Protoplasma (2010) 240:83–97
DOI 10.1007/s00709-009-0094-z

ORIGINAL ARTICLE

Morpho-physiological and biochemical responses in the floating lamina of *Trapa natans* exposed to molybdenum

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Received: 10 September 2009 / Accepted: 20 November 2009 / Published online: 12 December 2009

Abstract The response to molybdenum (Mo) was studied in the metal-tolerant hydrophyte *Trapa natans* L. Previously, it was shown that the plant accumulates Mn in the floating lamina by means of phenolic compounds and responded with acclimation responses of the chloroplast. Since the involvement of phenolics has been proposed also in Mo resistance, we tested the response of *T. natans* to increasing doses (5, 50, 150, 600 μM) of Mo using the photosynthetic apparatus as an indicator of cellular stress. Only 5 μM Mo did not cause evident modifications with respect to controls. Conversely, 50 to 600 μM Mo induced progressively marked alterations of the lamina morphology. The chloroplast ultrastructure showed disorganisation of the thylakoid system, and correspondingly, the photosynthetic pigment pattern was altered with a fall-down in photosynthesis. Microspectrofluorimetry indicated alterations of photosystem II, with differences among the three cell layers (first and second palisade and spongy tissues). While the highest dose caused plant death, 50 and 150 μM Mo-treated plants underwent partial recovery, and the plant survived up to the end of the vegetative season. However, reproduction was unsuccessful. Mo treatment did not induce increase in total phenolics, but only in anthocyanin. In contrast to Mn, detoxification of Mo by chelation inside vacuoles, possibly by anthocyanins, is suggested to be an insufficient mechanism to reduce Mo toxicity, which probably includes an impairment of nitrogen metabolism. However, the metal was accumulated in the lamina. On the whole, *T. natans* showed limited capabilities to survive Mo excess as compared with Mn.

Keywords Chloroplast · Hydrophytes · Microspectrofluorimetry · Molybdenum · Photosystem II · *Trapa*

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Introduction

Many heavy metals are essential micronutrients for plants because of their involvement in numerous metabolic processes, because they are constituents of numerous enzymes and other proteins. However, they become toxic if their concentration exceeds a specific critical point (Hall 2002). Nevertheless, some plant species can grow and reproduce in metal-contaminated environments, by developing several mechanisms that make them resistant against the metal by avoidance and/or tolerance (reviewed by Hall 2002). These strategies involve a combination of species-specific modifications of plant morphology and metabolism, including: metal exclusion from the protoplast (Heumann 2002); metal compartmentalisation inside the vacuole (Rauser 1995; Baldisserotto et al. 2004, 2007);

production of chelating compounds (Hale et al. 2001; Steinke et al. 2008). Metals can be also partially integrated in the metabolism as constituents of metallo-proteins which can participate in the regulation of the metal availability in the cell (Hall 2002; Graham and Stangoulis 2003). Among heavy metals, molybdenum (Mo) occurs in a wide range of metalloenzymes in bacteria, fungi, plants, and animals, where it forms the active part of these enzymes (Mendel and Bittner 2006). In plants, Mo is involved in nitrogen metabolism and assimilation (Mendel and Hansch 2002). Mo is assimilated by plants as molybdate ion (Kuper et al. 2000). Mo unavailability, scarceness or excess represent dramatic problems for plant nutrition (Mendel and Bittner 2006). Scarceness is widely studied, and visual symptoms of Mo deficiency are different in young and old leaves (Hewitt and Bolle-Jones 1952; Kaiser et al. 2005). Conversely, while exceeding Mo is a quite studied topic in reports about animal nutrition and health (Barceloux 1999; Gardner et al. 2003; Majak et al. 2006), it is very poorly studied in plants. Ruiz et al. (2007) studied Mo excess (9.6 mg l^{-1}) in sunflower leaves by comparison with Se and Al excess for NO_3^- assimilation and found that Mo was the less toxic element, with no significant changes in biomass production, nitrate reductase (NR) activity, and NO_3^- foliar concentration. Moreover, Chatterjee and Nautiyal (2001) and Nautiyal and Chatterjee (2004) studied both deficiency and excess of Mo in *Triticum aestivum* ($2{\cdot}10^{-5}$ and 10 mg l^{-1}, respectively) and in *Cicer arietinum* ($1{\cdot}10^{-5}$ and 2 mg l^{-1}, respectively) and reported that Mo excess leads to more drastic and anticipated toxicity effects than those caused by deficiency. Brune et al. (1995) found that barley seedlings treated with Mo (38 mg l^{-1}) preferentially accumulated and compartmentalised the metal inside vacuoles of both epidermal and mesophyll cells of the leaves. At the best of our knowledge, literature on Mo excess concerns only terrestrial plants, while no report is available for the effect of Mo excess in aquatic plants.

We have undertaken a series of studies on the metal tolerance of *Trapa natans* (water chestnut), an annual floating-leaved aquatic angiosperm that populates natural wetlands. It is characterised by floating rosettes with leaves formed by bright green laminae and reddish, inflated petioles. The rosette is anchored to the mud through a submerged flexuous stem which presents abundant feathery leaves and numerous finely branched roots at the lower extremity. The plant can live in a wide range of nutrient levels, metals included, being able to bioaccumulate them both in fruits and leaves (Maljuga 1947; Rai and Sinha 2001; Kumar et al. 2002; Takamura et al. 2003; Baldisserotto et al. 2004, 2007). In previous studies, it was shown that the Mn resistance of this plant is linked to induction of chelating phenolics in the floating leaves (Baldisserotto et al. 2004, 2007). Leaves, in fact, are rich in phenolic compounds, anthocyanin included (Hoque and Arima 2002; Baldisserotto et al. 2004, 2007). Anthocyanin accumulation in leaves has been observed in crops exposed to high levels of Mo (Gupta 1997; Kaiser et al. 2005); Hale et al. (2001) suggested that anthocyanins may play a role in the mechanisms reducing the toxic effects of the metal. *Brassica* species treated with excess Mo (30 or 60 mg l^{-1}) sequestrated the metal inside vacuoles, probably as a Mo–anthocyanin complex (Hale et al. 2001). In the present work, the effect of excess Mo is investigated in *T. natans* leaves in order to evaluate if its phenolic compounds, anthocyanin included, can protect the plant against Mo. Four increasing doses of Mo have been employed. The photosynthetic apparatus is used as an indicator of cellular stress, and emphasis has been given to ultrastructural and physiologic aspects of the chloroplast.

Materials and methods

Plant material

T. natans L. (Mirtales, Lythraceae) plants grown in their natural environment in a canal near Ferrara (Northern Italy) were employed in this study. In toto plants were sampled at the end of June 2005. The sampling site was situated far from urban areas and was surrounded by cultivated fields. Mo concentration in water was about 0.02 mg l^{-1}, pointing to absence of Mo pollution (see "Measurements of Mo concentration" in this section).

The harvested plants were transferred to the Botanical Garden of Ferrara and set into ten teflon-covered ponds with a capacity of 180 l. The bottom of the ponds had previously been covered with mud, and the ponds were subsequently filled with freshwater. The ponds were located in the open sunlight (1,800 μmol $m^{-2}s^{-1}$ at 12:30 P.M.) without shading plants growing above, analogous to the natural environment.

Ten plants comparable in dimension (not shown) were acclimated in each pond for 1 day. Subsequently, on the basis of literature data on Mo-treated plants and considering our past results on Mn resistance of *Trapa* (Baldisserotto et al. 2004, 2007), $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (Merck, Darmstadt, Germany) was added to eight ponds until Mo (VI) reached the concentrations of 5 (0.48 mg l^{-1}), 50 (4.8 mg l^{-1}), 150 (14 mg l^{-1}), and 600 μM (58 mg l^{-1}); two ponds were maintained as controls. The experiment was performed in duplicate. Plants were analysed for 10 days after Mo treatment. During the 10-day treatment, the light intensity at 12:30 P.M. was about 1,800 μmol $m^{-2}s^{-1}$ and the air temperature 30±2°C. It has been ascertained that before treatments, Mo levels were negligible in all ponds (0.02± 0.01 mg l^{-1}; see "Measurements of Mo concentration" in this section).

Treated and control plants were observed up to the subsequent vegetative season, and germination ability of the seeds and viability of the shoots were evaluated. Chestnuts were, in fact, transferred into new untreated ponds.

Observations were performed on the young floating laminae of the second verticil of rosettes, unless it is differently specified. Controls were analysed in parallel. At the moment of Mo treatment, these laminae were the most external leaflets of the plant bud.

Dimension and CO_2 gas exchange measurements were performed for each dose of Mo treatment. The other analyses were performed for 50 to 600 μM Mo-treated samples, especially focussed on 50 and 150 μM, because 5 μM Mo treatment did not cause evident effects with respect to control.

Morphological examinations

Dimension measurements of the photosynthetic apparatus Laminae, five per pond, were sampled for determination of dimension after 3 and 10 days of cultivation in treated and control conditions. For in toto rosettes (five per pond), the averaged diameter and number of leaves per rosette were calculated.

Transmission electron microscopy After 3 and 10 days of cultivation, small pieces (2 mm^2) of floating laminae were cut, rinsed with Na-K-phosphate buffer 0.1 M (pH 7.2). Fixation, infiltration and embedding were performed as in Baldisserotto et al. (2007). Ultra-thin sections were observed with a Hitachi H800 electron microscope (Electron Microscopy Centre, Ferrara University, Italy).

