

Selective effects of H₂O₂ on cyanobacterial photosynthesis

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Abstract

The sensitivity of phytoplankton species for hydrogen peroxide (H₂O₂) was analyzed by pulse amplitude modulated (PAM) fluorometry. The inhibition of photosynthesis was more severe in five tested cyanobacterial species than in three green algal species and one diatom species. Hence the inhibitory effect of H₂O₂ is especially pronounced for cyanobacteria. A specific damage of the photosynthetic apparatus was demonstrated by changes in 77 K fluorescence emission spectra. Different handling of oxidative stress and different cell structure are responsible for the different susceptibility to H₂O₂ between cyanobacteria and other phytoplankton species. This principle may be potentially employed in the development of new agents to combat cyanobacterial bloom formation in water reservoirs.

Additional key words: chlorophyll fluorescence induction; diatoms; fluorescence emission spectra; green algae; species differences.

Introduction

Hydrogen peroxide is a commonly encountered component in the aquatic environment (Häkkinen *et al.* 2004). In natural conditions it originates predominantly as a side-product of oxygenic photosynthesis when exposure to high irradiance coincides with a limitation in replenishment of the conventional electron sinks, *i.e.* carbon dioxide or nitrate, and oxygen then serves as electron acceptor instead. Organisms are equipped with enzymes that reduce H₂O₂ and prevent damage by this reactive compound. However, in the case of increased H₂O₂ loading of the aquatic environment, H₂O₂ may act as an algicide (Quimby *et al.* 1988). In addition to its production in oxygenic photosynthesis, H₂O₂ also originates from photo-catalyzed processing of photosensitive compounds (Cooper *et al.* 1988). Not much is known on the biological impact of H₂O₂ despite its common occurrence.

A few indications that microalgal species differ in susceptibility towards H₂O₂ have been reported (Kay *et al.* 1984, Barroin and Feuillade 1986). In a previous paper we demonstrated that H₂O₂ concentrations affecting photosynthesis in the cyanobacterium *Microcystis*

aeruginosa are about ten times lower than those harmful to the green algae *Pseudokirchneriella subcapitata* (Drábková *et al.* 2007). A difference in sensitivity of algal groups to H₂O₂ could potentially be used to combat toxic cyanobacterial species. High concentrations of H₂O₂ in the aquatic environment might cause shifts in the relative abundance of phytoplankton classes, suppressing cyanobacteria more than green algae and diatoms.

The cause of the high sensitivity of cyanobacteria to H₂O₂ has not been analyzed in detail before. Our previous study suggests damage to the photosynthetic apparatus as the mode of action; besides a decreased photosynthetic yield we also observed significant differences in the fluorescence parameter F₀, which was strongly increased in *M. aeruginosa* with hydrogen peroxide present, while it was decreased in green algae and diatoms (Drábková *et al.* 2007). However, whether this observation holds true for more cyanobacterial species remained uncertain. Also the underlying mechanism of the high susceptibility of cyanobacteria towards H₂O₂ awaits further investigation.

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As reported in few previous studies, elevated F_0 values were mainly connected with damage by heat stress or salinity in thylakoids of higher plants and green algae (González-Moreno *et al.* 1997, Haldimann and Feller 2005). A similar increase of F_0 was also reported for the cyanobacterium *Spirulina platensis* after heat stress (Wen *et al.* 2005). In that study, additional measurements of fluorescence emission spectra showed detachment of phycobilisome (PBS) from the thylakoid membranes as cause. In our working hypothesis, the elevated F_0 and also the high susceptibility of cyanobacteria for H_2O_2 may

