1981; GILMOUR *et al.*, 1982).

Key words: *Dunaliella acidophila*, Golgi complex, Microanalysis, Salt stress, Ultrastructure.

The physioecological features of this genus have been extensively studied by numerous reseachers, also for the promising commercial perspectives of this alga as a source of β-carotene, glycerol and unsaturated fatty acids (BOROWITZKA & BOROWITZKA, 1989).

D. acidophila (KALINA) MASSYUK, one of the few nonmarine members of this genus, has been isolated for the first time by KALINA (1965) and described as *Spermatozopsis acidophila* species nova. Subsequently MASSYUK (1973) placed this species into the genus *Dunaliella* for the absence of a twisted shape of the cell, a typical feature of *Spermatozopsis,* and for the presence of a pyrenoid. The differences between these two genera have been made clear by MELKONIAN AND PREISIG (1984).

In Italy *D. acidophila* has been isolated in six acid sites at a pH range from 1.0 to 1.8 (PINTO & TADDEI, 1978). The alga is particularly resistant to high sulphuric acid concentrations, being able to grow at pH as low as 0.2, if slowly preadapted (FUGGI *et al.*,1988a).

The biochemistry, particularly the lipid content (POLLIO *et al.*, 1988; DELLA GRECA *et al.*, 1989), and the physiology of *D. acidophila* have been investigated during the last years. *D. acidophila,* as other members of this genus from marine habitats, accumulates glycerol when grown in media supplemented with different solutes (FUGGI *et al.*, 1988b). Even when the alga is grown at its optimal pH 1.1, the cytoplasmic pH is close to 7.0 (GIMMLER *et al,*. 1989) and the membrane potential result to be positive (GIMMLER *et al.*, 1991; 1992).

Apart from the early works of TREZZI *et al.* (1964, 1965), in the last two decades the fine structural features of *Dunaliella* species from saline habitats, have been investigated for various purposes. Particularly, fine structure observations were carried out on the cellular mitosis of *D. bioculata* (MARANO, 1976), on the cell coat of *D. tertiolecta* (OLIVEIRA *et al.*, 1980), *D. bioculata* (CHARDAD, 1987) and on the flagellar apparatus *of D. salina* (HYAMS & CHASEY, 1974; EYDEN, 1975; MARANO, 1976).

The ultrastructure of *D. acidophila* has been studied only in a preliminar way (ALBERTANO *et al.*, 1981; SANTISI & ALBERTANO, 1984), but there are no available studies dealing with the effects of ionic stress on the fine structure of this alga.

In this paper the ultrastructure of *D. acidophila* cells subjected to iper-osmotical stress was studied by means of TEM and SEM electron microscopy and X-ray microanalysis of cells.

MATERIAL AND METHODS

Axenic cultures of *Dunaliella acidophila* were obtained from a soil sample collected in June 1991 at Pisciarelli, Naples, Italy (1° 41'47"E 40°49'46"N, coordinates referred to Rome, Monte Mario, according to 1:25000 map of Istituto Geografico Militare (IGM) of Italy).

The alga was maintained in Allen modified medium previously described (ALBERTANO *et al.*, 1981) at 23 °C and at pH 1.1. The photoperiod was 16 h light: 8 h dark. Algae in exponential growth phase were inoculated in 100ml Erlenmeyer flasks for the experiments of salt tolerances.O.6 M Na_2SO_4 or 0.5 M NaCl were added to the basal growth medium before the inoculum of algae. During the experiments an irradiance of 100μmE $m^{-2}s^{-1}$ was provided by Philips fluorescent lamps, at the same photoperiod. Cultures were gently bubbled with 3% CO_2 in air.

After seven days the algae were centrifugated and the pellets were collected and prepared for X-ray microanalysis and for electron microscopy.

For light microscopy, untreated cells from logarithmic phase of growth were observed using a Zeiss Standard GFL light microscope, equipped with phase contrast optics and photographic unit.

For scanning electron microscopy (SEM) the pellets were fixed in 3% buffered glutaraldehyde and then dehydrated in a ethanol series. After the intermediate medium Freon 113, the material was treated for critical point dry in CO_2, and mounted on Al-stubs. Metalization was carried out with carbon and gold. Observations were carried out by means of a Scanning Cambridge 250 Mark III at 21 Kv. For microanalysis purposes, C-stubs were used and the metalization of samples was carried out with carbon alone. The equipment used was a Link AN 10000 device.