Physiological analyses

CO_2 gas exchange measurements After 3 and 10 days of cultivation, net photosynthesis was measured. Analyses were performed at 12.30 P.M. with an LCA-4 (ADC Co. Ltd., Hoddesdon, UK) open-system infrared gas analyser, at ambient CO_2 concentration (370±10 μl l^{-1}) and light intensity (1,800 μmol $m^{-2}s^{-1}$) (Baldisserotto et al. 2007). The analyses were also performed on laminae belonging to the third verticil of rosettes.

Room temperature microspectrofluorimetry In accordance with Pancaldi et al. (2002), fluorescence emission spectra were recorded using a microspectrofluorimeter (RCS, Florence, Italy), associated with a Zeiss Axiophot photomicroscope. All spectra were recorded in vivo at room temperature (25°C), as described in Baldisserotto et al. (2007), on cell groups belonging to the first and second layer of the palisade tissue and to the spongy tissue. The light emission was collected by the objective lens and deviated to the detector system. Autolab software (RCS, Florence, Italy) was employed for instrument and data management (Pancaldi et al. 2002). For each cell type, at least five spectra were recorded.

Elaboration of spectra was performed with Microcal Origin 6.0 software (Microcal Software Inc.), as reported by Baldisserotto and co-workers (2007). For each deconvoluted fluorescence spectrum, the area subtended under each Gaussian component was used to evaluate the relative fluorescence intensity of the corresponding emitter (Pancaldi et al. 2002). The band attribution is reported in Table 1. Inner antenna system of PSII ($CP_{43–47}$) is usually the main emission source at room temperature, when proper energy transfer processes occur. Since individual emissions from CP_{43} (around 685 nm) and CP_{47} (around 695 nm) are not always resolved, in this paper they are jointly discussed as $CP_{43–47}$ (Baldisserotto et al. 2004, 2007). Due to the complex origin of fluorescence signals, absolute intensities were not used for quantitative evaluations but were interpreted as functions of the efficiency of energy transfer processes among Chl–protein complexes (Pancaldi et al. 2002). These values were used to evaluate the fluorescence emission ratios (RCII/$CP_{43–47}$ and LHCII/PSII, where PSII is the sum of reaction centre of PSII (RCII) and $CP_{43–47}$) indicative of the assembly state of the PSII Chl–protein complexes (Pancaldi et al. 2002). Events preventing the energy transfer to the RCII increase the relative emission from RCII and/or LHCII (Vassiliev et al. 1995; Pancaldi et al. 2002). RCII/$CP_{43–47}$ is an assembly index of the PSII core complex, while LHCII/PSII is an index of the assembly state of the peripheral antenna with the PSII core (Pancaldi et al. 2002; Baldisserotto et al. 2004, 2007; Ferroni et al. 2009). Even subtle changes can be highlighted by variations in these fluorescence emission ratios (Pancaldi et al. 2002; Ferroni et al. 2007a, b).

Biochemical analyses

Photosynthetic pigment analysis After 3 and 10 days of cultivation, leaves from three different control and treated

Table 1 Attribution of fluorescence emission bands

λ (nm)	Attribution	References
665–675	Uncoupled chlorophyll	Santabarbara et al. 2001
676–680	RCII	Ignatov and Litvin 1994, 1998
683–697	CP_{43-47}	Alfonso et al. 2004; Groot et al. 1999
699–715	LHCII	Vassiliev et al. 1995
720–750	LHCI and PSI	Krause and Weis 1991

plants were cut into small pieces for extraction of photosynthetic pigments as in Baldisserotto et al. (2007). Pigment concentrations were calculated as proposed by Lichtenthaler (1987).

Total phenolic compounds and anthocyanin assay For quantification of total phenolic compounds, portions of three control and treated laminae from different plants were extracted as in Baldisserotto et al. (2004). The Folin–Ciocalteu reaction for the detection of phenolics in extracts was employed (Velioglu et al. 1998). The reaction and the spectrophotometric quantification (Pharmacia Ultrospec Spectrophotometer) were performed as in Baldisserotto and co-workers (2004). Results were expressed as equivalent mg of coumaric acid (Sigma Chemicals Co., St. Louis, MO, USA).

For anthocyanin quantification, portions of three control and treated laminae from different plants were ground in acidic methanol (1% HCl *w*/*v*) with a tissue mixer (Sorvall Omni-Mixer) after 3 and 10 days of cultivation, and extractions were performed as in Baldisserotto and co-workers (2007). The absorbance of the extracts was measured at 530 nm (absorption peak of anthocyanin) and 657 nm (absorption peak of Chlorophyll (Chl) phaeo-derivatives in acidic methanol) employing the same spectrophotometer described above following the equations reported in Mancinelli et al. (1991). Concentration of anthocyanin was evaluated using a molar extinction coefficient of $\mathrm{Log}_{10}\ \varepsilon$=4.48 (Strack et al. 1989).

Measurements of Mo concentration After 3 and 10 days of cultivation, measurement of Mo concentration was performed in six control laminae (three per pond) and in six treated laminae with 50 and 150 μM Mo. All laminae were repeatedly washed in tap water. Mo concentration was also measured in the cultivation water. Laminae were dried for 24 h at 100°C and subsequently ground in a mortar before digestion. Water samples were analysed without any additional treatment.

All reagents were of analytical grade. Digestion procedure was performed as reported in Baldisserotto et al. (2007). Atomic emission was performed using a Perkin–Elmer Optima 3100 XL inductively coupled plasma optic spectrometer (ICP), equipped with axial torch, segmented array charge coupled device detector, Babington-type nebulizer with cyclonic spray chamber for sample introduction. A Perkin–Elmer AS 91 Tray F autosampler was also used. Replicates were never less than three. All samples were analysed relative to aqueous calibration standard.

Analysis of various forms of nitrogen After 10 days of cultivation, nitrogen analyses were performed for control and 150 μM Mo-treated laminae. Second verticil laminae of control and treated rosettes were ground with a tissue mixer (Sorvall Omni-Mixer) in 0.5 M K_2SO_4 (Merck). Nitrogen extraction was performed under continuous stirring at room temperature for 90 min. After centrifugation at 1,000×*g* for 2 min, extracts were mechanically filtered with 0.45-μm diameter filtres to harvest all the dissolved material. In parallel, the same analyses were triggered for laminae of plants cultivated for 10 days in 125 μM NH_4^+ (as $(NH_4)_2SO_4$) to verify if the results were due to Mo or to NH_4^+ ions added. In fact, 150 μM Mo treatment required the employment of $(NH_4)_6Mo_7O_{24}{\cdot}4\ H_2O$, i.e., 125 μM NH_4^+. Extracts were analysed colorimetrically using a continuous flow analyser (FlowSys, Systea, Roma, Italy) (Bragazza and Limpens 2004).

Statistical analysis

Statistical treatment of data was performed using Statistica (StatSoft). Multiple comparisons were performed with ANOVA followed by post hoc Tukey's HSD test (significance level, 5%). Asterisks are used to identify the levels of significance: *$P\leq0.05$; **$P\leq0.01$, ***$P\leq0.001$.

Results

Morphologic observations

Macroscopic morphology

Mo treatment induced different responses at the macroscopic level in both in toto rosettes and leaves. Control and 5 μM Mo-treated plants were characterised by rosettes similar in the macroscopic morphology and in the rhythmic production of new leaves during the whole vegetative season (Fig. 1a, b); they both developed normal flowers and fruits. Their seeds germinated in Spring 2006 and formed vital and well-conformed plants (Fig. 1c). Differently, already after 3 days of treatment with 50 μM Mo, rosettes showed signs of toxicity, since laminae lost the bright green colouration typical of controls and dark spots were visible in leaves of the second and third verticils (Fig. 1d). Moreover, treated rosettes showed two different kinds of leaf morphology after 10-day treatment: (1) young leaves belonging to the bud and to the first two verticils were similar to those of controls; (2) older leaves (starting from the third verticil) had yellowish areas to be linked to chlorosis, beside the dark spots that had previously appeared (Fig. 1e). After 30 days, rosettes restored an aspect similar to that of controls, in which all leaves were bright green (not shown). Treated plants developed flowers and fruits, but seeds produced rosettes that quickly became

very altered and almost died by 1.5 months (Fig. 1f). Treatment with 150 μM Mo caused stronger alterations to the laminae, and after only 3 days, large dark spots occupied almost the entire lamina surface, except for veins, and the laminae of the bud and the first verticil grew somehow orthotropically with respect to the water surface (Fig. 1g). Toxic signs became more and more evident after 10 days (Fig. 1h). However, young leaves of the bud and of the first and second verticils were similar to those of controls, while leaves from the third verticil were almost completely dark brown and underwent disintegration when they were touched (Fig. 1h). Moreover, leaves starting from the third verticil detached easily at the petiole basis because the insertion point on the rosette was extremely weak. Therefore, the rosettes had only few leaves after 40 days, and many plants died (70–80%; not shown). The survived plants produced flowers and fruits, but seeds originated only abortive seedlings with rosettes that could not float (Fig. 1i). Finally, leaves treated with the highest dose (600 μM) in only 2 days assumed an orthotropic position, and many of them were chlorotic, red-spotted or curled (Fig. 1j). After 3 days, leaves did not normally float, even if petioles were still enlarged (Fig. 1k). Moreover, after 7-day treatment, the entire upper surface of laminae assumed a red-brown colouration, while petioles easily detached from the stem (not shown). After 10 days of Mo-exposure, rosettes lost all leaves, were submerged and died in a few days (Fig. 1l).

Dimension measurements of photosynthetic apparatus

Rosettes and leaves treated for 3 days with all doses of Mo showed dimensional parameters similar to those of the corresponding controls (Table 2). A relevant, but not significant, decrease in the number of leaves per rosette (−36%) was observed after 10 days of treatment only for 150 μM Mo-treated plants (Table 2).