Materials and methods

Toxicity tests: Cultures used in toxicity tests were as follows: the cyanobacteria *Microcystis aeruginosa* (PCC 7806), *Synechococcus nidulans* (CCALA 188), *Aphanothece clathrata* (CCALA 014), *Cyanobium gracile* (PCC 6307), and *Trichormus variabilis* (CCALA 204); the green algae *Pseudokirchneriella subcapitata* (CCALA 433, formerly known as *Selenastrum capricornutum* or *Raphidocelis subcapitata*), *Scenedesmus quadricauda* (CCALA 463), and *Chlamydomonas reinhardtii* (UTEX 2246); and the diatom *Navicula seminulum* (CCALA 384). Before use in the test, cultures were grown in Erlenmeyer flasks at 27 °C, an irradiance of 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and a light/dark cycle of 16/8 h for 3–5 d to achieve the exponential growth phase. As a growth medium we used a 1 : 1 mixture of medium Z (Zehnder in Staub 1961) and medium BB (Bristol modified by Bold 1949) diluted to 50 %. After pre-cultivation, the algal cultures were diluted with fresh medium to achieve cell densities sufficient for proper measurement with the PAM fluorimeter, but as low as possible not to decrease the sensitivity of the biotest. The cell densities of *P. subcapitata*, *N. seminulum*, and *M. aeruginosa* were 2×10^5 , 2×10^5 , and 5×10^5 cells per cm^3 , respectively, which represented approximately equal biovolume and also similar initial F_0 fluorescence values. In order to ensure the measurements of all tests with the same PAM settings, the density of other species was adjusted to achieve approximately equal density according to the initial F_0 fluorescence values.

H_2O_2 (Aldrich, 35 mass % in H_2O) was diluted immediately before addition; concentrations at the beginning of the test were 10.0, 2.5, and 0.5 g m^{-3} (corresponding to 300, 75, and 15 μM final concentrations, respectively); medium without H_2O_2 was used as a control. Tests were performed in transparent 96-well micro-plates. A volume of 300 mm^3 per well was used, 4 replicates were run for each concentration and control. The tests ran for 3 h at 26–28 °C using an incident irradiance of 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The limited duration of the incubation time balanced the effectiveness of the treatment as a function of the dose and the progressing decomposition of H_2O_2 .

PAM fluorescence measurements were performed using

result from a similar mechanism. In cyanobacteria, the PBS functions as light-harvesting complex (LHC) for photosystem 2 (PS2) and they are situated peripheral on the cytoplasm facing surface of the thylakoidal membrane, contrary to the functionally comparable LHC complex in green algae and diatoms, which is embedded inside the thylakoid membranes (Grossman *et al.* 1995).

The present study analyses the responses of cyanobacteria, green algae, and diatom to H_2O_2 using pulse amplitude modulated fluorometry and 77 K fluorometry.

a PAM-CONTROL fluorometer (*H. Waltz*, Effeltrich, Germany). The cells were dark adapted for 15 min before measurements. The parameters measured were: F_0 , the minimal fluorescence signal of dark-adapted cells, which was used as an indirect indicator of biomass, and F_m , the maximal signal of dark-adapted cells obtained with a saturating radiation pulse. These parameters allow the calculation of the maximal yield of PS2 as F_v/F_m and the capacity of dark-adapted cells to convert photon energy into chemical energy as $(F_m - F_0)/F_m$. This nomenclature is according to Van Kooten and Snel (1990). F_v/F_m is biomass independent and can be used as an indicator for the general level of fitness of photosynthetic organisms.

77 K fluorescence emission spectra: *M. aeruginosa* and *P. subcapitata* were chosen for the measurement of fluorescence emission spectra because these strains showed the highest sensitivity among the tested phytoplankton species. Two-fold cell densities (relative to the standard used in the PAM measurements described above) and micro-plates with 5 cm^3 volume in wells were used in the test to obtain sufficient algae for 77 K spectrometry. Other test conditions remained essentially as reported above. After 3-h exposure, samples were taken with a glass capillary with thick walls (2 mm inner diameter) and immediately frozen in liquid nitrogen. Simultaneously 300 mm^3 aliquots were taken and measured by PAM fluorometry. Low temperature fluorescence emission measurements were performed on an *Aminco-Bowman 500* series 2 luminescence spectrophotometer equipped with a Dewar flask filled with liquid nitrogen that was placed in the measuring chamber of the instrument. Excitation of photosynthetic pigments was at 435 nm both for green algae and cyanobacteria and at 615 nm for the cyanobacterium only; at this wavelength the PBS pigment cyanophycobilin is specifically excited. Fluorescence emission was recorded from 620 to 740 nm. Experimental noise was reduced by 5 times recording of each spectrum and data averaging.

