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Response of oxidative stress enzymes in charophyte *Nitellopsis obtusa* exposed to allochthonous leaf extracts from beech *Fagus sylvatica*

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Leaf litter is one of the allochthonous sources of dissolved organic carbon (DOC) in freshwater ecosystems. Throughout the degradation of leaves, a lot of chemical compounds are set free into water, which may promote oxidative stress via generation of reactive oxygen species and / or by disturbance of the intracellular oxidative balance in aquatic organisms. We investigated concentration- and time-dependent relationships for the activities of oxidative stress enzymes in macrophytic algae cells of *Nitellopsis obtusa*, induced by beech *Fagus sylvatica* leaf extracts, respectively, 0.11–55.0 mg L^{-1} DOC (after 24 h) and 1.1 mg L^{-1} DOC after (15 min – 24 h). The oxidative stress enzymes included catalase (CAT – E. C. 1.11.1.6), glutathione reductase (GR – E. C. 1.6.4.2), cytosolic and microsomal glutathione S-transferase (GST – E. C. 2.5.1.18). A significant increase in sGST and mGST activities after a 24-h exposure to 1.1–11.0 mg L^{-1} and to 5.5 mg L^{-1} DOC of beech leaf extracts was detected. sGST activity significantly increased in the whole period of exposure, while mGST activity decreased below control levels when exposed for 1–4 h. Glutathione reductase activity increased significantly at all DOC concentrations of beech leaf extracts after 24 h of exposure. The highest elevation of GR activity was observed in extracts containing 5.5 mg L^{-1} DOC and the lowest activity increment at 0.11 mg L^{-1} DOC. A statistically significant increase in GR activity started after 1 h (at 1.1 mg L^{-1} DOC) and was evident until the end of the 24-h exposure. The activity of catalase increased significantly only at the lowest (1.1 mg L^{-1}) DOC concentration after 24 h of exposure. Beech leaf extracts induced a statistically significant time-dependent elevation of CAT activity during the whole period of exposure. In general, an increase of antioxidative enzyme activities in charophyte *Nitellopsis obtusa* indicated that the defence mechanisms against oxidative stress were induced when cells had been exposed to beech leaf extracts.

Key words: *Nitellopsis obtusa, Fagus sylvatica*, oxidative stress, catalase, glutathione S-transferase, glutathione reductase, dissolved organic carbon, leaf litter

INTRODUCTION

Aquatic ecosystems experience various influences from terrestrial surroundings [1]. In particular, leaf litter is one of the main sources of dissolved organic carbon (DOC) and nutrients in freshwater ecosystems. When leaves are falling into water and degrade, biotic and abiotic processes are involved in the formation and removal of dissolved organic matter [2]. One of the terrestrial species that enter via leaf litter fall into the aquatic environment is beech *Fagus sylvatica*. Beech leaves are known to contain phenolic compounds, have high nitrogen concentrations, and low concentrations

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of carbon-based plant protection compounds such as tannins and lignin [3]. The effect of leaf litter substances on aquatic plants is not well understood and few studies have been carried out; e. g., Kamara and Pflugmacher (2006) investigated the impact of extracts from reed *Phragmites australis* and *Quercus robur* leaves on the antioxidative system and photosynthetic rate of the aquatic macrophyte *Ceratophyllum demersum*.

During leaf degradation, many chemical compounds are released into water and may influence aquatic organisms. Part of these compounds are quinoid structures capable of generating reactive oxygen species (ROS); for example, they influence the photosynthesis of aquatic plants by capturing electrons from the plastoquinone chain between PSII and PSI [4]. Enhanced production of ROS (e. g. $O_2^{\cdot}$, O_2^{-}, H_2O_2, and $HO^{\cdot}$) is able to break up cell homeostasis and may cause lesions of different cell components. To avoid cellular damage caused by these ROS, cells have developed a protective system involving antioxidative enzymes, including catalase (CAT), glutathione reductase (GR) and glutathione S-transferases (GST) in order to reduce ROS concentrations and toxic metabolites within cells. CAT reduces hydrogen peroxide in water. GR is part of the oxido-reduction cycle involving glutathione and ascorbate [5, 6]. More precisely, GR catalyses the reduction of glutathione disulfide (GSSG) in a NADPH-dependent reaction to glutathione and, therefore, plays a pivotal role in both protection against oxidative damage and adjustment processes of metabolic pathways [7]. Beside the biotransformation of exogenous xenobiotics, the glutathione S-transferase is important in detoxifying endogenous toxic lipid peroxidation products [8, 9]. These findings suggest that leaf litter extracts might be an important environmental factor influencing specific plant species and thus community structure within freshwater ecosystems.