TEM observations

Control samples showed some scanty flaky inclusions inside vacuoles of cells of the upper epidermis and the first palisade tissue (Fig. 2a). These inclusions were not found in vacuoles of the second palisade tissue (Fig. 2b). Plastid ultrastructure was different in cells from each mesophyll layers (Fig. 2c–e). In fact, plastids of the first layer of the palisade were characterised by stroma thylakoids, small grana and, when present, small starch granules. Plastids of the second palisade layer contained numerous membranes and large starch granules (Fig. 2c, d). Spongy tissue contained amylo-chloroplasts (Fig. 2e). Plastoglobules were always scarce (Figs. 2c–e and 3). Laminae treated with 50 μM Mo contained dark vacuolar deposits inside the cells of the first palisade tissue and of the upper epidermis, more evident than in controls (Figs. 2a and 4). Dark concretions were more abundant after 10 days of treatment than after 3 days (Fig. 4). Dark precipitates were globular, different from those observed in controls (Fig. 2a and 4). Precipitates were never observed in cells of the second palisade tissue (Fig. 4b, d). These aspects were observed also in 150 μM Mo-treated samples (not shown). However, plastids showed different aspects in 50 and 150 μM Mo-treated laminae (Figs. 5 and 6). In 50 μM, plastids were characterised by a not altered morphology in cells of all mesophyll layers after 3 days of Mo exposure and mithocondria were numerous, pointing to active metabolism (Fig. 5a–c). Thylakoids and starch granules were abundant and rather well deposed (Fig. 5a–c). Only the number of plastoglobules per plastid increased in treated samples (Fig. 3). Conversely, 10-day treatment induced alteration of plastid ultrastructure; few thylakoids were present and plastogloubules increased in number with respect to controls and to 3-day-treated samples (Figs. 3 and 5d, e). Large starch granules were still present, even with an altered matrix, to be linked to unbalanced photosynthetic activity, especially inside cells of the second palisade layer (Fig. 5e). In 150 μM Mo-treated samples, plastids appeared altered after 3 days and, more evidently, after 10 days of treatment in cells of both the first and the second palisade layers (Fig. 6). In particular, after 3 days, plastids from these two-cell layers were characterised by well-deposited thylakoids (Fig. 6a–c). Stroma regions, devoid of membranes, were filled with plastoglobules, while starch granules were not present inside plastids belonging to cells of the first palisade layer (Fig. 6a, b). Conversely, plastids from the second layer of the palisade contained starch granules: some of them were elongated with a uniform texture, and others were similar to controls, with characteristic streaks (Fig. 6c). On the other hand, plastids from both layers of the palisade tissue indistinctly showed the same alterations after 10 days of Mo exposure (Fig. 6d, e). The organelles, in fact, contained few thylakoids conformed as in controls or, alternatively, swollen membranes and numerous, sometimes large, plastoglobules (Figs. 2c, d, 3 and 6d, e). Starch granules were absent or very small (Fig. 6d, e). Finally, samples from 600 μM Mo-treated laminae were observed only 3 days after treatment, as Mo exposure was very strong, and 10 days of cultivation in metal-polluted waters were sufficient to induce plant death. All samples appeared extremely damaged, with altered cell ultrastructure (Fig. 7a). Moreover, plastids from the first layer of palisade tissue lacked starch and contained many thylakoids that appeared strongly degraded or curled in an anomalous dark stroma (Fig. 7b). Plastids from the second palisade layer and from the spongy tissue showed minor

ultrastructural alterations, as membranes were abundant and starch occupied a large plastid area, similarly to controls (Figs. 2d, e and 7c, d).

Physiological parameters

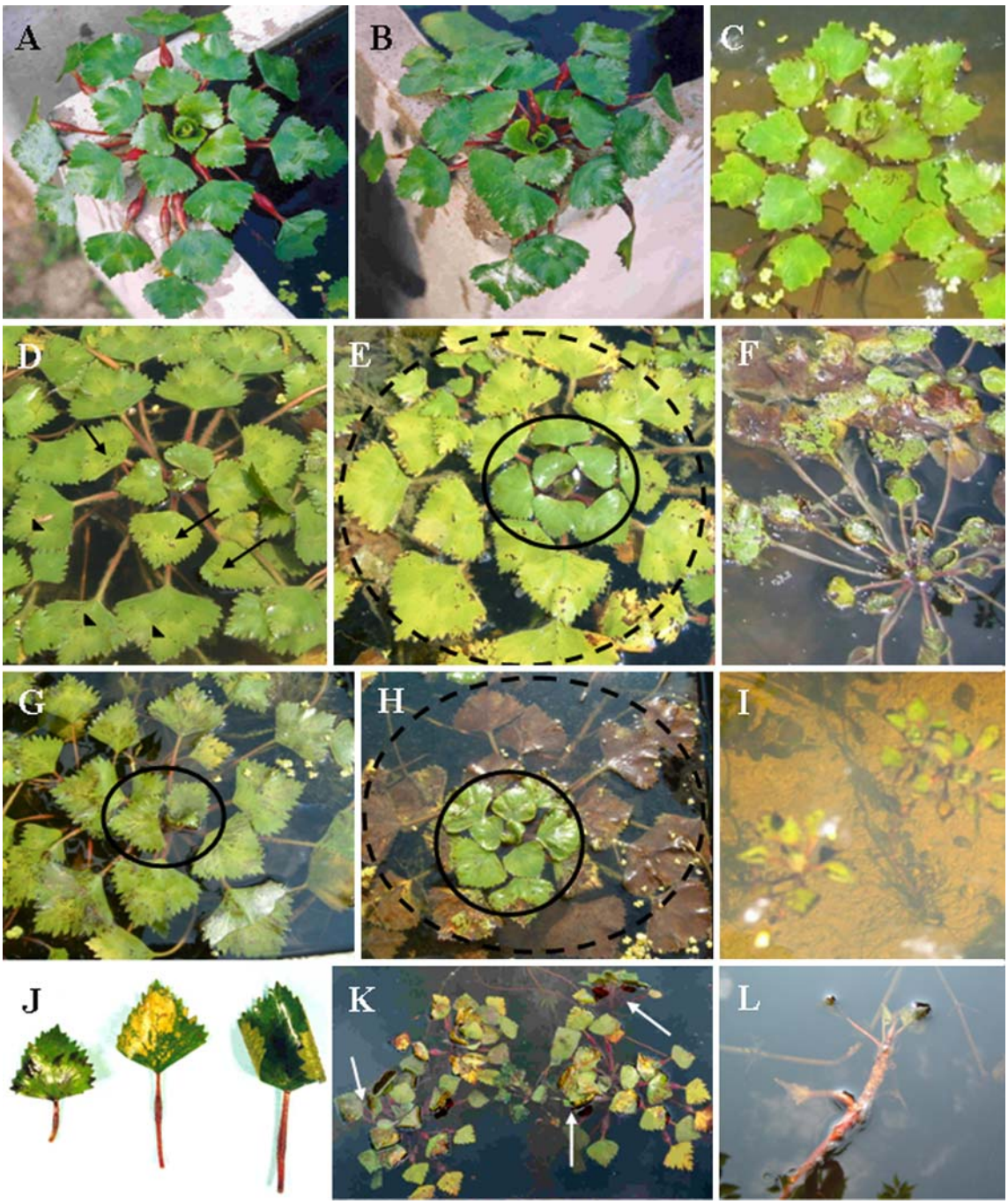
CO_2 gas exchange measurements

Photosynthesis was never significantly influenced by Mo exposure in 5 μM treated plants with respect to controls (Table 3). Conversely, after 3 and 10 days of 50 μM Mo treatment, both laminae of the second and third verticils showed a strong decrease (about 60% and 70%, respectively) in net photosynthesis if compared with controls (Table 3). This decrease became dramatic for plants after 150 μM Mo treatment. In fact, after 3 days, the laminae of both the second and third verticils were characterised by a CO_2 exchange to be linked to respiration. However, 10 days after Mo exposure, laminae of the second verticil, which at the third day of treatment belonged to the bud, showed a 47% decrease in net photosynthesis compared with controls. Conversely, laminae of the third verticil, which were derived from leaves of the second verticil after 3 days of Mo exposure, still had a negative net photosynthesis (Table 3). Finally, laminae from 600 μM treated plants showed a dramatic reduction 3 days after treatment.

Room temperature microspectrofluorimetry

No microspectrofluorimetric analyses were performed on 600 μM Mo-treated samples. The Gaussian deconvolution of spectra from control and 50 and 150 μM Mo-treated samples showed the presence of all typical Chl–protein components of PSII (Table 1) in plastids of each cell layer of the mesophyll. Fluorescence emission ratios are reported in Fig. 8. For 50 μM Mo-treated laminae, the RCII/$CP_{43–47}$ ratio showed an increasing trend for plastids of the first and second layer of the palisade tissue with respect to controls (0.56 and 0.37 for 3-day-treated, 0.86 and 0.57 for 10-day-treated samples, respectively, versus 0.45 and 0.23, respectively, for controls). Conversely, the LHCII/PSII ratio showed a different behaviour. In treated samples, plastids from the first palisade tissue showed a very high value of the ratio after 3 days (0.43; 0.13 for controls), but it underwent a reduction to values (0.19) similar to those of controls after 10 days (0.13). Concomitantly, plastids from the second palisade tissue did not show differences with respect to controls (0.18 and 0.21 for 3- and 10-day-treated samples, respectively, versus 0.24 for controls), while plastids from the spongy tissue increased their LHCII/PSII ratio values, especially after 10 days of treatment (0.39 for treated, 0.17 for controls). A different situation was observed for 150-μM-treated samples. A dramatic decrease in the RCII/$CP_{43–47}$ ratio was observed in the plastids of the first layer of palisade after 3 days of treatment (0.08; 0.45 for controls). This value increased after 10 days (0.23), even if it remained halved with respect to controls. This was not observed in plastids of the second layer of palisade, which showed an increase in the RCII/$CP_{43–47}$ ratio after 3 days of treatment (0.47; 0.23 for controls) and a value similar to that of controls after 10 days (0.29). Finally, minor differences were observed in the RCII/$CP_{43–47}$ ratio in plastids of the spongy tissue of treated samples with respect to controls (0.14 and 0.10 for 3- and 10-day-treated samples, versus 0.19 for controls). Differently, the LHCII/PSII ratio appeared not to be influenced by the metal after 3 days of Mo exposure in plastids of all cell mesophyll layers (0.16, 0.25, 0.21, respectively, for treated samples, versus 0.13, 0.24, 0.17, respectively, for controls), while it was homogeneously increased after 10 days (0.28, 0.38, 0.24, respectively, for treated samples, i.e., about two times in plastid of the first layer of palisade tissue and 1.5 times in plastids of the second palisade and the spongy tissue).