For transmission electron microscopy (TEM) pellets of *D. acidophila* were fixed in 3% glutaraldehyde in phosphate buffer (pH 7.2) for 3h at room temperature, collected by mild centrifugation and washed in the same buffer. Post-fixation was carried out with 1% osmium tetroxide solution for 1h. All the samples were dehydrated in a graded ethanol series followed by three rapid changes in propilene oxide, and embedded in Polarbed 812. Sections were taken with a LKB Ultrotome III equipped with a diamond knife, stained with a 2% uranyl

acetate in H_2O and lead citrate, and viewed on a Philips M 301 or Siemens Elmiskop IA electron microscopes.

RESULTS

A sample of the material from which *Dunaliella acidophila* was isolated has been examined with X-ray microanalysis. In Fig.1 is reported the spectrum of major components of the sample.

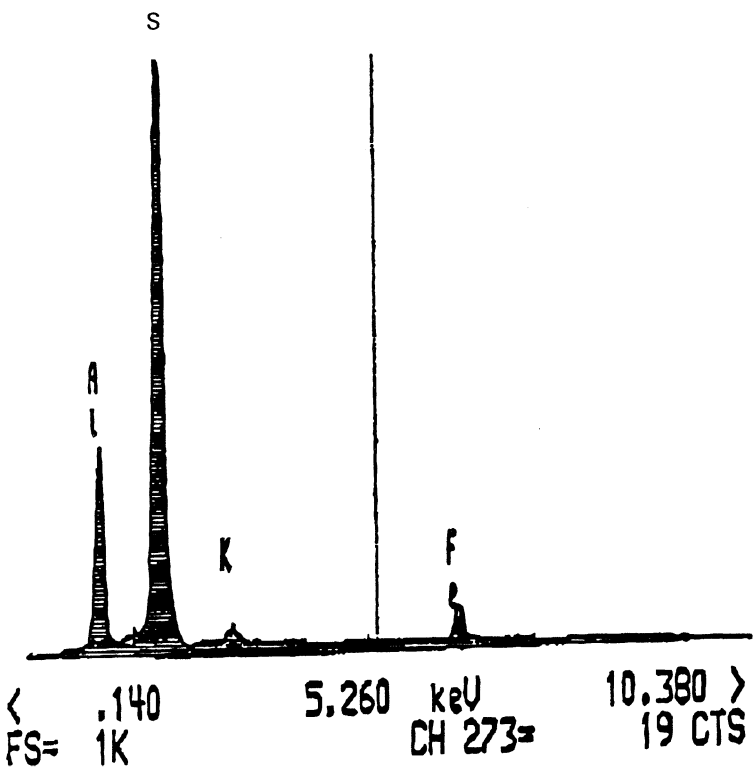

Fig. 1 - X-ray microanalysis diagram of a soil sample from which *D. acidophila* has been isolated. Sulphur (S) largely predominates on all the other elements, but also aluminium (Al) and, to a lesser extent, iron (Fe) and potassium (K) are present in significative amounts.

As can be seen sulphur predominates on all the other elements and aluminium is also aboundant, whilst sodium and chloride are scarcely present. The alga was subjected to chronical shocks in media containing sodium sulphate or sodium chloride to verify if the variation of the osmotic pressure caused adaptations at the fine structural level. X-ray microanalysis of *D. acidophila* cells treated with salt concentrations (0.6 M Na_2SO_4 or 0.5 M NaCl) evidenced no significant variation of ionic content within the cells (not shown).

Phase contrast and S.E.M. observations showed viables aggregates simulating mating types of cells (Plate I, 1-2). Biflagellate cells of *D. acidophila* are ellipsoidal to ovoid, with an acute or quite rounded apex (Plate I, 2). The fine structure of cells shows flagella with the typical 9+2 pattern of microtubules (Plate III, 9). In longitudinal section cytoplasmic microtubules run parallel beneath the plasma membrane and cell coat, and appear to originate mainly in the region of the flagellar bases (Plate I, 3-4).

