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Increase of starch accumulation in the duckweed *Lemna minor* under abiotic stress

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Abstract

Abiotic stresses often result in suppression of photosynthesis and plant growth. Using the duckweed species *Lemna minor* and subjecting these plants to abiotic stress *viz.*, (1) application of heavy metals, (2) application of salt (NaCl), and (3) lack of phosphate, we showed that photosynthesis was inhibited to a lesser degree than plant growth. This became evident by detecting the accumulation of starch under these conditions: (1) Cadmium ions and other heavy metals induced the accumulation of starch after 4 days of treatment at concentrations when growth was almost completely suppressed (e. g. 80 %). (2) Application of NaCl at a concentration of 150 mM also resulted in accumulation of starch but the highest level could be observed only after 7 days. (3) Depletion of phosphate in the growth medium had similar effects leading to starch accumulation after 14 days of treatment. Starch can accumulate to approximately 50% of dry mass under the three different conditions. We suggest the following common molecular mechanism: The stress factor suppresses growth more effectively than photosynthesis. The resulting surplus of carbohydrates is then stored as starch. This hypothesis has biotechnological relevance since stressors may be applied for increasing starch accumulation in duckweed and thus could be used to optimize bioethanol production from this aquatic crop.

Key words: Duckweed, *Lemna minor*, Lemnaceae, Starch accumulation, stress responses

The species *Lemna minor* L. belongs to a family of floating, aquatic plants (Lemnaceae). Plants of this family represent not only the smallest Angiosperms but also the fastest growing flowering plants. Several species increase their biomass within one week by a factor of 10 or 20 [9]. The production of high amount of duckweed biomass in the areas that are unfit for traditional agriculture led to the idea of developing these plants as a new crop for animal feed and fodder considering their high protein content [1], and for the production of bioethanol based on their carbohydrate levels [8]. At the same time, plants have to take up nutrients from the water body to support their fast growth. Therefore, duckweeds can be used for cleaning eutrophic water sources like municipal or animal waste water [3].

In the present paper we report the increase in starch accumulation under different abiotic stress conditions: in the presence of heavy metals (Cadmium), high salt concentration (NaCl), or under limited nutrient (phosphate) availability. Looking at the mechanism underlying these effects, instead of inhibiting growth under condition of inhibited photosynthesis, it could be shown that growth is inhibited stronger (or earlier) than photosynthesis and the resulting surplus of carbohydrates that is not being used for growth is therefore stored as starch.

L. minor L, clone 9441 was obtained from the stock collection of the Institute of Plant Physiology, University of Jena, Germany. This clone was originally collected in 1946 in Marburg, Germany [6]. The plants were pre-cultivated under axenic conditions at 25 ± 1°C in 300 mL Erlenmeyer flasks containing 180 mL sterile nutrient medium. They were exposed to continuous white light at 100 µmol m^{-2} s^{-1} photosynthetically active radiation from fluorescence tubes TLD 36W/86 (Philips, Eindhoven, Netherlands) following the ISO 20079 protocol [6]. According to the ISO 20079 protocol, the plants were conditioned to the nutrient medium for four weeks during this pre-cultivation phase in order to ensure reproducible results. A modified Schenk-Hildebrand medium [7] was employed with the following composition: $CaCl_2 \cdot 2H_2O$ 0.68 mM, KNO_3 12.4 mM, $MgSO_4 \cdot 7H_2O$ 0.81 mM, $(NH_4)H_2PO_4$ 1.3 mM, $MnSO_4 \cdot H_2O$ 30 µM, H_3BO_3 40 µM, $ZnSO_4 \cdot 7H_2O$ 1.74 µM, KI 3.0 µM, $CuSO_4 \cdot 5H_2O$ 0.4 µM, $Na_2MoO_4 \cdot 2H_2O$ 0.21µM, $CoCl_2 \cdot 6H_2O$ 0.21 µM, FeNaEDTA 27.0 µM, $Na_2EDTA \cdot 2H_2O$ 2.74 µM. The pH of the medium was adjusted to 5.5. During pre-

cultivation sufficient biomass was produced for the subsequent treatment under stress conditions.