Fig. 1 Macroscopic morphology of *Trapa natans* rosettes or laminae ▶ of control (**a**), 5 (**b**, **c**), 50 (**d**–**f**), 150 (**g**–**i**) and 600 μM (**j**–**l**) Mo-treated samples. **a** A rosette of control samples after 10 days of experiment showing bright green, fan-shaped laminae with red, inflated petioles. **b** A rosette of 5 μM Mo-treated samples after 10 days of experiment presenting laminae and petioles similar in feature to those of corresponding controls (see **a**). **c** A rosette grown for 1 month in Spring 2006, germinated from seeds produced during 2005 vegetative season by 5 μM Mo-treated samples, showing green laminae. **d** A rosette of 50 μM Mo-treated samples after 3 days of treatment showing dark spots over laminae from the second (*arrow*) and third (*arrowheads*) verticils. **e** A rosette of 50 μM Mo-treated samples after 10 days of treatment. The rosette shows bright green young laminae belonging to leaves of the bud and the first and second verticils (*inner solid circle*), while altered older laminae starting from the third verticil (*outer dashed circle*) with *wide yellowish areas and dark spots*. **f** A rosette grown for 1 month in Spring 2006, germinated from seeds produced during 2005 vegetative season by 50 μM Mo-treated samples, showing very altered laminae. **g** A rosette of 150 μM Mo-treated samples after 3 days of treatment. *Large dark spots* are visible over all laminae of rosette and laminae belonging to the bud and to the first verticil (*circle*) take an orthotropic position with respect to the water surface. **h** A rosette of 150 μM Mo-treated samples after 10 days of treatment, with bright green laminae belonging to the bud and to the first and second verticils (*inner solid circle*) and very altered, brown laminae starting from the third verticil (*outer dashed circle*). **i** Three rosettes grown for 1 month in Spring 2006, germinated from seeds produced during 2005 vegetative season by 150 μM Mo-treated samples. They do not reach the water surface and always remain submerged. **j** Three leaves from 600 μM Mo-treated samples after 2 days of treatment: dark-red spots (*left*), chlorotic areas (*middle*) or curled lamina margins (*right*) are visible. **k** Rosettes of 600 μM Mo-treated samples after 3 days of treatment do not normally float, showing submerged areas (*arrows*). **l** Residual portion of a rosette from 600 μM Mo-treated samples after 10 days of treatment



Biochemical parameters

Photosynthetic pigment quantification

Data from pigment analyses of control and Mo-treated plants are reported in Table 4. After 3 days of treatment, laminae belonging to the second verticil of 50 μM Mo-treated rosettes showed a not significantly altered pigment content with respect to controls, although Chl*a* slightly decreased (about 17%). Differently, 10-day treatment did not alter carotenoids (Car) content but induced an overall decrease in Chl content, especially Chl*a* (20%). Conversely, 150 μM Mo caused a general decrease in all photosynthetic pigment content, especially after 10 days of treatment. In detail, no difference was observed in Chl*b* and Car content after 3 days of metal exposure. Finally, data of pigment analyses of 600 μM Mo refer only to plants treated for 3 days. All photosynthetic pigments strongly decreased. Trends of photosynthetic pigment concentrations led to interesting molar ratios for all doses and time of treatment. In fact,

Table 2 Dimension parameters of in toto rosettes and of floating laminae of *Trapa natans*

Time of examination and parameter	Mean value in[a]				
	Control	5 μM Mo	50 μM Mo	150 μM Mo	600 μM Mo
3 days					
In toto rosette					
Diameter (mm)	199±67	195±59	193±54	196±58	205±67
Leaves/rosette	15.0±2.2	15.3±2.7	14.1±2.1	13.0±2.5	11.8±3.3
Lamina					
Length (mm)	42±10	41±10	42±8	43±9	44±11
Width (mm)	48±15	47±13	46±11	45±13	48±9
Area (mm^2)	107±58	96±50	96±45	104±49	106±46
10 days					
In toto rosette					
Diameter (mm)	197±68	201±54	196±68	148±68	nd
Leaves/rosette	14.9±2.3	15.2±3.3	15.7±4.7	9.4±3.7	nd
Lamina					
Length (mm)	37±10	40±7	35±9	38±10	nd
Width (mm)	42±15	45±9	42±11	46±12	nd
Area (mm^2)	84±47	90±34	77±39	96±42	nd

nd not determined

[a] Values are means with standard deviations (n=10)

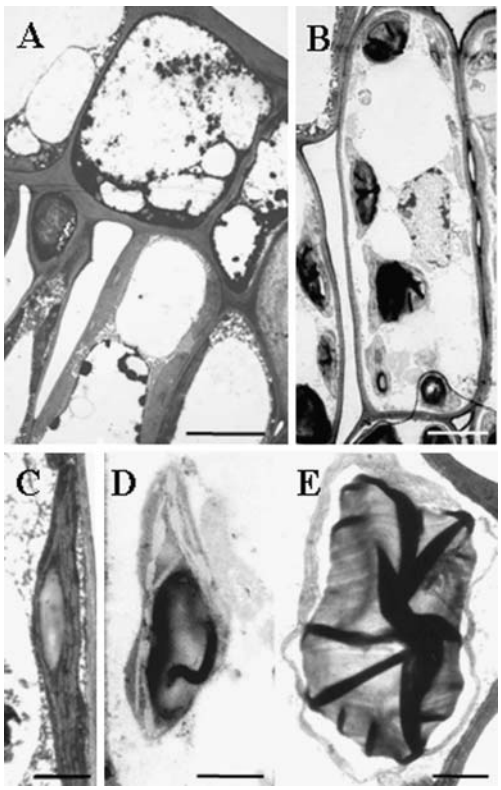


Fig. 2 Transmission electron micrographs of cells (**a**, **b**) and plastids (**c**–**e**) from control *Trapa natans* lamina samples. **a** Cell section from upper epidermis and the first palisade layer of the mesophyll showing some vacuolar flaky electrondense inclusions. **b** Section of cells belonging to the second layer of palisade tissue: no electrondense material is observed inside vacuoles. **c** Plastid of a cell belonging to the first layer of palisade tissue with thylakoids organised in small grana and starch. **d** Plastid of a cell belonging to the second layer of palisade tissue with stroma and grana thylakoids and starch. **e** Amylo-chloroplast of a cell belonging to the spongy tissue. *Bars* in **a** 5 μm; in **b** 3 μm; in **c**–**e** 1 μm

after 3 days of Mo treatment, the Chl*a*/*b* ratio exponentially decreased as a function of the dose employed (R^2=0.98) and appeared to be preferentially linked to the decrease in Chl*a*. A different decrease was observed after 10 days of treatment, since 50 μM Mo showed a reduction of about 10% versus only 4% of 150 μM Mo with respect to control. Also, the Chl(*a*+*b*)/Car molar ratio showed a decreasing trend, from 17% for 50 μM Mo-treated samples up to 28% for 600 μM Mo-treated samples after 3 days of treatment, and 23% for 150 μM Mo-treated samples after 10 days of metal exposure.