The Golgi complex is composed of a single dictyosome lying beneath the flagellar bases and nucleus. The dictyosome possess cisternae with the forming face lying towards the plasma membrane and the maturation one towards the nucleus. Sometimes the proximal stack of cisternae become nearly bowled-shaped, enclosing a mass of cytoplasm with ribosomes (Plate II, 5). Few coated and smooth vesicles scattered in the area of Golgi complex were observed. The former are small and are characterized by the presence on their surface of an electron-dense, fuzzy coat. The latter, which are present at the periphery of dictyosome, are larger and electron transparent. A single rough ER profile is also observed between the basal bodies and dictyosome forming face (Plate II, 6-7).

The nucleus lies betwecn the Golgi complex and the chloroplast and is provided by a nuclear envelope which encloses the nucleoplasm with nucleolus and chromatin masses, often adhering to the inner membrane. The nucleus is surrounded by chloroplast arms which extend towards the flagellar pole as far as the Golgi region (Plates II-III, 5-9).

In the ribosome-rich cytoplasm, a few mithocondria and electron-transparent vacuoles surrounded by a single membrane are observed (Plate III, 10).

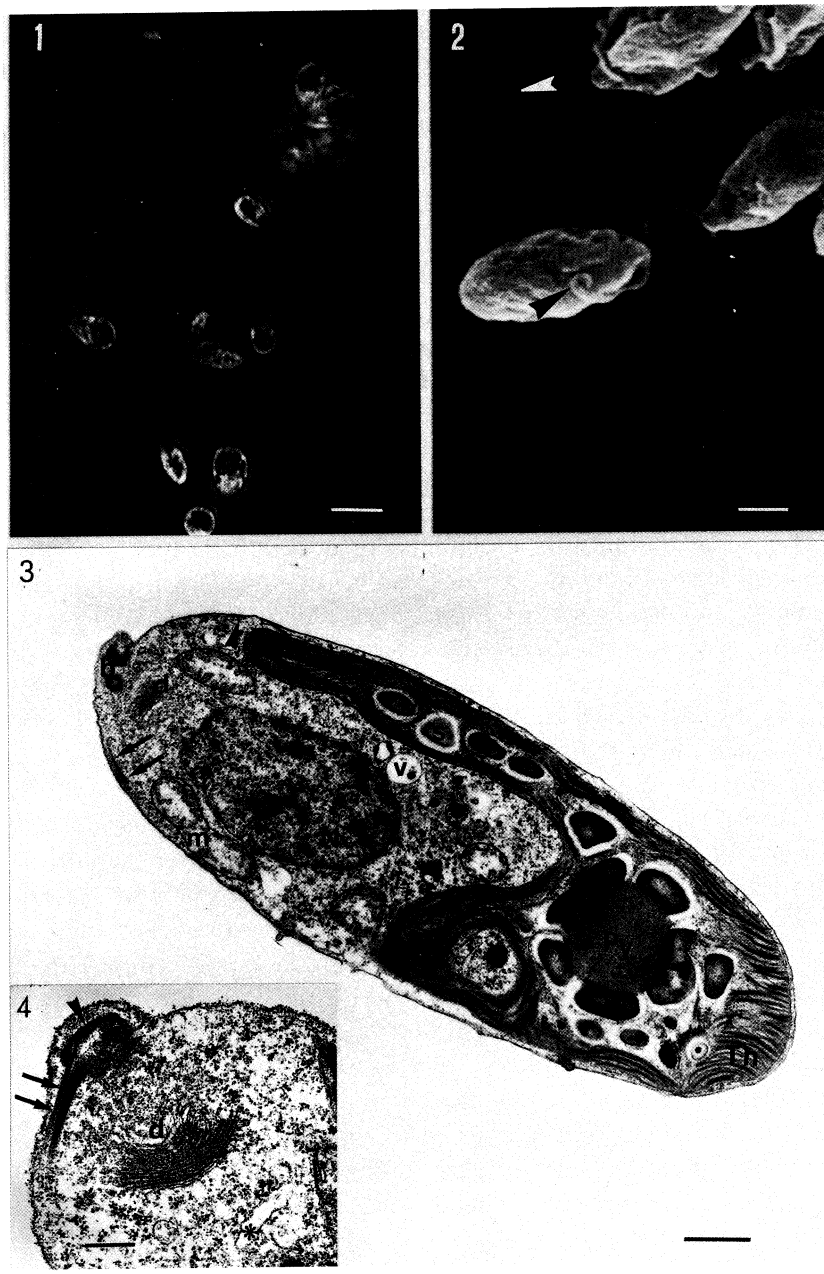
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Plate I - (1) Living cells of *D. acidophila* observed with phase contrast microscope. Bar = 10 mm.