Data analysis: Mean values of F_v/F_m were calculated and graphed, to determine the EC_{50} values (50 % inhibition concentration) and their 95 % confidence intervals. EC_{50}

values were obtained by a linear interpolation method. Sensitivity of eukaryotic algae *versus* cyanobacteria has

Results

H₂O₂ effects in nine species: The parameters F_v/F_m in controls untreated by H₂O₂ were 0.343, 0.191, 0.134, 0.165, and 0.218 for the cyanobacteria and 0.642, 0.704, 0.363, and 0.504 for the green algae and the diatom, respectively. In all cyanobacteria tested F_v/F_m was inhibited by more than 50 % at concentrations from 0.5 to 2.5 g(H₂O₂) m⁻³ (Fig. 2). In contrast, in 2 out of 3 green algal species and the diatom a concentration of 2.5 g(H₂O₂) m⁻³ caused no effect. A small inhibition of up to 20 % was observed in the green alga *P. subcapitata* (Fig. 1). Despite some differences among the cyanobacterial species, the effects of H₂O₂ exposure for all cyanobacterial species largely differed from the effects in the green algae and the diatom used in this study. Comparison of the EC₅₀ values (Table 1) demonstrated statistically significant differences at $p < 0.05$.

The parameter F_0 in controls untreated by H₂O₂ was 444, 472, 522, 380, and 343 for the cyanobacteria, and 546, 477, 330, and 629 for the green algae and the

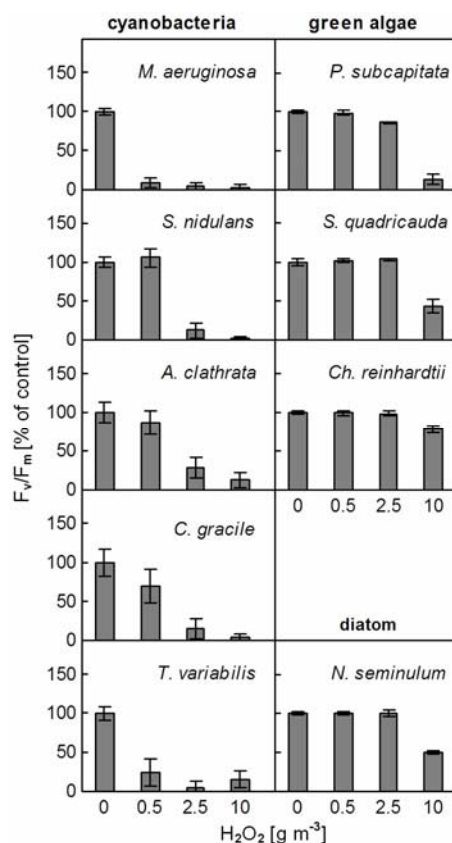


Fig. 1. Inhibition [%] of the chlorophyll fluorescence parameter F_v/F_m (photosynthetic yield) 3 h after exposure to three different H₂O₂ concentrations, compared to untreated controls, in 9 phytoplankton species.

been compared by Mann-Whitney U test by use of the acquired EC₅₀ values.

diatom, respectively. Significant elevation of the F_0 parameter of up to 300 % of the control occurred in all cyanobacterial species (Fig. 2). In contrast, there was no such increase or even a decrease of F_0 for the green algae and the diatom.

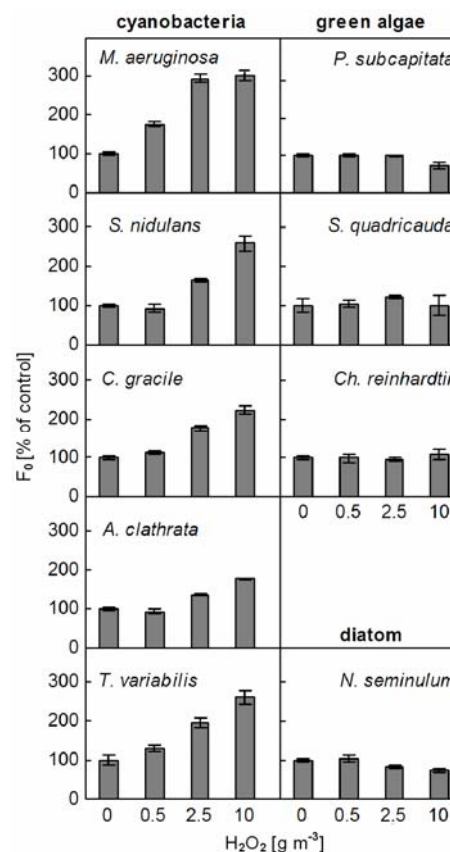


Fig. 2. Changes [%] observed for the fluorescence parameter F_0 3 h after exposure to three different concentrations of H₂O₂ added to the incubation medium, compared to untreated controls, in 9 phytoplankton species.