Phenolic compounds and anthocyanin assay

For total phenolics, there was no significant difference between extracts from control, 50 and 150 μM treated

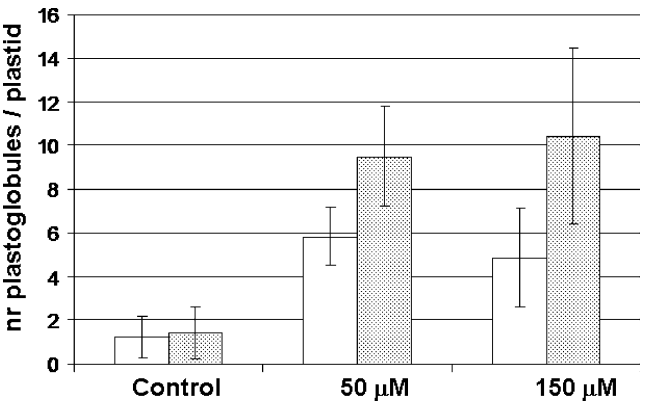


Fig. 3 Number of plastoglobules per plastid in control and 50 and 150 μM Mo-treated *Trapa natans* samples after three (*white area*) and ten (*dotted area*) days of experiment. Values are means ± SD (n=10)

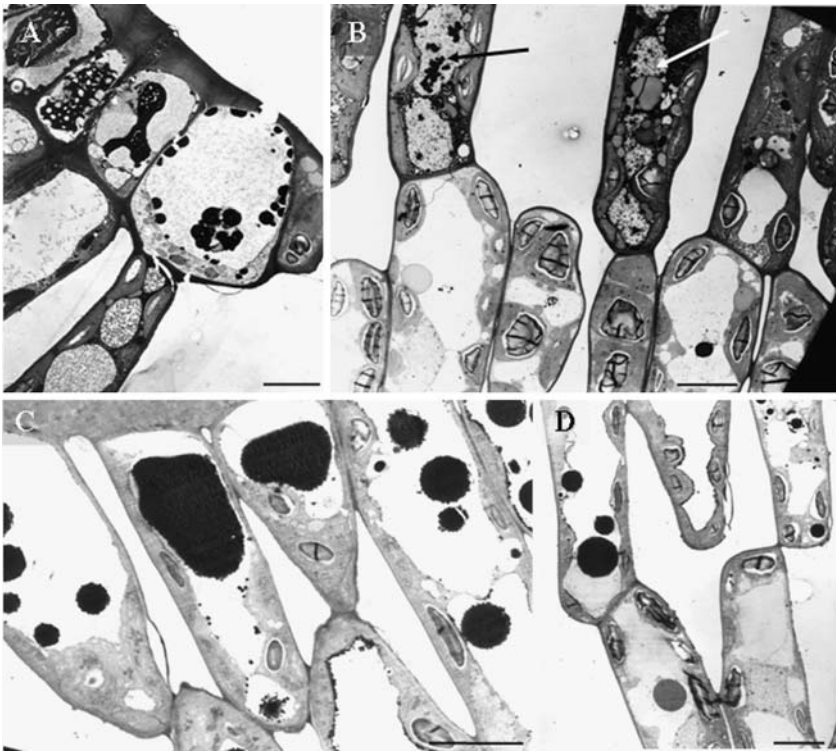


Fig. 4 Transmission electron micrographs of *Trapa natans* lamina cells belonging to 50 μM Mo-treated samples after three (**a**, **b**) and ten (**c**, **d**) days of experiment. **a** Section of cells of the upper epidermis showing dark globular material inside vacuoles. **b** Section of cells of the first and second layers of the palisade tissue: electrondense vacuolar inclusions are present in cells of the first layer the palisade tissue (*arrow*). **c** Section of cells of the first palisade layer of the mesophyll: abundant dark vacuolar precipitates have a globular feature. **d** Section of cells of the first and second layers of the palisade tissue: dark vacuolar inclusions are visible inside vacuoles of cells belonging to the first layer of the palisade. *Bars*, 5 μm

laminae both after 3 and 10 days of Mo exposure (Table 5). An increase was observed after 3 days of treatment only for 600 μM treated plants (Table 5). Differently, anthocyanin analysis showed a dose-dependent increasing trend in treated laminae, except for the 50 μM Mo-treated samples, which only after 3 days showed an unchanged content with respect to control (Table 5).

Mo quantification

Since 600 μM treated samples were dramatically affected by Mo, ICP spectroscopy was performed only for 50 and 150 μM treated samples. This analysis highlighted a very high accumulation of Mo in laminae of both treated samples after 3 and 10 days of cultivation (Table 6). In

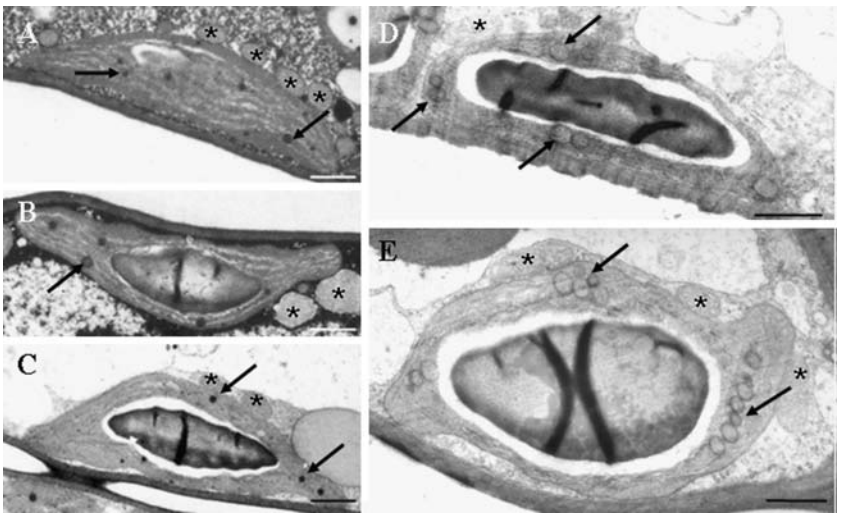


Fig. 5 Transmission electron micrographs of plastids belonging to lamina cells of *Trapa natans* treated with 50 μM Mo after three (**a**–**c**) and ten (**d**, **e**) days of experiment. **a**, **b** Plastids of cells belonging to the first layer of palisade tissue characterised by thylakoid membranes, starch grains and plastoglobules. Numerous mitochondria are visible near the plastids. **c** Plastid of a cell belonging to the second layer of palisade tissue with thylakoids, starch grains and plastoglobules. Two mitochondria are visible near the plastid. **d**, **e** Plastids of cells of the second layer of palisade tissue with few thylakoid membranes and numerous plastoglobules; large starch grains have slightly altered matrix. *Arrows* plastoglobules; *asterisks* mithocondria. *Bars* 1 μm

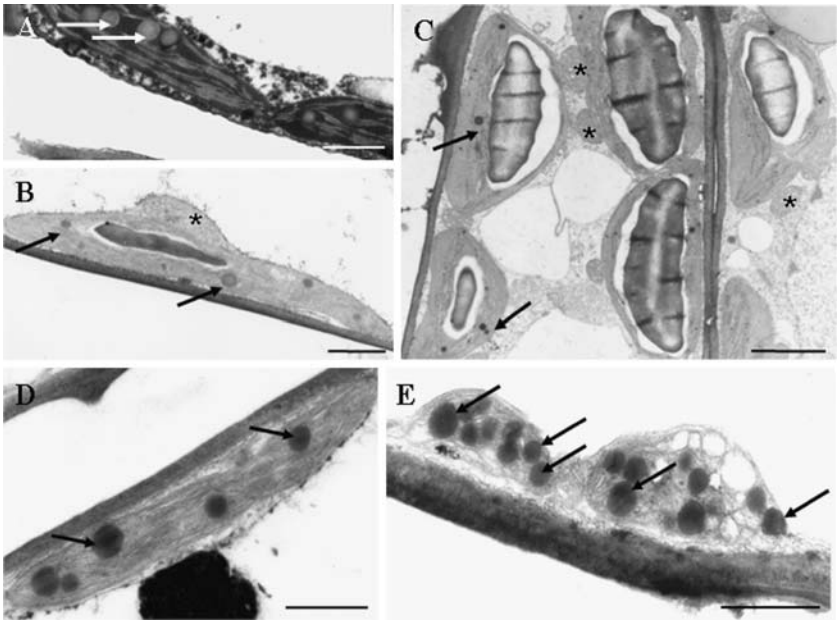


Fig. 6 Transmission electron micrographs of plastids belonging to lamina cells of *Trapa natans* treated with 150 μM Mo after three (**a**–**c**) and ten (**d**, **e**) days of experiment. **a** Plastid of a cell belonging to the first layer of palisade tissue characterised by thylakoid membranes and plastoglobules; no starch grain is visible. **b**, **c** Plastids of cells belonging to the second layer of palisade tissue with thylakoid membranes and plastoglobules; starch granules are elongated with uniform texture (**b**) or characterised by typical streaks (**c**). **d** Plastid of a cell belonging to the first layer of palisade tissue with few well-deposited thylakoid membranes and numerous plastoglobules. **e** Plastid of a cell belonging to the second layer of palisade tissue with few swollen thylakoid membranes and very numerous plastoglobules. *Arrows* plastoglobules; *asterisks* mithocondria. *Bars* in **a**, **b**, **d**, **e** 1 μm; in **c** 2 μm

detail, Mo concentration was increased of about 50 times in 50 μM and 90 times in 150 μM Mo-treated laminae if compared with controls after 3 days of treatment. Moreover, it was very interesting that extremely high content of Mo in 150 μM Mo-treated laminae after 10 days (about 4,800 times with respect to controls and 35 times with respect to 3-day-treated laminae), versus the rather high content in 50 μM treated samples (120 times with respect to controls and only 2.5 times with respect to 3-day-treated samples).