Macrophytic green algae *Nitellopsis obtusa* form underwater benthic plantations in fresh and brackish waters. Charophytes, or stoneworts, are easily recognized by their typical morphology. The stem of the plant consists of several 'giant' cells, up to several centimetres long, connected through nodes. The long uneven-length branches appear angular at each node. Stoneworts are considered as good indicators of water quality [10]. The bioelectrical response of a charophyte cell is rapid and highly sensitive to chemical compounds, and can be used as a relevant endpoint to assess the toxicity of aquatic samples [11–13].

The aim of the study was to investigate concentration- and time-dependent relationships for the activities of oxidative stress enzymes in macrophytic algae cells of *Nitellopsis obtusa* induced by beech *Fagus sylvatica* leaf extracts.

MATERIAL AND METHODS

Plant material

The freshwater charophyte algae *Nitellopsis obtusa* (Desv.) was harvested in Lake Švenčius (Lithuania) by hook from a depth of about 5 m in autumn 2005. Plants were transported to the laboratory in plastic bags filled with lake water. Prior to experimentation, internodal cells were separated from neighbouring cells and were kept at room temperature (20 ± 2 °C) in glass aquariums filled with medium A consisting of equal parts of non-chlorinated tap water, lake water and artificial pond water (APW) containing (mM): 0.1 KH_2PO_4, 1.0 $NaHCO_3$, 0.4 $CaCl_2$, 0.1 $Mg(NO_3)_2$, and 0.1 $MgSO_4$ (pH 7.0–7.4) [14]. Most of the cells were about 1 mm in diameter and up to 15 cm long.

Preparation of beech leaf extracts

Fallen leaves of beech (*Fagus sylvatica*) from the top litter layer were collected within the catchment area of Lake Müggelsee (Germany) during the same season as Charophyte collection. These leaves were air-dried for two days in order to obtain a uniform humidity level and then ground with a Mill homogenizer (1094 Tecator). An amount of 100 g ground leaf material was soaked in 1.5 L of artificial freshwater (AFW) (milli-Q water, $CaCl_2$ 0.2 g L^{-1}, $NaHCO_3$ 0.103 g L^{-1} and sea-salt 0.1 g L^{-1}) [15] in separate plastic containers and stirred for 24 h at room temperature. The resulting mixture was centrifuged (L-60 Ultracentrifuge, Beckman LL-TB-003A) at 20 000× g for 10 min at 4 °C to remove suspended material. The supernatant was filtered through a 0.8 µm cellulose-nitrate membrane filter (Sartorius AG, Germany), and total dissolved organic carbon was determined according to DIN EN 1484 (1998).

Experimental setup

Cells of *Nitellopsis obtusa* were exposed to beech leaf extracts of various DOC concentrations for 24 h. The treatments contained 0.11, 1.10, 5.5, 11.0 and 55.0 mg L^{-1} DOC. Alternatively, to investigate time-dependent activity changes, the cells were exposed to beech leaf extract of 1.1 mg L^{-1} DOC for 15 min, 0.5, 1, 1.5, 2, 4 and 24 h. In control experiments, only water medium (no leaf extract) was added. The control (dilution) medium consisted of AFW [15]. All exposures were done in quintuplicate where each replicate contained 25 algae cells. The temperature was maintained at 20 ± 1 °C and the photoperiod at 14 : 10 (dark / light). After exposure, the *N. obtusa* cells were frozen in liquid nitrogen.

Enzyme preparation

Protein extraction was done according to Pflugmacher and Steinberg [16] by homogenizing each sample (25 *N. obtusa* cells) in a glass potter, adding 10 mL

ice-cold 0.1 M sodium phosphate buffer (pH 6.5) containing 1.4 mM dithioerythritol (DTE) and 1 mM EDTA. After removing cell debris by centrifugation at 10 000× g for 10 min, the supernatant was centrifuged again at 40 000× g for 60 min to collect the membrane-bound protein fraction (e. g. microsomes). The microsomes were resuspended in 1 mL of sodium phosphate buffer (20 mM, pH 7.0) containing 20% glycerol, homogenized in a glass potter and shock-frozen in liquid nitrogen. The first protein precipitation was achieved by a 35% ammonium sulphate saturation followed by a centrifugation at 20 000× g for 20 min where the pellet was discarded. The second protein precipitation was achieved by an 80% ammonium sulphate saturation followed by a centrifugation at 30 000× g for 30 min. The pellet from the last precipitation step was suspended in sodium phosphate buffer (20 mM, pH 7.0) and the resulting protein extract was desalted using NAP-5 columns (*GE* Healthcare Life Sciences).

