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Dedicated to Prof. dr. sc. ZVONIMIR DEVIDÉ on the occasion of his 80th birthday

Effect of calcium chloride and calcium bromide on chloroplasts of *Lemna minor* L.

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Saturated water solutions of $CaCl_2$, $CaBr_2$ and a 1:1 mixture of them are commonly used as »high density brines« for pressure control in oil wells. To investigate the effect of these chemicals on chloroplast morphology and their sedimentation profile in a sucrose gradient, duckweed, *Lemna minor* L., was chosen as a test organism. By light microscopy, round to oval chloroplasts in control plants and in plants grown on media supplemented with 0.025 and 0.050 mol dm^{-3} of tested salts were observed. However, in plants grown on media containing higher concentrations of the salts ested (0.075 and 0.1 mol dm^{-3}) most of the chloroplasts were of irregular shape, and bigger. The sedimentation profile of the control and the treated chloroplasts (based on chlorophyll determination in 200-µl fractions) also differed. Control samples showed three peaks, while treated samples showed an additional peak (fractions 5–8). One of the peaks obtained in the treated samples was sharper and more intensive than the peak present at the same position in the control extracts. The results obtained showed changes in chloroplast morphology and sedimentation profile after treatment with the higher concentrations (0.075 and 0.1 mol dm^{-3}) of the salts tested that could be the consequence of starch accumulation in chloroplasts.

Key words: duckweed, *Lemna minor*, calcium chloride, calcium bromide, chloroplast, starch accumulation

Introduction

Saturated water solutions of $CaCl_2$, $CaBr_2$ and a 1:1 mixture of them are commonly used as »high density brines« (also called »heavy brines«) for pressure control in oil wells during

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special operations in the exploration and production of natural gas and crude oil (SCHMIDT et al. 1983). Accidental spills of these solutions could pollute ground water and soil.

Aquatic macrophytes are among the first organisms reached by pollutants released in the water. Most of them are highly sensitive to a wide range of polluting substances and have been used as test organisms for the assessment of effects on the aquatic environment (WANG 1991, LEWIS 1995).

The duckweeds (family *Lemnaceae*) are small, vascular floating macrophytes that grow rapidly and reproduce vegetatively. The ease with which they can be cultivated and their availability for manipulation in aseptic laboratory conditions make them suitable organisms for toxicity evaluation (WANG 1986, WANG 1990). Rapid vegetative reproduction enables the establishment of *in vitro* duckweed lines of similar ages, appropriate for the comparison of treated and control plants. The typical test end-points are growth rate (expressed as frond production, fresh and dry weight and frond area), change in pigment content (WANG 1991) and ultrastructural changes (SEVERI 1991, MUANAMPUTU ZIMAFUALA et al. 1997).

The influence of $CaCl_2$, $CaBr_2$ and a 1 : 1 mixture of them on the growth and photosynthetic pigment content of *Lemna minor* L. has already been investigated (TKALEC et al. 1998, VUJEVIĆ et al. 2000). During the 14 days of exposure, the solutions tested, in a concentration of 0.025 mol dm^{-3}, promoted duckweed growth, while concentrations of 0.05 and 0.075 mol dm^{-3} did not affect the growth significantly. The highest concentration applied (0.1 mol dm^{-3}) reduced the growth. With increasing concentration of the chemicals tested, the chlorophyll *a*, chlorophyll *b* and carotenoid content was correspondingly higher.

The purpose of the present study was to evaluate the influence of »high density brines« on the morphology of chloroplasts and their sedimentation profile in a sucrose gradient.

Materials and methods

Chemicals

Saturated water solutions of $CaCl_2$, $CaBr_2$ (concentrations 481.3 g dm^{-3} and 1065 g dm^{-3}, respectively) and a 1 : 1 mixture of them, commonly used as oil industry »high density brines«, were used as stock solutions in our investigation.

Plant material

Lemna minor L. (family *Lemnaceae*) was collected from the Botanical Garden of the Faculty of Science, University of Zagreb. Plants were sterilized with 50% (v/v) ethanol solution and 0.1% (w/v) mercuric chloride water solution (KRAJNČIČ and DEVIDÉ 1980). They were then maintained as stock cultures on Pirson-Seidel's nutrient medium (PIRSON and SEIDEL 1950) under axenic conditions.