Quantification of various forms of nitrogen

Nitrogen quantification was performed only on 150 μM treated laminae after 10 day of Mo exposure, as they

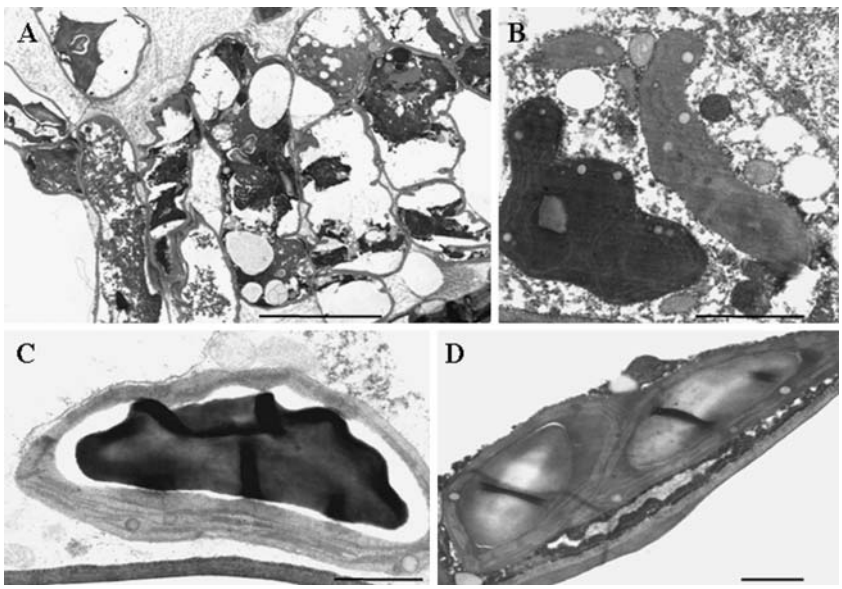


Fig. 7 Transmission electron micrographs of cells (**a**) and plastids (**b**–**d**) from lamina samples of *Trapa natans* treated with 600 μM Mo after 3 days of experiment. **a** Altered cells of lamina section with deformed organelles and dark cytoplasm. **b** Plastids of a cell of the first layer of the palisade tissue with anomalous elongated or curled thylakoid membranes and dark stroma. **c** Plastid of a cell belonging to the second layer of the palisade showing large starch granules and abundant well-deposed thylakoids. **d** Plastid of a cell of the spongy tissue characterised by numerous thylakoid membranes and quite large starch grains. *Bars* in **a** 10 μm; in **b** 2 μm; in **c**, **d** 1 μm

Table 3 Net photosynthesis rate in the floating laminae of *Trapa natans*

Sample	Mean value for sample at[a]			
	3 days		10 days	
	2nd verticil	3rd verticil	2nd verticil	3rd verticil
Control	17.54±1.36	17.99±2.10	17.25±1.94	18.10±1.92
5 μM Mo	17.92±0.84	17.16±1.77	17.62±1.87	17.89±2.01
50 μM Mo	6.88±0.82***	4.90±1.10***	7.20±1.07**	5.43±0.21***
150 μM Mo	−0.35±2.28***	−0.27±1.87***	9.12±0.80**	−0.52±3.01***
600 μM Mo	−0.22±1.91***[b]	−2.81±0.95***[c]	nd	nd

nd not determined

[a] Values are given as micromoles of CO_2 per square metre per second and are means with standard deviations (n=3)

[b] Red spotted laminae

[c] Chlorotic laminae

**P<0.01, treated samples significantly different from control samples; ***P<0.001, treated samples significantly different from control samples

seemed to show the most interesting responses to the metal. Data are reported in Fig. 9. The analysis showed a strong increase in NH_4^+ ion content (about three times) and even stronger in NO_2^- content (more than eight times) in Mo-treated samples with respect to controls. Concomitantly, a decrease in the NO_3^- concentration occurred in treated laminae with respect to controls. A different behaviour was observed for laminae after NH_4^+ treatment. No difference was observed for NO_2^- content between control and NH_4^+-exposed samples, while the NH_4^+ concentration enhanced and NO_3^- decreased in NH_4^+-treated samples with respect to controls. On the whole, all the nitrogen forms were less abundant after NH_4^+ treatment than after Mo exposure.

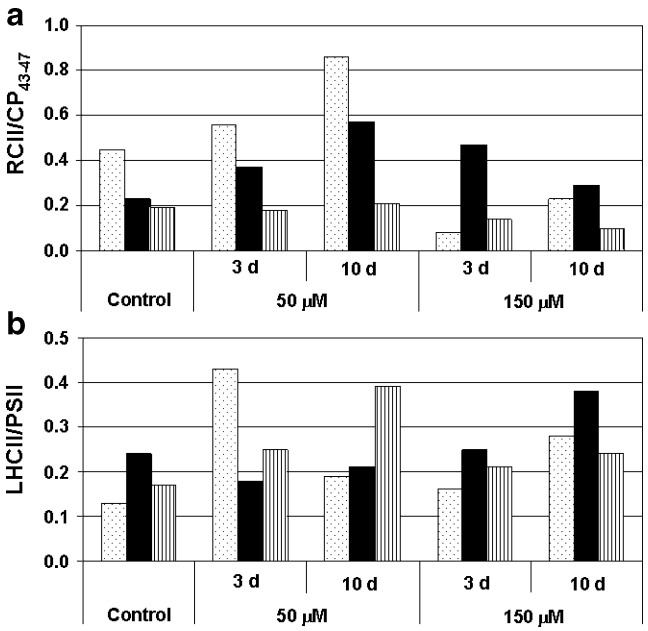


Fig. 8 Histogram with fluorescence emission ratios, RCII/CP_{43-47} (**a**) and LHCII/PSII (**b**), calculated from plastids of *Trapa natans* lamina samples treated with 50 and 150 μM Mo after 3 and 10 days of experiment. Control in parallel. The fluorescence ratios were calculated from microspectrofluorimetric analyses separately performed on plastids belonging to the first (*dotted area*) and second (*black area*) layers of the palisade tissue and to the spongy tissue (*vertical lines*). Both ratios have been calculated from areas under Gaussian curves corresponding to RCII, CP_{43-47} and LHCII. PSII is the sum of RCII and CP_{43-47}

Discussion

In previous studies, it was reported that *T. natans* can tolerate Mn concentrations as high as 1 mM (Baldisserotto et al. 2007), which suggested that the plant could be a resistant species also against other metals. Here, we show that this hypothesis holds only partly good, even as shown in this study case on the response to a metal—Mo—which is expected to share similar protecting mechanisms with Mn.

T. natans exposed to four increasing doses of Mo showed dose-dependent signs of sufferance. Only treatment with 5 μM Mo, a concentration about 16 times higher than that present in control waters, did not induce any disease in *T. natans*, as the plant normally vegetated and produced seeds that developed similarly to those of control samples. All the other doses induced strong responses. In particular, the effect of the highest treatment (600 μM) was dramatic and leaves rapidly showed strong macro- and microscopic alterations, which became more and more relevant in few days, finally inducing the plant death. In 3-day-treated samples, the content in photosynthetic pigments significantly decreased, and the cell ultrastructure in laminae was very altered. The photosynthetic activity was rapidly and irreversibly lost. Differently, treatment with 50 and 150 μM

Table 4 Photosynthetic pigments concentration and photosynthetic pigment molar ratios in the floating laminae of *Trapa natans*

Parameter and time of analysis	Mean value in[a]			
	Control	50 μM Mo	150 μM Mo	600 μM Mo
Chl*a*[b]				
3 days	3.88±0.40	3.21±0.48	3.02±0.15*	1.48±0.18***
10 days	2.92±0.18	2.32±0.30*	1.49±0.08***	nd
Chl*b*[b]				
3 days	0.57±0.08	0.50±0.09	0.54±0.03	0.31±0.05**
10 days	0.46±0.05	0.40±0.07	0.24±0.03**	nd
Car[b]				
3 days	1.60±0.17	1.60±0.29	1.49±0.08	0.90±0.17**
10 days	1.36±0.12	1.30±0.16	1.02±0.07*	nd
Chl(*a*+*b*)				
3 days	4.45±0.40	3.71±0.47	3.56±0.14*	1.79±0.25***
10 days	3.38±0.18	2.72±0.29**	1.73±0.12***	nd
Chl*a*/*b*				
3 days	6.80±0.98	6.53±1.31	5.63±0.39	4.88±0.70
10 days	6.34±0.78	5.87±1.25	6.12±0.74	nd
Chl(*a*+*b*)/Car				
3 days	2.78±0.40	2.32±0.51	2.39±0.17	1.99±0.29
10 days	2.48±0.27	2.08±0.34	1.70±0.16*	nd

nd not determined

[a] Values are means with standard deviations (n=3)

[b] Values are given as nanomoles per milligramme (dry weight)

*P<0.05, treated samples significantly different from control samples; **P<0.01, treated samples significantly different from control samples; ***P<0.001, treated samples significantly different from control samples

Mo did not cause a complete decay of photosynthesis; in fact, plants underwent a partial recovery and produced fruits at the end of vegetative season. However, these fruits produced seeds that did not successfully develop, pointing to alterations due to treatments. Consequently, major attention has been focussed on the responses of the laminae belonging to plants treated with these doses of Mo. Both concentrations induced different macroscopic responses in young or old laminae belonging, respectively, to the second and third verticils of rosettes, especially with 150 μM Mo. In fact, only the younger laminae could recover. Similarly, CO_2 gas exchange analysis pointed to major alterations of photosynthetic performance in laminae of the third verticil after 3 and 10 days of Mo exposure. At the same time, photosynthesis appeared less affected in laminae of the second verticil of rosettes. This is in line with the reports indicating the different response to metals of old and young leaves (Hewitt and Bolle-Jones 1952; Luna et al. 1994; Vinit-Dunand et al. 2002). Thereby, further analyses were performed on laminae of leaves of the second verticil for a characterisation of the response to Mo excess of the photosynthetic apparatus in *T. natans*.