77 K fluorescence emission spectra: To elucidate the cause of the elevated F_0 value in cyanobacteria after exposure to H₂O₂ the PAM fluorometry parameters and fluorescence emission at 77 K were measured in the same samples (Figs. 3 and 4). Fig. 3A shows emission spectra for the green alga *P. subcapitata* from chlorophyll (Chl) *a* excitation at 435 nm. The control cells exhibited emission peaks at 688 nm (PS2) and 716 nm (photosystem 1, PS1). The signal at about 664 nm is related to emission from the Chl *a/b* LHC antenna. The spectra were normalized to the peak of 716 nm. The H₂O₂ concentration of 0.5 g(H₂O₂) m⁻³ did not exhibit any significant change of fluorescence emission spectra and only a small change of

the spectra was observed at $2.5 \text{ g(H}_2\text{O}_2) \text{ m}^{-3}$. At a concentration of $10.0 \text{ g(H}_2\text{O}_2) \text{ m}^{-3}$ a strong decrease of the fluorescence peak from PS2 was observed. These results for the green alga *P. subcapitata* match the changes observed for F_v/F_m and also F_0 in the PAM fluorometry assays (Fig. 4).

Table 1. Toxicity as EC_{50} values [g m^{-3}] for H_2O_2 (concentration which causes 50 % inhibition of the photosynthetic yield, F_v/F_m). The test comprised 9 phytoplanktonic species from 3 different taxonomic groups. C.I. – confidence intervals, *extrapolated value.

		$\text{EC}_{50} \pm \text{C.I.}$
Cyanobacteria	<i>Microcystis aeruginosa</i>	0.27 ± 0.03
	<i>Synechococcus nidulans</i>	1.69 ± 0.22
	<i>Aphanothece clathrata</i>	1.74 ± 0.49
	<i>Cyanobium gracile</i>	1.14 ± 0.64
	<i>Trichormus variabilis</i>	0.35 ± 0.13
Green algae	<i>Pseudokirchneriella subcapitata</i>	6.22 ± 0.46
	<i>Scenedesmus quadricauda</i>	9.25 ± 1.22
	<i>Chlamydomonas reinhardtii</i>	$21.04 \pm 6.55^*$
Diatom	<i>Navicula seminulum</i>	10.00 ± 0.40

Under the same excitation wavelength of 435 nm the control in the cyanobacterium *M. aeruginosa* showed the emission peak at 685 nm for PS2 and a peak at 720 nm for PS1 (Fig. 3B). Curves were normalized to the peak at 720 nm. The 435 nm excitation also exhibited emission at a wavelength of 664 nm indicative for free PBS emission, though the 435 nm wavelength is in particular selected for excitation of Chls. The signal pertinent in *Microcystis* was also seen in the cyanobacterium *Calothrix*, but was absent in the cyanobacterium *Synechocystis* PCC6803 (data not shown). It suggests that the strain of *Microcystis* used in this work contains some phycoerythrin or phycourobilin, these are PBS pigments that absorb more in the green and blue domains of the spectrum, and hence become excited and give rise to PBS emission. Decrease of the 685 nm peak fluorescence is apparent in samples at H_2O_2 concentrations of 2.5 and $10.0 \text{ g(H}_2\text{O}_2) \text{ m}^{-3}$, bleaching of the PBS is evident as well. In contrast to what was observed for the green algae, the 77 K and PAM results did not fully match for *Microcystis*. In the latter, the effect of H_2O_2 was seen as a strong decrease of F_v/F_m and a significant increase of F_0 already at a concentration of $0.5 \text{ g(H}_2\text{O}_2) \text{ m}^{-3}$ in the PAM results (Fig. 4), but not in the 77 K spectra (Fig. 3B).