The main phase of cultivation used for determining the growth rates employed the same conditions as described for the pre-cultivation, except that 400 mL glass beakers containing 300 mL autoclaved Schenk-Hildebrand medium covered with glass plates were used. Fronds were randomly selected from the pre-culture as inoculum for initiating the main (measurement) phase of cultivation. Fronds were inoculated in control medium under the same conditions as describe above. Alternatively, different concentrations of $CdCl_2$ (Sigma, Munich, Germany), or 150 mM NaCl (Roth, Karlsruhe, Germany) were applied or $(NH_4)H_2PO_4$ was replaced by equimolar concentrations of NH_4Cl (Sigma, Munich, Germany) to induce abiotic stress. The main cultivation phase lasted for maximal 14 days. Six plant (inoculum) samples were examined in each case, harvested at the time points given.

For the determination of starch, plant material (200 mg fresh weight) was homogenised in 4 mL 18 % (w/v) HCl. The homogenate was shaken for 60 min at 5°C and centrifuged for 20 min at 5,000 *g*. An aliquot of the diluted supernatant was mixed with equal volume of Lugol´s solution (0.5% w/v KI and 0.25% w/v I_2 in water) and absorbance was measured spectrophotometrically at 605 nm and 530 nm. The amount of starch per dry weight was calculated as described before [2].

The influence of Cd^{2+} ions on the starch content was investigated after 2, 4 and 7 days of treatment (Fig. 1). There was a certain transient increase in starch accumulation also in the control with maximum starch content between 2 and 4 days after the start of the experiment. Application of Cd^{2+} resulted in a much stronger increase of starch accumulation reaching a maximum level after 4 days. Ten µM of Cd^{2+} had the highest impact resulting in approximately 50 % starch on a dry weight basis. Lower and higher concentrations of Cd^{2+} were less effective.

Cultivation in salt-containing nutrient medium also resulted in accumulation of starch in *L. minor*. Maximum impact was observed in plants exposed for 7 days to 150 mM NaCl (Fig. 2). The maximum level of starch accumulated is similar to that after application of 10 µM Cd^{2+}. Lower concentrations of NaCl were less effective, whereas higher concentrations tend to kill the plants, at least after 14 days of cultivation (data not shown).

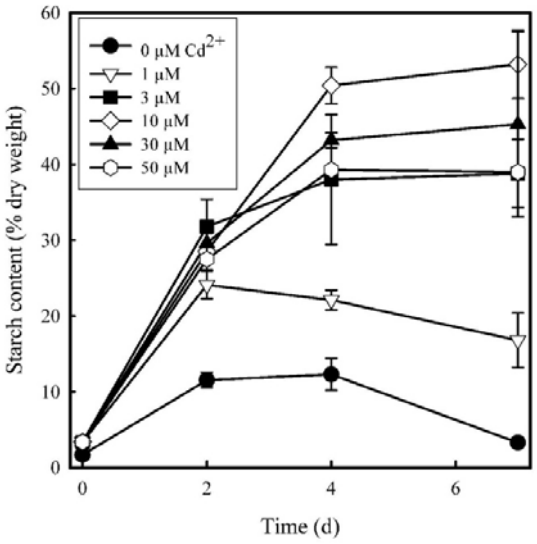

Figure 1. Accumulation of starch in *Lemna minor* under the influence of Cadmium. Different concentrations of Cd^{2+} were applied as indicated and the starch content was measured at the given time points. Data were presented as mean ± standard error from six parallel samples.

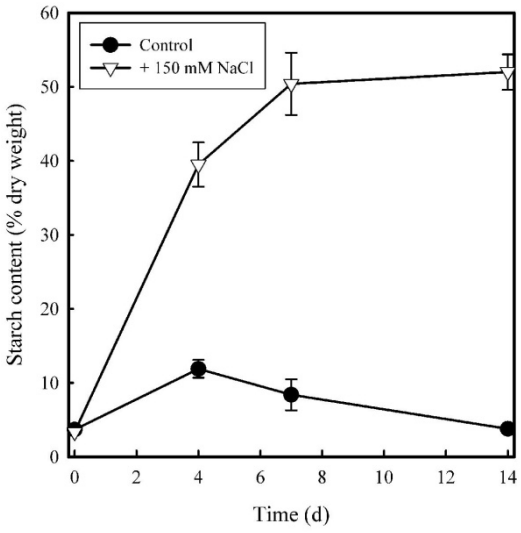

Figure 2: Accumulation of starch in *Lemna minor* in the presence of NaCl (150 mM). Plants were transferred at time zero to nutrient medium with (150 mM) or without (control) NaCl. The starch content was measured at the given time point. For further explanations see Fig. 1.