Mo quantification showed a very high storage of the metal in laminae from 50 and 150 μM Mo-treated samples,

Table 5 Total phenolic and anthocyanins concentrations in the floating laminae of *Trapa natans*

Sample	Mean value of[a]			
	Total phenolics[b]		Anthocyanins[c]	
	3 days	10 days	3 days	10 days
Control	29.6±1.4	29.4±1.3	0.43±0.03	0.46±0.04
50 μM Mo	30.4±1.4	30.9±1.5	0.49±0.05	0.61±0.08*
150 μM Mo	31.7±1.2	31.0±1.1	0.78±0.08**	0.64±0.10*
600 μM Mo	36.1±1.4**	nd	0.78±0.09**	nd

nd not determined

[a] Values are mean with standard deviation (n=3)

[b] Values are given as equivalent coumaric acid milligrammes per gramme (dry weight)

[c] Values are given as nanomoles per milligramme (dry weight)

*P<0.05, treated samples significantly different from control samples; **P<0.01, treated samples significantly different from control samples

Table 6 Concentration of molybdenum in the floating laminae of *Trapa natans*

Sample	Mean value at[a]	
	3 days	10 days
Control	6.62±1.65	4.28±1.32
50 μM Mo	330±25***	509±37***
150 μM Mo	594±46***	20,400±1,976***

[a] Values are given as micrograms per gramme (dry weight) and are means with standard deviation (n=3)

***P<0.001, treated samples significantly different from control samples

up to about 120 and 4,800 times, respectively. Since Baldisserotto and co-workers (2004, 2007) found that the treatment with exceeding Mn let *T. natans* overproduce phenolic compounds for metal detoxification inside vacuoles of mesophyll cells, a possible involvement of phenolics has been investigated also in response to Mo. However, the quantification of total phenolic compound did not highlight differences among control and 50 and 150 μM Mo-treated samples. It appears that in *T. natans*, the phenolic compounds could not be directly or completely involved in Mo detoxification. However, it could be proposed a role of anthocyanins in reducing toxic effects triggered by Mo, as suggested by Hale and co-workers (2001) for *Brassica* species. In fact, anthocyanins were more concentrated in treated samples than in controls. As anthocyanins are usually abundant in vacuoles of epidermal cells (Kubo et al. 1995; Markham et al. 2000), the ultrastructural observations of dark precipitates in vacuoles of cells of the upper epidermis of treated samples could be probably due to anthocyanin-chelated Mo. Dark precipitates in the second palisade tissue could be linked to the presence of phenolic compounds and probably of other Mo-chelating molecules, such as organic acids (Steinke et al. 2008). However, it seems that the chelation inside vacuoles is not a very effective mechanism for Mo detoxification in *T. natans*.

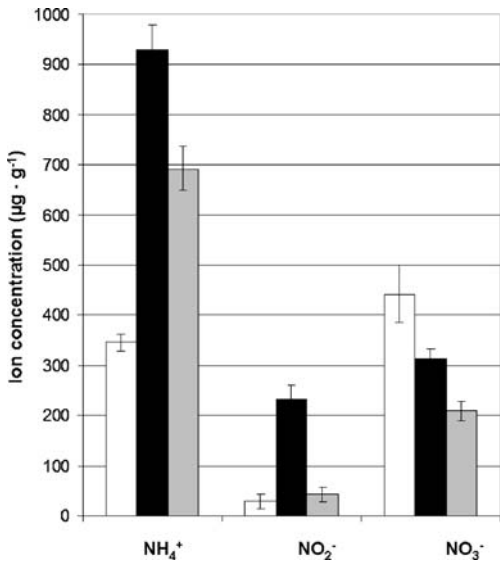


Fig. 9 Histogram with results from nitrogen form analysis performed on lamina samples of *Trapa natans* plants treated with 150 μM Mo for 10 days (*black area*). Control (*white area*) and 125 μM NH_4^+-treated samples (*light grey area*) in parallel. Values are means ± SD (n=3)

The response of plastids in lamina cells belonging to the second verticil in plants treated with 50 and 150 μM Mo was also analysed. In 3-day 50 μM Mo-treated samples, despite the normal assembly of thylakoid membranes and accumulation of starch in cells of all mesophyll layers, disassembly and partial loss of PSII were observed for plastids of both palisade layers. In fact, the high RCII/$CP_{43–47}$ ratio in treated samples, coupled with the decrease in the Chl (a+b)/Car ratio, indicated a failure in energy transfer and thus disassembly of PSII core (Baldisserotto et al. 2007; Ferroni et al. 2007a, b). The partial loss of pigment–protein complexes was suggested by an incipient decrease in the photosynthetic pigment content and was accompanied by a strong increase in the LHCII/PSII ratio in the plastids of the first palisade layer (Ferroni et al. 2007a). These considerations could be also confirmed by the increase in the number of plastoglobules per plastid, as they are involved in thylakoid disassembly and play active roles in several metabolic and stress-response pathways (Austin et al. 2006; Brehelin et al. 2007). Imbalance in pigment content and energy transfer among pigment–protein complexes of PSII can justify the low photosynthetic rate. Differently, plastids from the spongy tissue showed RCII/$CP_{43–47}$ ratio similar to that of controls, pointing to good energy transfer of complexes, but this cellular layer provides only a minor contribution to the photosynthetic yield of the lamina (Aalto and Juurola 2002). Subsequently, at 10 days, the high RCII/$CP_{43–47}$ ratio still indicated disassembly of PSII in the palisade layers. However, LHCII/PSII ratios with values similar to those of controls pointed to a sort of rearrangement for recovery of energy transfer among complexes. These aspects were coupled with alterations in ultrastructure, but only with slight differences in photosynthetic pigment composition. Different from that observed after 3 days of Mo treatment, the most affected plastids belonged to cells of the spongy tissue.

Treatment with 150 μM Mo induced, instead, very strong effects on the photosynthetic apparatus. The fall in RCII/$CP_{43–47}$ ratio in plastids of the first palisade layer after 3 days of metal exposure pointed to the loss of RCII and was linked to the very damaged ultrastructure of plastids and the loss of Chl*a* (Baldisserotto et al. 2004; Ferroni et al. 2007a). In parallel, plastids from the second palisade layer showed doubled RCII/$CP_{43–47}$ ratio with respect to control and suggested disassembly of PSII complexes in visibly

altered chloroplasts. The decrease in the photosynthetic pigment molar ratios also supported this observation (Baldisserotto et al. 2007; Ferroni et al. 2007a, b). Conversely, the LHCII/PSII ratio was not strongly affected. Interestingly, the microspectrofluorimetric responses obtained after 10 days of experiment could suggest an attempt to recover effective energy transfer among complexes, as also the Chl*a*/*b* ratio suggested. This molar ratio, in fact, is a good index for antennae size (Anderson 1986; Yamazaki et al. 2006). Moreover, photosynthesis rate showed an increased value with respect to that recorded after 3 days of treatment, although remaining at a low level.

All aspects considered in this work point to a limited capability of *T. natans* in the resistance to Mo exposure by comparison with Mn (Baldisserotto et al. 2004, 2007). Only the lowest dose employed allowed the plant to survive, while the others caused time-limited life or rapid death. Damages could be induced not only directly by the metal ion but also by alterations of metabolic pathways linked to Mo presence. In fact, Mo is an important co-factor of the NR (Mendel and Hansch 2002; Mendel and Bittner 2006), and nitrogen metabolism was strongly altered, as shown by anomalies in NO_3^-, NO_2^- and NH_4^+ concentrations. Different from that observed by Srivastava (1980) and by Sairam and co-workers (1995), who found that an excessive Mo application can depress the NR activity and thus reduce plant growth, preliminary results obtained in *T. natans* suggested an overproduction of NO_2^- by NR. Nitrite is normally transferred from the cytoplasm, where it is toxic if it exceeds a specific threshold level, into plastids, where nitrite reductase (NiR) operates the conversion of nitrite to ammonia (Cabello et al. 1998; Ferrario-Mery et al. 2008). The high ammonia concentration measured in the lamina of *T. natans* exposed to Mo suggested a high NiR activity, even if it was not sufficient to reduce all nitrite ions produced by NR. This could lead to accumulation of the ion, very probably inside plastids, where it becomes toxic at high concentrations as well (Cabello et al. 1998; Ferrario-Mery et al. 2008).

This work shows that, although *T. natans* shows marked bioaccumulation abilities for Mo as for Mn, the concentration that the plant can tolerate without evident impairment of its fitness is very different for the two metals. In contrast to Mn, detoxification of Mo by chelation inside vacuoles is suggested to be an insufficient mechanism to reduce Mo toxicity at doses higher than 5 μM.

Acknowledgements The authors thank E. Ferroni for help in language editing. Funding for study was provided by the Fondo per gli Investimenti della Ricerca di Base (FIRB2001) of the Italian MIUR (Ministero per l'Istruzione, l'Università e la Ricerca) and by the University of Ferrara.

Conflict of interest The authors declare that they have no conflict of interest.