Fig. 3C shows the same sample of *M. aeruginosa* but now under PBS excitation at 615 nm. An emission peak is seen at 650 nm (phycocyanin – PC) with a shoulder around 664 nm (allophycocyanin – APC) and a distinct peak at 685 nm, which results possibly from two emitters,

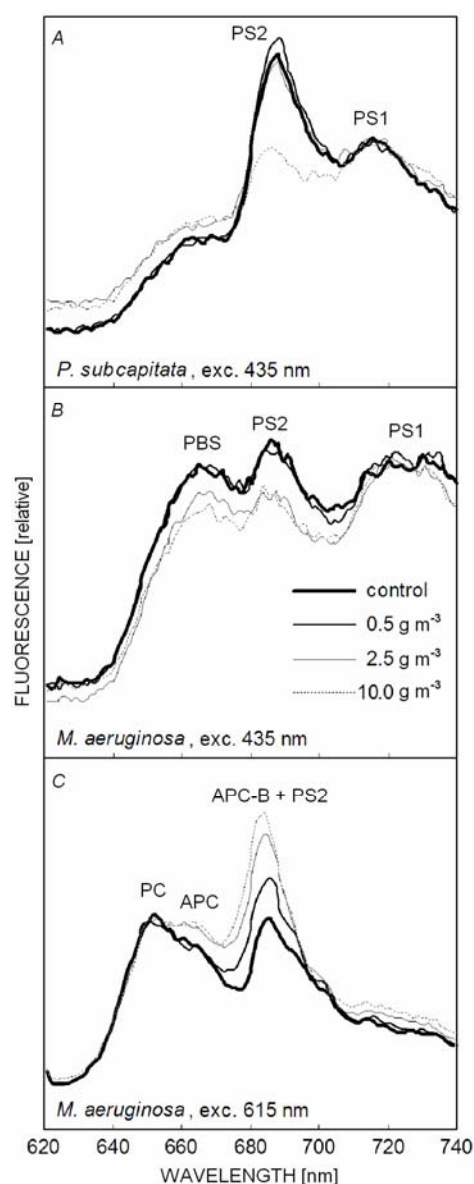


Fig. 3. 77 K fluorescence emission spectra of the green alga *P. subcapitata* excited at 435 nm (A) and of the cyanobacterium *M. aeruginosa* excited at 435 nm (B) or 615 nm (C). Spectra were measured 3 h after the addition of H_2O_2 .

the terminal emitter of the PBS and from PS2. The fluorescence intensity in samples affected by H_2O_2 strongly increases especially at 685 nm. The detachment of the PBS from PS2 that is suggested as the cause for the increase in the 685 nm fluorescence is also the plausible reason for the high F_0 values in the PAM experiment. The effects that result from increasing concentrations of H_2O_2 show a gradual dose dependent response both in the 77 K (Fig. 3C) and PAM experiments (Fig. 4).

Discussion

Susceptibility of cyanobacteria to external H₂O₂ exposure has been indicated in a few earlier studies. Kay *et al.* (1984) reported that the green alga *Ankistrodesmus* was sensitive to 6.4–10.2 g(H₂O₂) m⁻³ while an effective dose for growth inhibition of the cyanobacteria *Microcystis* and *Raphidiopsis* was 3.4 and 1.7 g(H₂O₂) m⁻³, respectively. A concentration of 15 g(H₂O₂) m⁻³ had no effect on growth and pigment decomposition in the green alga *Pandorina morum*, whereas the cyanobacterium *Oscillatoria rubescens* was destroyed at 1.5 g(H₂O₂) m⁻³ (Barroin and Feuillade 1986). Sodium carbonate peroxyhydrate (2 Na₂CO₃×3 H₂O₂) completely inhibited the growth of *Oscillatoria* cf. *chalybea* at 100 mM [equivalent to 1 g(H₂O₂) m⁻³] while a 10 times higher concentration was required for complete inhibition of growth for the green algae *P. subcapitata* and *Pediastrum simplex* (Schradler *et al.* 1998). A 10-fold higher sensitivity towards H₂O₂ for the cyanobacterium *M. aeruginosa* than for both the green alga *P. subcapitata* and the diatom *N. seminulum* was concluded from F_v/F_m values in PAM experiments (Drábková *et al.* 2007). The present results extend these findings to 5 different cyanobacteria and underline the major difference in sensitivity between the highly susceptible cyanobacteria and the relatively resistant green algae.

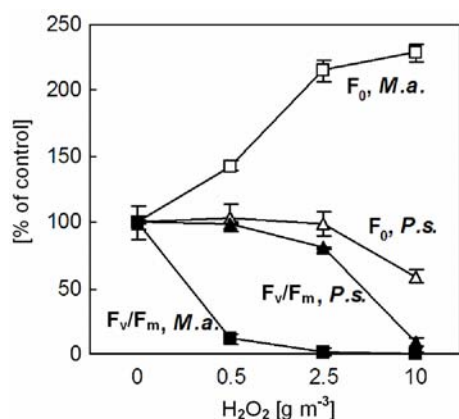


Fig. 4. Changes of the fluorescence parameters F_v/F_m (photosynthetic yield) and F₀; comparison of the green alga *P. subcapitata* (*P.s.*) and the cyanobacterium *M. aeruginosa* (*M.a.*). Data were obtained 3 h after the onset of exposure to H₂O₂.