Experimental cultures were started by picking healthy colonies with 2–3 fronds from stock cultures and transferring them into 100 ml Erlenmeyer flasks containing 60 ml of modified Hoagland's nutrient medium (KRAJNČIČ 1974). »High density brines« were added to the nutrient medium in volumes appropriate to achieve 0.025; 0.050; 0.075 and 0.1 mol

dm^{-3} of tested chemicals ($CaCl_2$, $CaBr_2$ and a 1:1 mixture of them). The stock and experimental cultures were grown under 16 hours of light (80 $\mu E\ m^{-2}\ s^{-1}$) at 24 ± 2 °C.

After 14 days of cultivation, experimental and control cultures were used for microscopy and chloroplast isolation.

Microscopic observation

Fresh hand-cut sections of plant tissue were examined under light microscope (»Opton«, Karl Zeiss, Jena, Germany) with a camera. Photographs were taken on KB-50 film and processed in FR-3 developer (Fotokemika Zagreb, Croatia).

Chloroplast isolation

Fresh plant material cultivated on medium supplemented with 0.1 mol dm^{-3} of the chemicals tested was used for chloroplast isolation. All steps were performed using ice-cold reagents. Plant tissue (1 g) was ground by mortar and pestle in freshly prepared buffer (10 ml) containing 50 mM HEPES, 2 mM Na-EDTA, 2 mM sodium ascorbate, 0.35 M sucrose, pH 7.6, by the addition of about 50 mg PVP (MUÑOZ et al. 1990). After filtration through four layers of cheesecloth, the homogenate was centrifuged at 400*g* for 3 minutes at +4 °C. The supernatant was centrifuged at 3500*g* for 10 minutes at +4 °C and the pellet was gently resuspended with a soft paintbrush in 1 ml of the buffer previously used for extraction.

Density gradient centrifugation

Sucrose gradients were prepared following the procedure described by SPALDING et al. (1979) and MUÑOZ et al. (1990) with minor modifications. Solutions of 50%, 40%, 30% and 20% (w/w) were prepared dissolving sucrose in the buffer containing 50 mM Tris-HCl, 10 g dm^{-3} PVP-10 (Mr ~ 10000), 5 mM EDTA, and 2 mM 2-mercaptoethanol (pH 7.8). Such a nonlinear gradient was prepared in centrifuge tubes using a glass pipette, and incubated for 30 minutes at +4 °C. After that, the chloroplast suspensions were carefully layered on the top of the gradients and centrifuged in a »swing-out« rotor at 5000*g* for 30 minutes. After centrifugation, the tubes' contents were collected as 200 µl fractions in Ependorf tubes.

Chlorophyll and density determination

For chlorophyll extraction 1.3 ml of cold acetone (80% v/v) was added to each of the 200 µl fractions. After incubation (30 minutes at +4 °C), samples were centrifuged 10 minutes at 2500 *g*. Absorbency was measured at 645 nm and 663 nm against an 80% (v/v) acetone blank. Chlorophyll content was determined according to SCHULER and ZIELINSKI (1989).

The sucrose concentration (% w/v) in each fraction was estimated by refractometry (refractometer 32F, Karl Zeiss, Jena) and the density was calculated according to standard concentration/density conversion table.

The experiment was repeated three times per treatment and the results were expressed as a mean value.

Results

Light micrographs showed round to oval chloroplasts in duckweed cultivated for two weeks on nutrient medium without the addition of tested salts (control) and on media supplemented with 0.025 and 0.050 mol dm^{-3} of tested salts (Fig. 1a). However, in the plants grown in the presence of the higher concentrations of the salts tested (0.075 and 0.1 mol dm^{-3}) different morphological forms of chloroplasts were observed. Some of them were round to oval as in the control plants, but the most of them were of irregular shape and bigger than those found in the controls (Fig. 1b, 1c and 1d).