Enzymatic measurements in *N. obtusa* cells

Soluble (cytosolic) and microsomal (membrane bound) glutathione S-transferases were determined using the standard model substrate 1-chloro-2, 4-dinitrobenzene (CDNB) according to Habig et al. [17]. Catalase was determined in the cytosolic fraction according to Aebi [18]. Glutathione reductase activity in the soluble fraction (cytosolic) was measured photometrically according to Carlberg and Mannervik [19], based on the oxidation of NADPH to $NADP^+$ accompanied by a decrease in absorbance at 340 nm. Protein content in the samples was determined according to Bradford [20] with serum bovine albumin (Sigma) as a standard protein reference. The measurement of enzyme activity was expressed in nkat mg^{-1}protein.

Statistics

The values are expressed as means ± S. D. Significant differences between the control and exposures were subjected to one-way ANOVA followed by a Newman–Keuls test at $p < 0.05$ using Statistica v.5.5 [21].

RESULTS

The activity of cytosolic GST in *N. obtusa* cells when exposed to *F. sylvatica* leaf extracts for 24 h showed a significant increase of sGST at DOC concentrations 1.1, 5.5 and 11 mg L^{-1} by a factor of ≈1.5 as compared to control, whereas at DOC concentrations 0.11 and 55 mg L^{-1} the activity of sGST was similar to that of control (Fig. 1 A). Beech leaf extracts induced a significant elevation of microsomal GST (mGST) activity by a factor of 1.4 at a concentration of 5.5 mg L^{-1} DOC (Fig. 1 B). The other tested DOC concentrations showed no significant differences as compared to the controls.

The time-dependent enzymatic response of *N. obtusa* to *F. sylvatica* leaf extracts of 1.1 mg L^{-1} DOC concentration during 24 h revealed a significant increase in sGST activity (by a factor of 2.5) in exposures between 15 min and 4 h (Fig. 2 A). Even after 24 h of exposure to beech leaf extracts, the sGST activity of *N. obtusa* remained increased by a factor of 2 compared to control. The mGST activity was not significantly different from controls in exposures at 15 and 30 min, whereas a statistically significant decrease of mGST activity by a factor of 1.4 to 1.7 below control level was observed in the period between 1 and 4 h (Fig. 2 B). In a 24-h exposure, the activity of mGST in the algae preparations again increased up to the control level (Fig. 2 B).

The catalase activity within the DOC concentration-dependent algae exposures increased significantly in treatments of 1.1 mg L^{-1} DOC (Fig. 1 C). A statistically significant decrease of CAT activities (by a factor of 1.3 to 2.0) was observed as the DOC concentrations increased from 5.5 to 55 mg L^{-1}. Beech leaf extracts induced a statistically significant elevation of CAT activity in *N. obtusa* during the whole period of time-dependent exposure (Fig. 2 C). Overall, the CAT activity increased by a factor of 1.4 to 2.0 as compared to control, where the highest elevation (by a factor 2.0) was observed after 4 h of exposure to *F. sylvatica* leaf extracts (Fig. 2 C). At 24 h of exposure to beech leaf extract, the CAT activity tended to decrease again (Fig. 2 C).

The glutathione reductase activity in 24-h exposed algae cells increased significantly (by factors of 1.4 to 2.6) at all DOC concentrations of leaf extracts (Fig. 1 D). The highest elevation of GR (by a factor of 2.6) was observed at the DOC concentration 5.5 mg L^{-1} and the lowest one (by a factor of 1.4) at 0.11 mg L^{-1} (Fig. 1 D). Time-dependent exposure of *N. obtusa* to beech leaf extracts at 1.1 mg L^{-1} DOC concentration showed a significant increase in GR activity (by a factor of 2) after a 2-h exposure. After 4 h and 24 h, GR activity tended to decrease again, but still remained significantly different from that of control (Fig. 2 D).