(2) Scanning electron micrograph of two opposite cells. Note the curled flagellar tips (arrowheads). Bar = 3 mm.

(3) The fine structure of a untreated cell at the end of the exponential phase of growth. A coat (arrowhead) surrounds the organism. Flagellar bases (b) with an arising microtubular system (arrows) are showed. The chloroplast (C) with a pyrenoid (Py), starch grains (s) and thylakoid membranes (th) occupies the posterior end of the cell. The nucleus (N), mitochondria (m), vesicles (v) and a dictyosome (d) are lying in the cytoplasm. Heterocromatin masses are adhering to the inner membrane of the nuclear envelope. Bar = 1 mm.

(4) Inset showing the anterior end of the cell with microtubules (arrows) originating from flagellar bases (b), a dictyosome (d) and ER cisternae (asterisk). Arrowheads points to distal striated connecting fiber of the flagella. Bar = 200 nm.

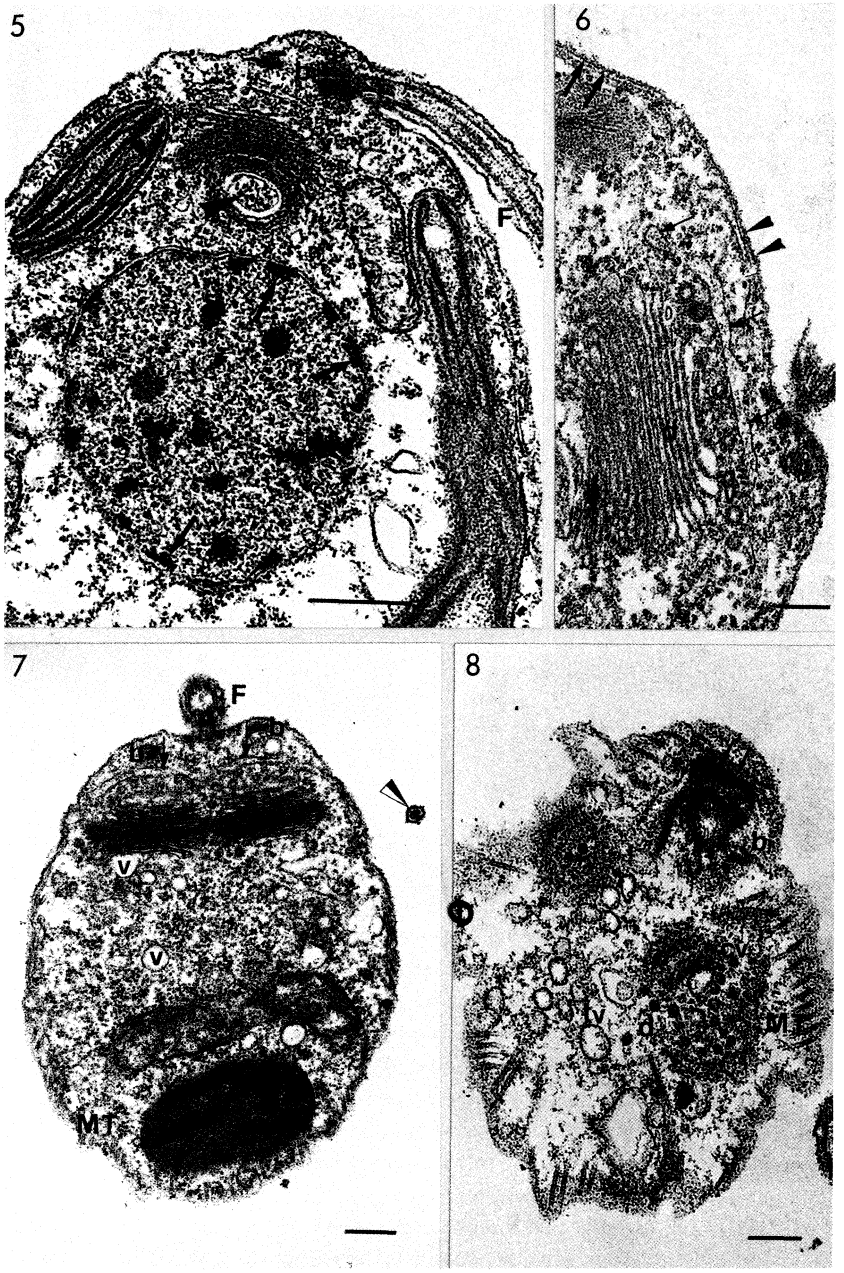
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Plate II - (5) Oblique section of a treated cell showing the flagellar pole with an emergent flagellum (F) and flagellar bases (b). The dictyosome (d) presents coated vesicles (cv) bowl-shaped cisternae enclosing a mass of cytoplasm (arrowhead). In the nucleoplasm the heterochromatin masses adhere to the nuclear envelope (arrows). Bar = 100 nm.