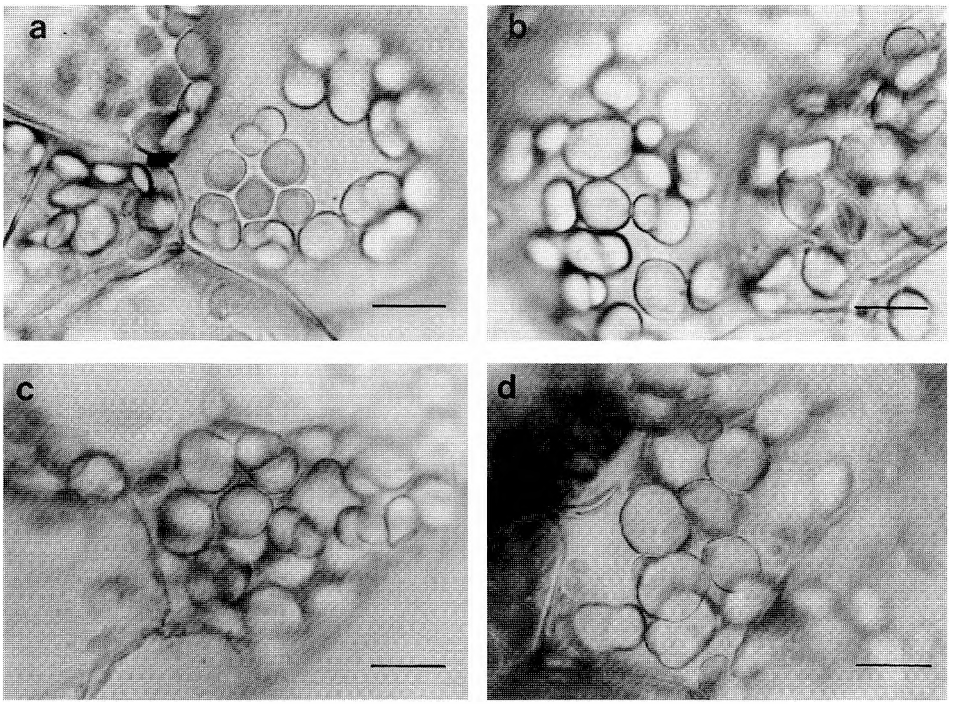

Fig. 1. Chloroplasts in the cells of control plants (a) and chloroplasts in the cells of plants cultivated on nutrient medium supplemented with 0.1 mol dm^{-3} $CaBr_2$ (b), $CaCl_2$ (c) and 1 : 1 mixture of $CaCl_2$ and $CaBr_2$ (d). Bars = 10 μm.

The control plants and the plants grown on the highest concentration tested (0.1 mol dm^{-3}) were chosen for chloroplast isolation and separation on the sucrose density gradients. After centrifugation, three broad, badly-defined green bands were observed in control gradients, while four bands could be seen in the samples treated with the salts tested. One of the bands of the treated samples was sharper and more intensive than the others and the peak present at the same position in the control sample. The middle two bands mainly contained intact chloroplasts, while in the other two (the lowest and the uppermost) only broken chloroplasts were microscopically observed.

The sedimentation profile of chloroplasts (determined as chlorophyll content in 200-µl fractions) is shown in Fig. 2. The chlorophyll peaks obtained from control plants were low and broad (fractions 3–4, 10–12, and 16–17), while the sedimentation profiles obtained from chloroplasts of plants grown on media containing the salts tested showed an additional peak in fractions 5–8 (fraction 5 for $CaCl_2$, fraction 6 for $CaBr_2$ and fractions 7–8 for 1 : 1 mixture) and a high, sharp peak in fractions 9–11.

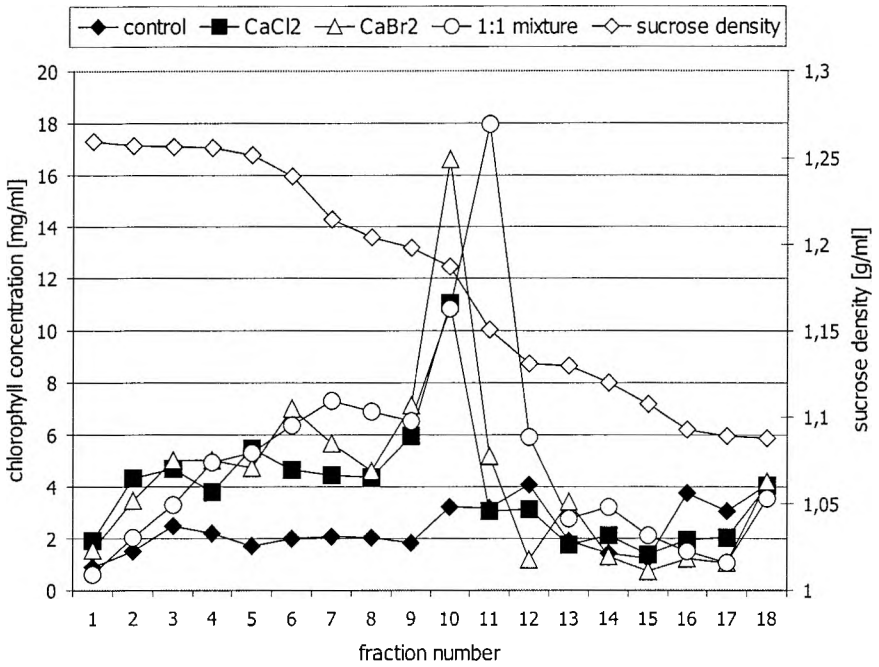

Fig. 2. Sedimentation profiles of chloroplasts (determined as chlorophyll content) from duckweed *Lemna minor* L. cultivated on control medium as well as on media containing $CaCl_2$, $CaBr_2$ and their 1:1 mixture.