DISCUSSION

The effect of artificially prepared *F. sylvatica* leaf litter extract on oxidative stress enzymes in charophyte *N. obtusa* in terms of both concentration (1.1–55 mg L^{-1} of DOC; 24 h) and time-dependent (15 min – 24 h; 1.1 mg L^{-1}) exposures was assessed. The substances obtained during the aquatic extraction from beech leaves were found to promote the antioxidative stress response in macrophytic algae cell.

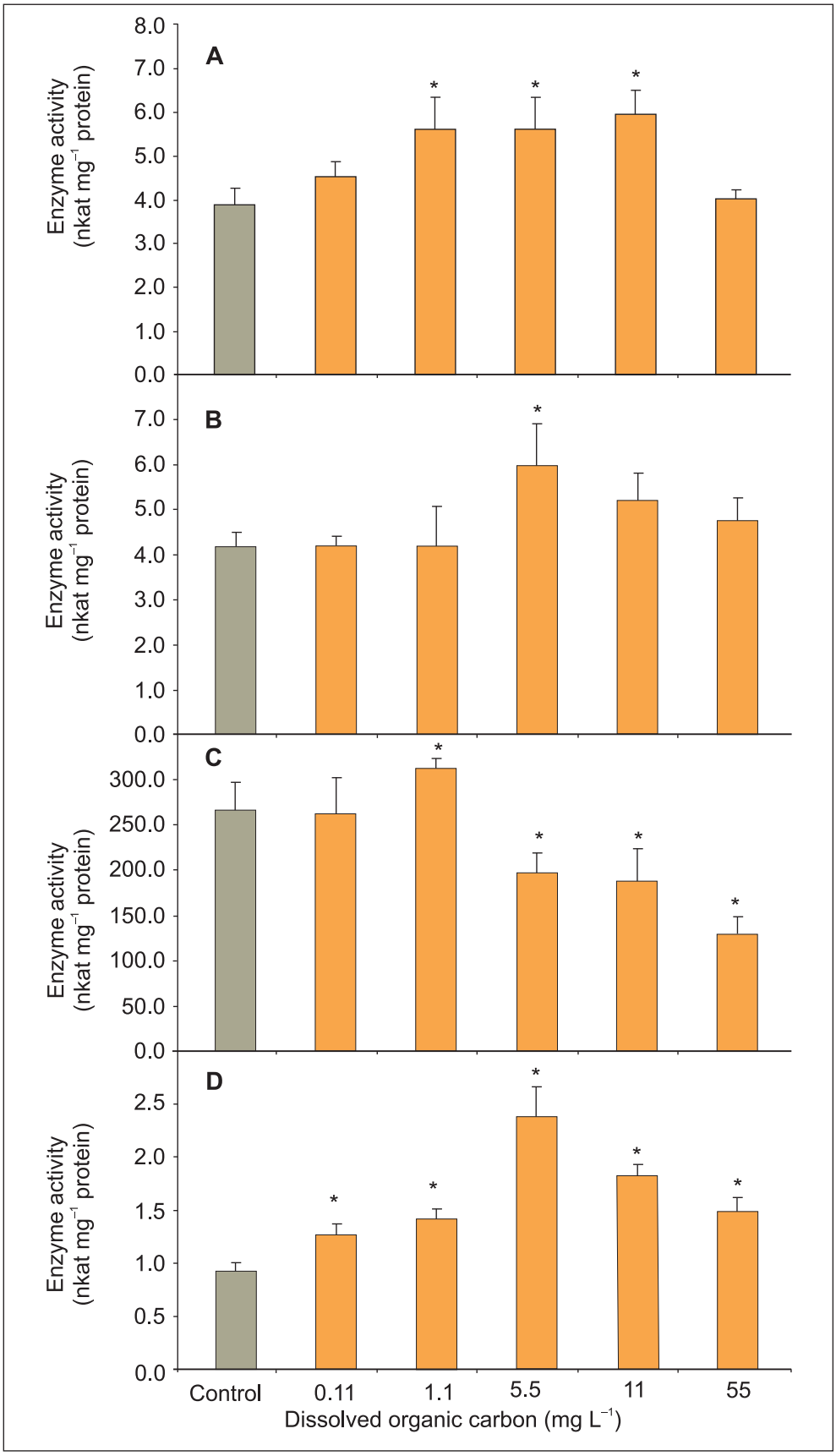

Fig. 1. Concentration–response relationships for activity of cytosolic (A) and microsomal glutathione S-transferase (B), catalase (C) and glutathione reductase (D) in *Nitellopsis obtusa* cells after 24 h of exposure to various DOC concentrations of leaf extract from beech (*Fagus sylvatica*). * Significantly different from the control, $n = 5$, $p < 0.05$