(6) The cell is surrounded by a plasmalemma (arrow) and a surface coat (arrowhead). A single ER cisterna (thin arrows) is superimposed to the dictyosome (d). A row of coated vesicles (v) lies between the dictyosome and ER profile. Bar = 100 nm.

(7) Two paired dictysomes with ER cisternae (thin arrow) and transparent vesicles (v) lie in the proximity of the flagellar bases (b). Other vesicles are scattered in the ribosome-rich cytoplasm. Arrowheads point to a cross section throughout a flagellar tip with two microtubules connected by electrondense material. Another flagellum (F) lies in a grove of the cellular apex. Bar = 300 nm.

(8) A tangential section of the flagellar pole of the cell showing two dictyosomes (d) with vesicles of two types (cv, tv) and microtubules (MT) around the cell. A flagellar base (b) and connecting fiber are also present. Bar = 250 nm.

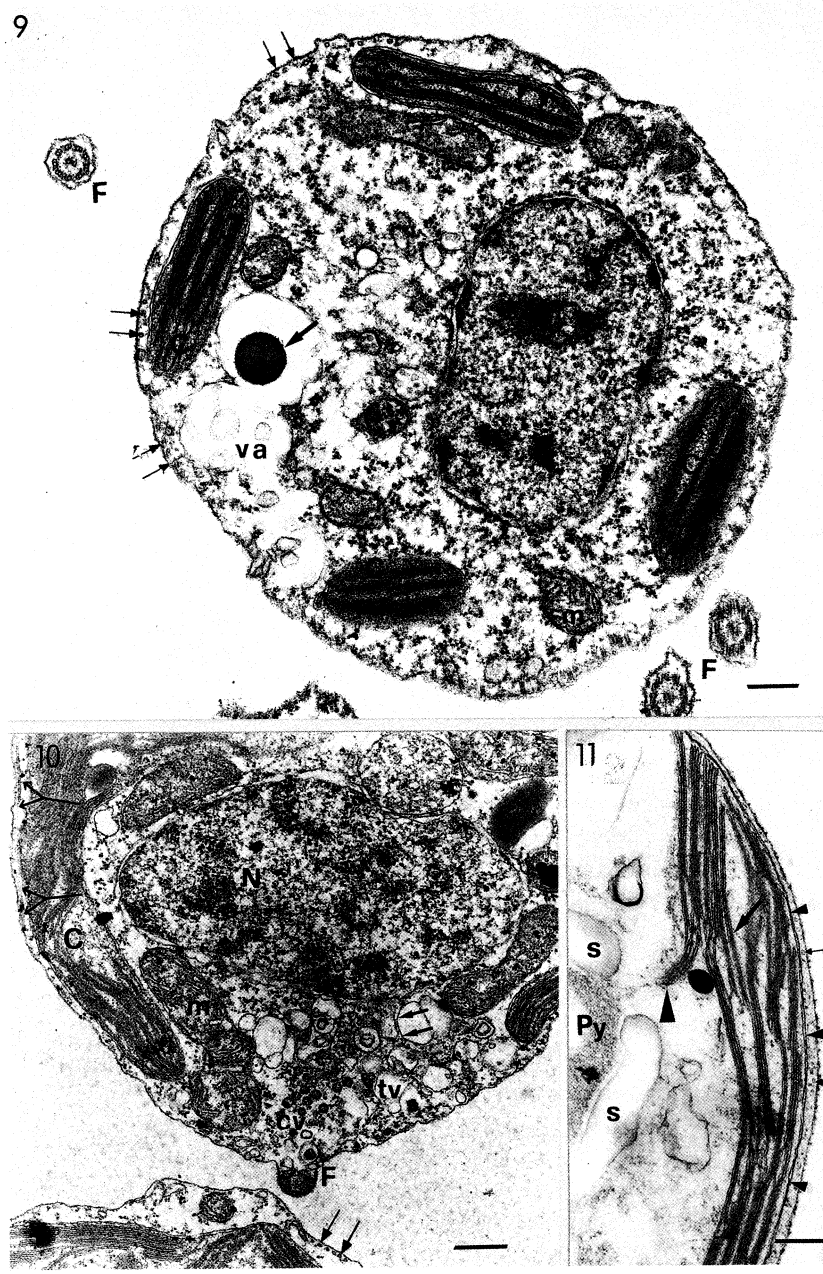
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Plate III - (9) A transection of a treated cell with four chloroplast arms (C), with thylakoid membranes in threes (arrowheads), typical of esponential phase of growth. Transparent vacuoles (va) with dense osmiophilic globules are present (arrow). Microtubules surround the cytoplasm beneath the plasma membrane (thin arrows). Around the cell flagella in transverse section are evident. Bar = 350 nm.