Discussion

The appearance of irregular chloroplasts after treatment with the constituents of »high density brines« present in the concentrations of 0.075 and 0.1 mol dm^{-3} might be due to the increased starch content. At this moment, this hypothesis is supported only by iodine staining of thin sections of control and treated plants and their comparison (ŽLENDER, unpublished results). However, it is still necessary to confirm the hypothesis by iodine staining followed by spectrophotometric assay and amylose estimation in the whole plant. Sugar accumulation in chloroplasts in response to applied osmotic stress and salinity was also documented by other authors (GORHAM et al. 1981, WANG et al. 1999). In our investigation the addition of the salts tested caused osmotic as well as salt stress. WANG et al. (1999) showed that osmotic stress induced by sorbitol or high salinity (120 mM NaCl) caused

starch accumulation in sweet potato cell cultures. The effect of salinity was also investigated by LECHNO et al. (1997). They treated cucumber seedlings with 0.1 M solution of NaCl or KCl and in swollen, enlarged chloroplasts large starch grains were noticed. Other substances could induce similar effects. For example, large deposits of starch were found in chloroplasts of *Rosa multiflora* L. plantlets developed on a culture medium containing 5% sucrose (CAPELLADES et al. 1991). The accumulation of large starch grains altering the shape of chloroplasts was noticed after the treatment of duckweed *Lemna minor* L. with aluminium (SEVERI 1991). Duckweed plants treated for 7 days with free oleic acid also accumulated starch grains in chloroplasts (MUANAMPUTU ZIMAFUALA et al. 1997), while chromium induced starch accumulation in some parts of bush bean plants (VÁZQUEZ et al. 1987).

It is well-known that starch accumulation is controlled by inorganic phosphate. Phosphate-dependent translocator regulates the movement of triosephosphates out of the chloroplasts. When phosphate is limiting, the triosephosphates are not readily transported out of the chloroplasts, but instead metabolised into starch (ARIOVICH and CRESSWELL 1983). *Panicum maximum* was observed to accumulate unusually large amounts of starch in the bundle sheath chloroplasts when starved of nitrate nitrogen and phosphate (ARIOVICH and CRESSWELL 1983). It is possible that calcium, which was also applied in our experiment, binds phosphate ions, making them unavailable for plants and in this way stimulates the starch accumulation in chloroplasts. However, this does not exclude any osmotic effect of the salts tested.

The appearance of more peaks after separation of the chloroplasts from the treated samples on sucrose density gradients than from the control, could also be explained by the accumulation of starch grains in treated chloroplasts, which changed the density and the shape of the organelles. However, three tested samples did not cause starch accumulation of the same intensity, so the obtained peaks were not precisely partitioned in the same fraction. Increase of total chlorophyll content in the treated plants has also been observed (Fig. 2). Similar effects have already been described by TKALEC et al. (1998) and VUJEVIĆ et al. (2000). They tested four concentrations of $CaCl_2$, $CaBr_2$ and a 1:1 mixture of them and noticed the increase of chlorophyll content with the increasing concentration of salts tested. This was explained by the presence of calcium ions in the incubation solutions, which can function as enzyme co-factors in the chlorophyll biosynthetic pathway. Although the chlorophyll content of treated plants was higher, this does not mean that the photosynthetic rate was higher too. TKALEC et al. (1998) and VUJEVIĆ et al. (2000) already observed growth reduction that could be the consequence of chloroplast damage caused by starch accumulation. That is, large deposits of starch could cause damage and disorientation of thylakoids and reduce the quantity of light reaching the photochemical centres.

It could be concluded that $CaCl_2$, $CaBr_2$ and a 1:1 mixture of them at the higher concentrations tested (0.075 and 0.1 mol dm^{-3}) induced changes in chloroplast morphology and their sedimentation profile in a sucrose gradient. Detailed ultrastructural characteristics of the treated chloroplasts as well as their precise sizes remain to be investigated by electronic microscopy.

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