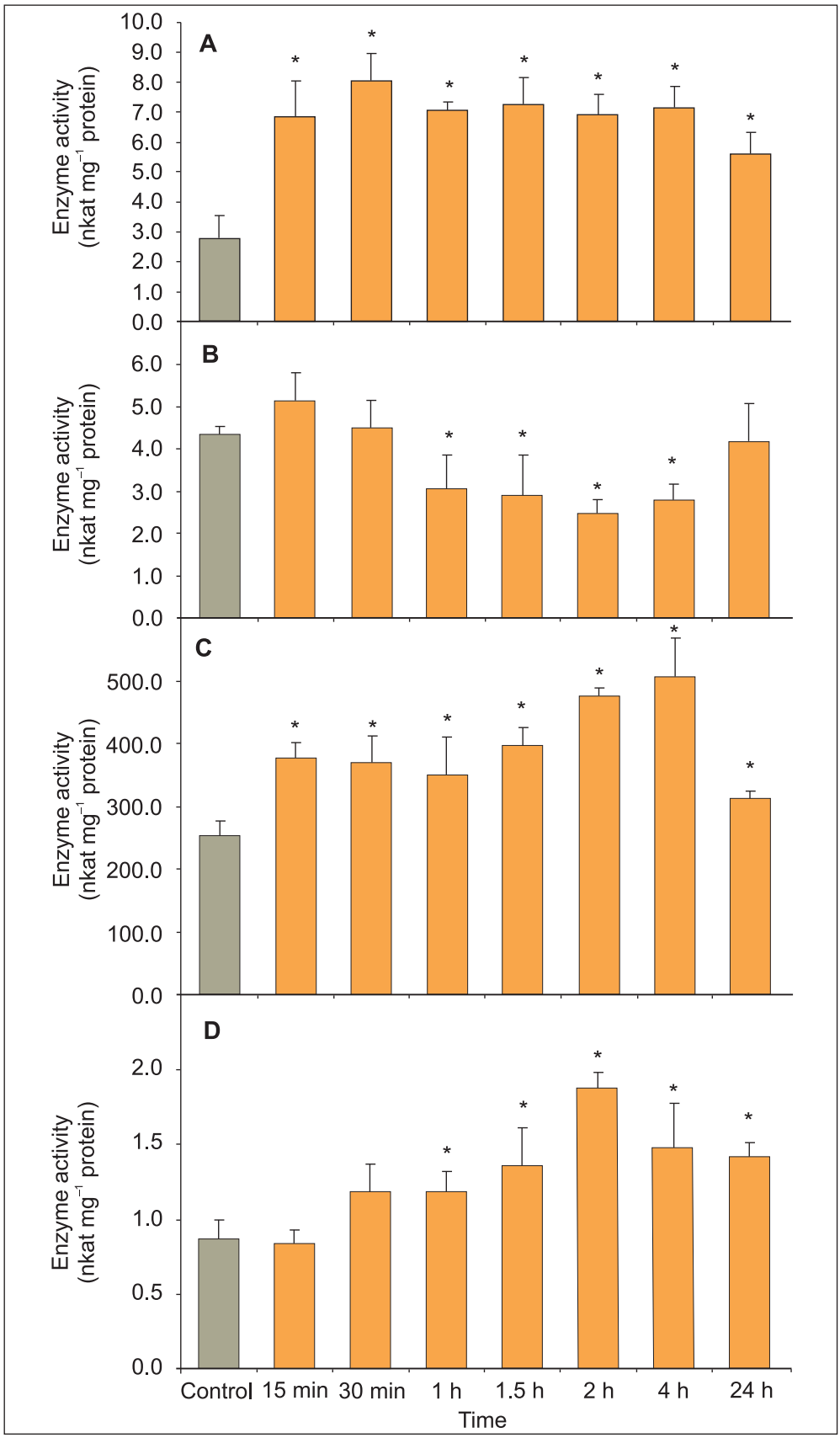

Fig. 2. Time–response relationships for activity of cytosolic (A) and microsomal glutathione S-transferase (B), catalase (C) and glutathione reductase (D) in *Nitellopsis obtusa* cells after exposure to 1.1 mg L^{-1} DOC from beech (*Fagus sylvatica*) leaf extract. * Significantly different from the control, $n = 5$, $p < 0.05$