(10) A Golgi area with coated and transparent vesicles (cv, tv). The latter are filled with membranous material and surrounded by a single membrane (arrows). Double arrows point to a microtubule running beneath the plasmalemma. Thin arrows point to a row of cytoscheletal microtubules in a adjacent cell. An emergent flagellum (F) is also present. Bar = 350 nm.

(11) The chloroplast in the exponential phase of growth. Thylakoid membranes are mainly in threes, with the presence connecting thylacoids (arrow). The chloroplast envelope (arrowhead) runs at a constant distance from the plasma membrane and cell coat; the space is occupied by cytoskeletal microtubules (little arrows). Large arrowheads point to three thylakoid membranes which penetrate in the pyrenoid matrix (Py) between starch grains (s). Bar = 300 nm.

A cup-shaped chloroplast, with a central pyrenoid, is the main organelle of the cytoplasmic matrix. Digitations characterize the organelle, simulating in transverse section several plastids. Thylakoid membranes lie in threes or more, in a ribosome-rich plastid stroma (Plate III, 9). The chloroplast envelope runs at a distance from the peripheral cytoplasm which is almost constant for the overall length (Plate III, 11). This space is occupied by microtubules and few ER cisternae. The pyrenoid matrix is in part traversed by single or double short thylakoids, whose thickness appears to be greater than the stromatic ones. Plastid lamellae are observed mainly at the periphery of the organelle, whereas large starch granules lie in correspondence of the axis of the plastid arms. Starch granules also surround the pyrenoid matrix (Plate I, 3).

A eyespot composed of a series of osmiophilic globules, lies at the anterior end of the chloroplast in a thylakoid-free area.

The main ultrastructural modifications observed in salt trated cells affect Golgi complex. Two paired dictyosomes are often observed.(Plate II, 7-8). Moreover, the stacked cisternae appear often more electron dense and their lumina rather thinner than the control (Plate II, 5-7-8). They are composed of 4-12 membranous elements which seem to release at the periphery numerous vesicles of coated type, often disposed on a single row (Plate II, 6). Moreover, many electron-transparent vesicles are observed lying in the cytoplasm between dictyosome and nucleus. Single or fused vesicles forming large vacuoles (Plate III, 9) may contain other components as vacuolar inclusions, portion of membranes and small vesicles (Plate III, 10) as well as granular or thread like material or osmiophilic globules.

DISCUSSION

In the hot spring area of Pisciarelli *D. acidophila* generally occurrs under thin crystallin layers covering large extensions of soil and rocks. The abundance of sulphur in superficial soil samples revealed by X-ray microanalysis is characteristic of this environment (PINTO & TADDEI, 1976). On the other hand, the low amounts of sodium and chloride found in the sample could explain the reduced tolerance of this organism to NaCl (FUGGI *et al.*, 1988a), respect to the species coming from marine environments. As pointed out by MELKONIAN & PREISIG (1984), *D. acidophila* seems to be the unique non-marine member of this

genus, the other four freshwater species described by MASSYUK (1973) being transferred in other genera.