References

Aalto T, Juurola E (2002) A three-dimensional model of CO_2 transport in airspaces and mesophyll cells of a silver birch leaf. Plant Cell Environ 25:1399–1409

Alfonso M, Montoya G, Cases R, Rodriguez R, Picorel R (2004) Core antenna complex, CP43 and CP47, of higher plant photosystem II. Spectral properties, pigment stoichiometry, and amino acid composition. Biochem 33:10494–10500

Anderson JM (1986) Photoregulation of the composition, function, and structure of thylakoid membranes. Ann Rev Plant Physiol Plant Mol Biol 37:93–136

Austin JR, Frost E, Vidi PA, Kessler F, Staehelin LA (2006) Plastoglobules are lipoprotein subcompartments of the chloroplast that are permanently coupled to thylakoid membranes and contain biosynthetic enzymes. Plant Cell 18:1693–1703

Baldisserotto C, Ferroni L, Medici V, Pagnoni A, Pellizzari M, Fasulo MP, Fagioli F, Bonora A, Pancaldi S (2004) Specific intra-tissue responses to manganese in the floating lamina of *Trapa natans* L. Plant Biol 6:578–589

Baldisserotto C, Ferroni L, Anfuso E, Pagnoni A, Fasulo MP, Pancaldi S (2007) Responses of *Trapa natans* L. floating laminae to high concentrations of manganese. Protoplasma 231:65–82

Barceloux D (1999) Molybdenum. J Toxicol Clin Toxicol 37:231–237

Bragazza L, Limpens J (2004) Dissolved organic nitrogen dominates in European bogs under increasing atmospheric N deposition. Global Biogeochem Cy 18:GB4018

Brehelin C, Kessler F, van Wijk KJ (2007) Plastoglobuies: versatile lipoprotein particles in plastids. Trends Plant Sci 12:260–266

Brune A, Urbach W, Dietz K-J (1995) Differential toxicity of heavy metals is partly related to a loss of preferential extraplasmic compartmentation: a comparison of Cd-, Mo-, Ni- and Zn-stress. New Phytol 129:403–409

Cabello P, de la Haba P, Gonzalez-Fontes A, Maldonado JM (1998) Induction of nitrate reductase, nitrite reductase, and glutamine synthetase isoforms in sunflower cotyledons as affected by nitrate, light, and plastid integrity. Protoplasma 201:1–7

Chatterjee C, Nautiyal N (2001) Molybdenum stress affects viability and vigor of wheat seeds. J Plant Nutr 24:1377–1386

Ferrario-Mery S, Meyer C, Hodges M (2008) Chloroplast nitrite uptake is enhanced in *Arabidopsis* PII mutant's. Febs Lett 582:1061–1066

Ferroni L, Baldisserotto C, Pantaleoni L, Billi P, Fasulo MP, Pancaldi S (2007a) High salinity alters chloroplast morpho-physiology in a freshwater *Kirchneriella* species (Selenastraceae) from Ethiopian Lake Awasa. Am J Bot 94:1972–1983

Ferroni L, Baldisserotto C, Zennaro V, Soldani C, Fasulo MP, Pancaldi S (2007b) Acclimation to darkness in the marine chlorophyte *Koliella antarctica* cultured under low salinity: hypotheses on its origin in the polar environment. Eur J Phycol 42:91–104

Ferroni L, Baldisserotto C, Pantaleoni L, Fasulo MP, Fagioli P, Pancaldi S (2009) Degreening of the unicellular alga *Euglena gracilis*: thylakoid composition, room temperature fluorescence spectra and chloroplast morphology. Plant Biol 11:631–641

Gardner W, Broersma K, Popp J, Mir Z, Mir O, Buckley W (2003) Copper and health status of cattle grazing high-molybdenum forage from a reclaimed mine tailing site. Can J Animal Sci 83:479–485

Graham RD, Stangoulis JCR (2003) Trace element uptake and distribution in plants. J Nutr 133:1502S–1505S

Groot M-L, Frese RN, de Weerd FL, Bromek K, Petterson Å, Peterman AJG, van Stokkum IHM, van Grondelle R, Dekker JP (1999) Spectroscopic properties of the CP43 core antenna protein of photosystem II. Biophys J 77:3328–3340

Gupta U (1997) Symptoms of molybdenum deficiency and toxicity in crops. In: UC G (ed) Molybdenum in agriculture. Cambridge University Press, Cambridge

Hale KL, McGrath SP, Lombi E, Stack SM, Terry N, Pickering IJ, George GN, Pilon-Smits EAH (2001) Molybdenum sequestration in *Brassica* species. A role for anthocyanins? Plant Physiol 126:1391–1402

Hall JL (2002) Cellular mechanisms for heavy metal detoxification and tolerance. J Exp Bot 53:1–11

Heumann HG (2002) Ultrastructural localization of zinc in zinc-tolerant *Armeria maritima* ssp halleri by autometallography. J Plant Physiol 159:191–203

Hewitt E, Bolle-Jones E (1952) Molybdenumas a plant nutrient. II. The effects of molybdenum deficiency on some horticultural and agricultural crop plants in sand culture. J Hort Sci 27:257–265

Hoque A, Arima S (2002) Overcoming phenolic accumulation during callus induction and in vitro organogenesis in water chestnut (*Trapa japonica* Flerov). In Vitro Cell Dev Plant 38:342–346

Ignatov NV, Litvin FF (1994) Photoinduced formation of pheophytin/chlorophyll-containing complexes during the greening of plant-leaves. Photosynth Res 42:27–35

Ignatov NV, Litvin FF (1998) A comparative study of the terminal stages of chlorophyll biosynthesis before and after water (D_2O) introduction into greening plant leaves. Photosynth Res 56:83–93

Kaiser BN, Gridley KL, Brady JN, Phillips T, Tyerman SD (2005) The role of molybdenum in agricultural plant production. Ann Bot 96:745–754

Krause GH, Weis DN (1991) Chlorophyll fluorescence and photosynthesis: the basics. Ann Rev Plant Physiol Plant Mol Biol 42:313–349

Kubo H, Nozue M, Kawasaki K, Yasuda Y (1995) Intravacuolar spherical bodies in *Polygonum cuspidatum*. Plant Cell Physiol 36:1453–1458

Kumar M, Chikara S, Chand MK, Bhatnagar AK (2002) Accumulation of lead, cadmium, zinc, and copper in the edible aquatic plants *Trapa bispinosa* Roxb. and *Nelumbo nucifera* Gaertn. B. Environ Contam Tox 69:649–654

Kuper J, Palmer T, Mendel RR, Schwarz G (2000) Mutations in the molybdenum cofactor biosynthetic protein Cnx1G from *Arabidopsis thaliana* define functions for molybdopterin binding, molybdenum insertion, and molybdenum cofactor stabilization. P Natl Acad Sci Am 97:6475–6480

Lichtenthaler HK (1987) Chlorophylls and carotenoids - pigments of photosynthetic biomembranes. Meth Enzymol 148:350–382

Luna C, Gonzalez C, Trippi V (1994) Oxidative damage caused by an excess of copper in oat leaves. Plant Cell Physiol 35:11–15

Majak W, Steinke D, Lysyk T, Ogilvie K, McGillivray J (2006) Efficacy of copper supplementation in the prevention of molybdenosis in cattle. Rangeland Ecol Manag 59:285–292

Maljuga DP (1947) Chemical composition of soils and plants as indicators of prospecting for metals. Izv Akad Nauk SSSR, Ser Geogr Geofiz 11:135–138

Mancinelli AL, Rossi F, Moroni DA (1991) Cryptochrome, phytochrome, and anthocyanin production. Plant Physiol 96:1079–1085

Markham KR, Gould KS, Winefield CS, Mitchell KA, Bloor SR, Boase MR (2000) Anthocyanic vacuolar inclusions - their nature and significance in flower colouration. Phytochem 55:327–336

Mendel RR, Bittner F (2006) Cell biology of molybdenum. Biochim Biophys Acta 1763:621–635

Mendel RR, Hansch R (2002) Molybdoenzymes and molybdenum cofactor in plants. J Exp Bot 53:1689–1698

Nautiyal N, Chatterjee C (2004) Molybdenum stress-induced changes in growth and yield of chickpea. J Plant Nutr 27:173–181

Pancaldi S, Baldisserotto C, Ferroni L, Bonora A, Fasulo MP (2002) Room temperature microspectrofluorimetry as a useful tool for studying the assembly of the PSII chlorophyll-protein complexes in single living cells of etiolated *Euglena gracilis* Klebs during the greening process. J Exp Bot 53:1753–1763

Rai UN, Sinha S (2001) Distributions of metals in aquatic edible plants: *Trapa natans* (Roxb.) Makino and *Ipomoea aquatica* Forsk. Environ Monit Assess 70:214–252

Rauser WE (1995) Phytochelatins and related peptides - structure, biosynthesis, and function. Plant Physiol 109:1141–1149

Ruiz JM, Rivero RM, Romero L (2007) Comparative effect of Al, Se, and Mo toxicity on NO3- assimilation in sunflower (*Helianthus annuus* L.) plants. J Environ Manag 83:207–212

Sairam RK, Till AR, Blair GJ (1995) Effect of sulfur and molybdenum levels on growth, nitrate-assimilation, and nutrient content of *Phalaris*. J Plant Nutr 18:2093–2103

Santabarbara S, Neverov KV, Garlaschi FF, Zucchelli G, Jennings RC (2001) Involvement of uncoupled antenna chlorophylls in fotoinhibition in thylakoids. Febs Lett 491:479–486

Srivastava HS (1980) Regulation of nitrate reductase-activity in higher-plants. Phytochem 19:725–733

Steinke D, Majak W, Sorensen T, Parvez M (2008) Chelation of molybdenum in *Medicago sativa* (Alfalfa) grown on reclaimed mine tailings. J Agr Food Chem 56:5437–5442

Strack D, Busch E, Klein E (1989) Anthocyanin patterns in european orchids and their taxonomic and phylogenetic relevance. Phytochem 28:2127–2139

Takamura N, Kadono Y, Fukushima M, Nakagawa M, Kim BHO (2003) Effects of aquatic macrophytes on water quality and phytoplankton communities in shallow lakes. Ecol Res 18:381–395

Vassiliev IR, Kolber Z, Wyman KD, Mauzerall D, Shukla VK, Falkowski PG (1995) Effects of iron limitation on photosystem-ii composition and light utilization in *Dunaliella tertiolecta*. Plant Physiol 109:963–972

Velioglu YS, Mazza G, Gao L, Oomah BD (1998) Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. J Agr Food Chem 46:4113–4117

Vinit-Dunand F, Epron D, Alaoui-Sosse B, Badot BM (2002) Effects of copper on growth and on photosynthesis of mature and expanding leaves in cucumber plants. Plant Sci 163:53–58

Yamazaki JY, Kozu A, Fukunaga Y (2006) Characterization of chlorophyll-protein complexes isolated from two marine green algae, *Bryopsis maxima* and *Ulva pertusa*, growing in the intertidal zone. Photosynth Res 89:19–25