During the concentration-dependent exposure, the catalase activity in *Nitellopsis obtusa* cells increased significantly at 1.1 mg L^{-1} DOC, but decreased below control levels at higher DOC concentrations at which the algae cell defense mechanisms seemed to be overstrained. Since GR is responsible for recycling of oxidized glutathione (GSSG) to its reduced form (GSH), the main role of GSH is expected in the oxidative stress response of *N. obtusa* exposed to beech leaf litter extracts, whereas CAT seems to play just a secondary role. As mentioned before, GST is involved in the oxidative stress response by detoxifying endogenous compounds generated via lipid peroxidation [9]. When checking the detoxification response of *N. obtusa* exposed to different DOC concentrations of beech leaf litter extracts, we found that sGST and mGST activities increased significantly within DOC concentrations of 1.1–11 mg L^{-1} and at 5.5 mg L^{-1}, respectively. Since our results show that during biotransformation the sGST and especially mGST activities are enhanced at the highest GR activity, they suggest that oxidative stress response through ROS scavenging via GSH and CAT is not enough to completely avoid lipid peroxidation. This is in accordance with the results obtained by Nimptsch and Pflugmacher [22] in *V. dubiana* exposed to leaf litter degradation extracts from oak and beech.

A similar pattern was detected in the time-dependent exposure of algae cells to 1.1 mg L^{-1} DOC of beech extract. Significantly increased CAT activities were observed during the whole exposure period, thus after 24 h CAT activity was still significantly high, but tended to reach the control level. On the other hand, GR activities did not increase significantly until 1 h of exposure, and only then increased above controls. This indicates that CAT is the first to participate in the oxidative stress response of charophyte *N. obtusa*, whereas GR is involved after 1 h of exposure. Since GR is dependent on the amount of GSSG, to recycle the GSH pool the reduced GSH should be oxidized first. This might explain the time shift between the increase in CAT activity and a subsequent increase in GR activity. The decrease of GR activity after 4–24 h and of CAT at 24 h might suggest an acclimation of the algae cells and a proper handling of ROS generated by phenolic substances contained within the leaf extracts. This pattern was observed also by Kamara and Pflugmacher [23, 24] in *C. demersum* exposed to oak and reed leaf extracts.

The results of the activities of soluble glutathione S-transferase (sGST) revealed a significant enhancement of sGST activity by a factor of ≈1.5, in the range of 1.1–11.0 mg L^{-1} DOC concentrations. This finding was similar to that in the aquatic macrophyte *Ceratophyllum demersum* exposed to different NOMs and quinones, where an elevation of sGST activity by a factor of 2–5 was observed [25]. sGST was also found to be significantly elevated in this aquatic plant upon exposure to litter extracts from *Quercus robur* and reed *Phragmites australis* [23]. sGST activity in *C. demersum* exposed to *P. australis* extract was decreased at higher DOC concentrations (10 and 100 mg L^{-1}) [23]; the same pattern (at the highest DOC concentration) was observed in the present study. An increase in GST activities is thus considered as a chemical oxidative stress signal, suggesting that the beech leaf extract used in this study triggered similar effects on the biotransformation system of *N. obtusa*.

The mGST activity showed a statistically significant increase in *N. obtusa* only at a DOC concentration of 5.5 mg L^{-1} after a 24-h exposure. A similar result was observed with *C. demersum*, when a significant increase in mGST activity was induced by the extracts of reed *P. australis* (up to 10 mg L^{-1} DOC) after 1 h of exposure and reached maximum levels after 24 h [24]. Exposure of *N. obtusa* to 1.1 mg L^{-1} DOC from *F. sylvatica* extract significantly increased sGST activity (by a factor of 2.5–2.9); the increase started after 15 min and continued for 4 h of exposure; however, sGST activity tended to decrease again to control levels at the end of the 24-h period, probably showing that *Nitellopsis obtusa* cells were slowly recovering from oxidative stress symptoms. *F. sylvatica* is well known for its relatively high tannin content as well as substances with aromatic structures when grown in suboptimal conditions and / or subjected to environmental stress [26–28]. These compounds are capable of generating reactive oxygen species by capturing electrons from the plastoquinone chain between PSII and PSI during photosynthesis in aquatic plants [4]. In *N. obtusa* exposed to *F. sylvatica* extract, mGST activity significantly decreased during 1 to 4 h and subsequently returned to control values in 24 h. As was observed in a previous study [24], mGST activity in *C. demersum* exposed to *P. australis* and *Q. robur* extracts for longer periods (48 h and 168 h) returned to control values, i. e., plants seem to acclimate to oxidative stress generated by leaf extracts.