H₂O₂ in addition to changes in F_v/F_m also demonstrated a large increase of the F₀ value in the cyanobacterial species, while there was a little decrease of F₀ in green algae. This substantial difference is explained firstly from differences in the photosynthetic apparatus. In PAM fluorometry, the measuring radiation has an excitation at 650 nm and most of the measuring radiation is absorbed by PBS in cyanobacteria. Therefore, F₀ fluorescence in cyanobacteria contains emissions from

phycobiliproteins and from PS2. PBS functions in F₀ fluorescence anomalies of cyanobacteria (Campbell *et al.* 1998). Our measurements of fluorescence emission spectra in cyanobacteria exposed to H₂O₂ revealed that the strongest increase in fluorescence emission comes from the 685 nm band. The peak at 685 nm can be attributed to both the PBS terminal emitter (allophycocyanin B) and to the small antenna of PS2 (Bald *et al.* 1996). A correlation between increasing F₀ values and 685 nm emission from 615 nm phycobilisome excitation is a good indication that detached PBS fluoresces *via* its terminal emitter, when functionally disconnected from PS2 by which exciton guidance from the PBS to the latter is impaired. In the green algae, a decrease in PS2 fluorescence emission was observed after exposure to the highest H₂O₂ concentration. This might be an indication of starting degradation of photosynthetic pigments and can also be attributed to the small decrease in F₀ observed in PAM fluorometry.

The effects of oxidative stress and H₂O₂ on photosynthesis in cyanobacteria has been studied in detail before (Samuilov *et al.* 2001, Yousef *et al.* 2003, Lupinková and Komenda 2004), however, the studies were performed at much higher H₂O₂ concentrations and algal densities and therefore those data are not easily comparable to our results. In the present study we used low H₂O₂ doses and algal densities that are realistic for the aquatic environment, which were at the lower limit for measurement of fluorescence emission at 77 K.

Our hypothesis to explain the high sensitivity of cyanobacteria to H₂O₂ is based on three elements. Firstly, cyanobacteria bear their light-harvesting complexes (*i.e.* the PBS) on the outside of the thylakoidal membrane directly exposed to the cytoplasm. Secondly, the typical prokaryote structure of cyanobacteria renders the photosynthetic apparatus more readily susceptible to externally added reagents than in systems with photosystems present in the cytosol embedded membrane of enclosed chloroplasts. Thirdly, cyanobacteria also have less elaborate H₂O₂ detoxification pathways, which is partly a function of their prevalence at low irradiances than in green algae.

The main H₂O₂ detoxification enzymes in cyanobacteria are catalase or catalase-peroxidase. In some cyanobacteria, the ascorbate peroxidase, thioredoxin peroxidase, and glutathione pathways were also found (Miyake *et al.* 1991, Yamamoto *et al.* 1999, Miller *et al.* 2000, Perelman *et al.* 2003). The enzymes most capable to destroy H₂O₂ in cyanobacteria are catalase or catalase-peroxidase; both are cytoplasmic enzymes capable to detoxify high H₂O₂ amounts. The irradiance-induced inactivation of catalase (Tytler *et al.* 1984, Tel-Or *et al.* 1986) makes cyanobacteria even more susceptible to peroxide at high irradiances, which is also in accordance with our previous results (Drábková *et al.* 2007).

Contrary, the main H₂O₂ detoxification enzyme in green algae is ascorbate peroxidase that besides catalase is present in peroxisomes. The ascorbate peroxidase enzyme might be present in the cytosol, associated with various cell compartments, and not inactivated by high irradiance (Asada 1992, Shigeoka *et al.* 2002). Ascorbate peroxidase activity was also found in some cyanobacterial species (Miyake *et al.* 1991), however, its activity is limited due to much lower intracellular concentration of ascorbate than that reported for chloroplasts (Tel-Or *et al.* 1986). Regarding these facts, we assume that the significant difference in sensitivity between cyanobacteria *versus* green algae only applies to H₂O₂ exposure and not necessarily to other reactive oxygen species for which the detoxification enzymes are different.