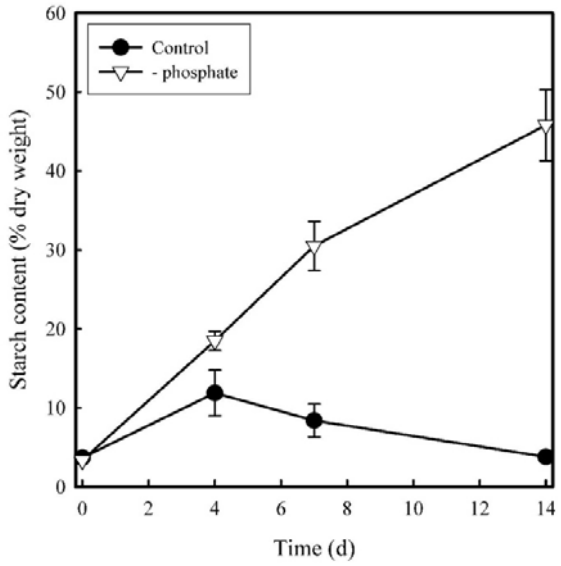

Figure 3: Accumulation of starch by cultivation of *Lemna minor* in complete nutrient medium (control) or in nutrient medium without phosphate ("- phosphate"). For further explanations see Fig. 1.

Growth is also strongly retarded in phosphate-free nutrient medium and starch is accumulated over a period of 14 days (Fig. 3). The maximum starch content on a dry weight basis is between 40 and 50 %.

From a biotechnological point of view there is a demand for plant biomass with high starch content. Starch is accumulated in *L. minor* under any of the three stress conditions investigated, i. e. application of heavy metals, saline conditions, and lack of nutrients. The accumulation of starch is important for practical applications as starch can be degraded to low molecular weight carbohydrates like glucose, maltose and oligosaccharides and finally fermented to bioethanol [5, 8]. The selection of suitable conditions for starch accumulation is very important for the subsequent biotechnological processing. In the presence of Cd^{2+} maximum starch is accumulated already after 4 days of treatment. However, use of large amounts of Cd^{2+} or other heavy metals might be disadvantageous both from an environmental point of view as well as for the subsequent biochemical processes such as starch hydrolysis and sugar fermentation. The investigations on the effect of other heavy metals on other species of duckweeds are on the way. Exposure to salt or to medium lacking phosphate also results in high levels of starch in *L. minor*. Moreover, sea water is easily available which has approximately fourfold higher NaCl content than that used in the present experiments. Lack of phosphate requires the longest period of incubation. This might be caused by the intracellular phosphate stores which can be used by the plants during the initial stages of their exposure to nutrient limiting conditions and hence the actual effect of lack of phosphate can be observed only after the internal store is exhausted. Real shortage of phosphate, therefore, has to be developed first by growth of the plants. For biomass production, instead of phosphate-free nutrient medium, spring source water with low mineral content can be used. The effect of lack of other nutrients on starch accumulation in *L. minor* has not yet been investigated.

As stress conditions inhibit growth (e.g. [2, 4]), a two-step process should be implemented. In an initial step, plants have to be cultivated under optimal growth conditions in order to obtain high amounts of biomass. In a second step starch accumulation can be induced under suitable stress conditions. During this second step there is no considerable increase in biomass but the starch content is elevated for further use. This is a crucial step and also needs much more optimization as the time required and the extent of starch accumulation play an important role in further practical applications. The present paper intends to bring forward the potential of duckweed for bioethanol production and to stimulate further research in this direction.

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