In conclusion, our results indicate that leaf extract from beech *Fagus sylvatica* is capable of imposing oxidative stress in green macrophytic algae *Nitellopsis obtusa* as reflected by the alteration of such enzymes as sGST, mGST, GR and CAT. Unexpectedly, relatively low DOC concentrations of beech leaf extract provoked oxidative stress. The effects had a tendency to decrease to normal (control) levels at elevated DOC concentrations (mainly after exposure for 24 h), thus showing the limit of the antioxidative ability of the charophyte *N. obtusa*.

Many aspects of this study still require further investigation. Nevertheless, this report highlights time- and concentration-dependent effects of beech leaf extract on the promotion of oxidative stress response in the charophyte *Nitellopsis obtusa*.

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MAURABRAGIO DUMBLIO (*NITELLOPSIS OBTUSA*) OKSIDACINIO STRESO FERMENTŲ ATSAKAS PAVEIKUS BUKO (*FAGUS SYLVATICA*) LAPŲ EKSTRAKTU

Santrauka

Lapų paklotė yra vienas svarbiausių alochtoninės ištirpusios organinės anglies (DOC) šaltinių vandens ekosistemose. Yrant lapams į vandenį patenka daug cheminių junginių, kurie gali veikti hidrobiontus inicijuodami reaktyvaus deguonies formų (ROS) susidarymą ir (arba) trikdydami ląstelių vidinį oksidacinį balansą. Mes tyrėme buko (*Fagus sylvatica*) lapų ekstrakto poveikio maurabragio dumblio (*Nitellopsis obtusa*) ląstelių oksidacinio streso fermentų aktyvumui priklausomybę nuo DOC koncentracijos (0,11–55,0 mg L^{-1} DOC; po 24 val.) ir ekspozicijos trukmės (15 min – 24 val.; 1,1 mg L^{-1} DOC). Tirti šie oksidacinio streso fermentai: citozolinė ir mikrosominė glutationo S-transferazė (GST – E. C. 2.5.1.18), katalazė (CAT – E. C. 1.11.1.6) ir glutationo reduktazė (GR – E. C. 1.6.4.2). Paveikus *N. obtusa* ląsteles įvairios koncentracijos ekstraktu nustatytas ryškiai padidėjęs GST aktyvumas. Po 24 val. statistiškai patikimas sGST ir mGST padidėjęs aktyvumas pastebėtas esant 1,1–11,0 ir 5,5 mg L^{-1} DOC koncentracijoms. Dumblių ląstelių sGST aktyvumas buvo gerokai padidėjęs per visą 24 val. trukmės eksperimentą, o mGST aktyvumas, atvirkščiai, buvo žemiau kontrolinio lygio veikiant 1–4 valandas. Glutationo reduktazės aktyvumas padidėjo veikiant

visų tirtų DOC koncentracijų buko lapų ekstraktu. Aukščiausias aktyvumas pastebėtas esant 5,5 mg L^{-1} DOC koncentracijai, o žemiausias – 0,11 mg L^{-1} DOC. Paveikus ląsteles buko lapų ekstraktu (1,1 mg L^{-1} DOC), jau po 1 val. aptiktas statistiškai patikimas aktyvumo pokytis. Katalazės aktyvumas *N. obtusa* ląstelėse statistiškai patikimai pakito esant 1,1 mg L^{-1} DOC. Veikiant buko lapų ekstraktu CAT aktyvumas statistiškai patikimai padidėjo viso 24 val. poveikio metu. Apibendrinant galima teigti, kad buko lapų ekstraktas aktyvuoja *N. obtusa* ląstelių oksidacinio streso fermentų kompensacinius mechanizmus.

Raktažodžiai: *Nitellopsis obtusa*, *Fagus sylvatica*, oksidacinis stresas, katalazė, glutationo reduktazė, glutationo S-transferazė