Looking at the typical attributes of the prokaryotic cyanobacterial cell structure, the cyanobacterial thylakoids are situated close to the cytoplasmic membrane

and are not organized in packed stack structures like in chloroplasts. This, along with the differences in H₂O₂ detoxification enzymes, makes the cyanobacterial photosynthetic apparatus more susceptible to external H₂O₂ attack. Contrary, in green algae the externally added H₂O₂ is faster degraded by ascorbate peroxidase in other parts of the cell before it can reach the sensitive parts of photosynthetic apparatus in sufficient concentration. This agrees well with our results, which show the effects of external H₂O₂ exposure on green algae only at such a high concentration (10 g m⁻³) that provokes destruction of photosynthetic pigments, which was recorded as a small decrease of fluorescence level.

The present results prove selectively higher effects on cyanobacteria by external attack of peroxides compared to green algae, which is a principle that may potentially be employed in the development of new agents to combat toxic cyanobacterial blooms formation in water reservoirs.

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Tuba, Z. (ed.): **Ecological Responses and Adaptation of Crops to Rising Atmospheric Carbon Dioxide**. – Food Products Press, an Imprint of the Haworth Press, Binghamton 2005. ISBN 13: 978-56022-121-0 (softbound), 13: 978-56022-120-3 (hardbound). 414 pp., USD 49.95 (softbound), 79.95 (hardbound).

The book was published simultaneously with volume 13 (2005) of the *Journal of Crop Improvement*. The volume Editor, a well-known Hungarian scientist, prepared a collection of 18 papers dealing with one of the most actual topics of the Nature in the 21st century. The effects of continuously rising atmospheric CO₂ concentration in atmosphere on plants are an interesting topic not only for science, but also for politicians and general public. The main questions are analysed in the introductory paper written by the Editor that precedes the other papers. About half of the papers, all peer-reviewed, of course, are review articles. The 50 authors of articles work in 14 countries (the USA 10, Italy 7, Ireland and Slovenia 5 each, Hungary and the UK 4 each, Finland, Germany, and Japan 3 each, Switzerland 2, Austria, Bulgaria, Denmark, and Sudan 1 each), hence the book is really internationally authored.

The papers deal with a wide plant material, mainly citrus, cotton, grain legumes, grapevine, green pepper, maize, pasture and cereal plants, poplar, potato, and rice. The basic questions are changes in climate (drought, floods, and extreme temperatures) and photosynthetic plant production introduced by changes in atmospheric concentration of CO₂. These changes are reflected in soil formation and composition, quality of plant products, species composition in plant communities, vegetation dynamics *etc.* in most climatic regions of the Earth. The changes may be positive (some increase in plant production and quality of products) or negative (possible stress effects) and depend also on other factors that are important for plant growth and metabolism (water relations, temperature, respiration types and rates, necessary

supply of mineral nutrients, especially nitrogen). One has also to distinguish short- and long-term effects, acclimation to CO₂ treatment, reactions in wild type and genetically modified plants, changes in growth and development of individual plants and plant communities, *etc.* In addition, some specific topics are dealt with, *e.g.* developmental models, effects on quality of products (content of glycoalkaloids in potato tubers, *etc.*), comparison of CO₂ effects in plant monoculture and bi-species mixture (*Trifolium* and *Trisetum*), use of a FACE (Free Air CO₂ Enrichment) system in this kind of research, effects on contents of photosynthetic pigments or ribulose-1,5-bisphosphate carboxylase/oxygenase, *etc.* Some review papers contain tables with interesting comparison of results of various authors (*e.g.* papers on pp. 73-89, 91-111).

As this is primarily an issue of journal, it was probably not necessary to unify different styles of the authors, shapes of figures, *etc.* I would only recommend unify the form of references (always italics for journal titles). An author index would be welcome, but it would certainly increase the number of pages reserved for this volume.

There are many books dealing with this crucial problem (cf. the book of Nátr reviewed in *Photosynthetica* **44**: 547, 2006), but the reviewed volume is very specific from the point of view of both plant physiology and agriculture. I recommend the book to all students and researchers who do not find the *Journal of Crop Improvement* in a near library. The main conclusions are certainly interesting for those who call themselves “ecologists” or for people of opposite opinion.

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