According to the classification proposed by HOFFMAN (1989) for soil algae *D. acidophila* can be considered an epilithic alga, which is subjected to continuous osmotic shocks caused by dramatic variation in water content of superficial soil during the day. As also reported in *D. parva* (HAJIBAGHERI *et al.*, 1986), X-ray microanalysis has evidenced a stability of cytoplasmic ionic environment in *D. acidophila* cells under chronic salt stress. This is in accord to FUGGI *et al.* (1988b), which found that glycerol synthesized by this alga under salt stress account for almost 100% of the osmotic balance of the cell, without any contribution by permeation of cells.

Cells of *D. acidophila* under salt stress seem to undergo ultrastructural changes mainly at Golgi complex level. The frequent presence of two dictysomes and the reduction of the lumen of cisternae observed in salt treated cells could be the consequence of an accelerated rate of metabolic activity, as reported for both animal and plant cells (DILLON 1981). The marked increase of vacuoles and small coated vesicles might be also involved in the osmotic relation of the cell. In salt-treated cells of *D. acidophila,* electron-transparent vesicles seem to migrate throughout the cytoplasm and fuse together into a complex of vacuoles. There are contrasting reports about the role of vacuoles in marine species of *Dunaliella* grown under high salt. The presence of large vacuoles was related to salinity changes and ionic balance in the cytoplasmic compartment of the cell (HAJIBAGHERI *et al.*, 1986). Moreover, BERUBE *et al.* (1991) found in *D. bioculata* an increase of Golgi vesicles in relation to the increase of external concentration of sodium chloride. On the other hand, HOSHAW & MALUF (1981) observed that in *D. tertiolecta* the number of vacuoles is more dependent on the ageing of the cell than on salt stress. In our experiments the cells were in a logarithmic phase of growth; therefore the increase of vacuoles found in *D. acidophila* seems to be related to the osmotic balance of the cell under salt stress.

The coated vesicles consist of "a lipid vesicle surrounded by a basket of protein" (KAMASEKI & KADOTA, 1969). The protein was named clathrin by PEARSE (1978) and the association of clathrin with these vesicles has been demonstrated by immunochemistry (CROZE *et al.*, 1982). These researchers also suggest that free vesicles are transported to points of

attachment to microtubules and then transferred to the cell surface.

In *D. acidophila* coated vesicles detached from the mature pole of the stacked Golgi cisternae have been observed in close proximity of plasma membrane but not in connection with cytoskeletal microtubules. As pointed out by FRIEND & FARQUHAR (1967), the coated vesicles associated to Golgi body transport acid phosphatase or possibly other acid hydrolases from the dictyosome to their site of action (the plasmalemma) or to lysosomes. These enzymes could be involved in glycerol metabolism of the cell, which is the result of a balance between synthesis and removal of this compound. The survival under high salt stress requires an increment of cellular metabolism due to the extra energy demand for the synthesis of organic osmotica (GIMMLER *et al*, 1981).

Further investigations are in progress to establish if the increase of both coated vesicles and vacuoles observed in *D. acidophila* cells grown under high salt is related to the enhanced turnover of glycerol.

Abstract

The ultrastructure of the green acidophilic flagellate *Dunaliella acidophila*, isolated from a soil sample near a hot spring and grown under 0.5 M NaCl or 0.6 M Na_2SO_4 has been studied by scanning electron microscopy (SEM), transmission electron microscopy (TEM). X-ray microanalysis of the soil sample has evidenced that sulphur and aluminium are largely present, whilst sodium and chloride have not been found in significant amounts. The concentration of sodium, sulphur and chloride within the cell does not change after seven days of exposure to sodium chloride or sodium sulphate. Significative changes at Golgi complex level of *D. acidophila* occurr when the alga is subjected to chronical salt stresses. Electron transparent vesicles become more aboundant in the Golgi area, and they tend to fuse forming large vacuoles, containing portion of membranes and granular or threadlike materìal. Small coated vesicles associated to Golgi appear to increase in salt-treated algae, particutarly at the level of the forming face of dictyosome.

The increase of electron transparent and coated vesicles could be involved in the osmotic balance of the alga.

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