TÜBİTAK

Turkish Journal of Botany

http://journals.tubitak.gov.tr/botany/

Research Article

Turk J Bot
(2017) 41: 464-475

doi:10.3906/bot-1608-33

Bioaccumulation and oxidative stress impact of Pb, Ni, Cu, and Cr heavy metals in two bryophyte species, *Pleurochaete squarrosa* and *Timmiella barbuloides*

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Received: 23.08.2016 • **Accepted/Published Online:** 03.04.2017 • **Final Version:** 28.09.2017

Abstract: This study was conducted to evaluate the bioaccumulation and oxidative stress impact of lead (Pb), nickel (Ni), copper (Cu), and chromium (Cr) in bryophyte species *Pleurochaete squarrosa* and *Timmiella barbuloides* exposed to Pb, Ni, Cu, and Cr at 1 mM concentration for 48 h. Bioaccumulation ratios in the non-Fenton metals Ni and Pb were found to be higher than in Fenton metals Cu and Cr for both moss species. Except for Cu, high levels of heavy metal accumulation caused a slight increase in dry weights of both species. Total chlorophyll and carotenoid contents of both species showed variations under Ni, Pb, and Cr stress; however, a Cu-induced decrease in the chl *a/b* ratio revealed prominent toxicity of the metal in both species. Copper and chromium provoked hydrogen peroxide (H_2O_2) increase in both species, but it was more prominent in *P. squarrosa*. Ni treatment also induced H_2O_2 accumulation in the species, but membrane damage was only observed in Cu-exposed samples of both species. Ni-, Pb-, Cu-, and Cr-induced oxidative stress was alleviated by different metal- and species-specific antioxidant components in both species. These findings indicate that both species are sensitive to Cu, and induced antioxidant components are not enough to overcome the phytotoxic effect of the metal. Accumulated Ni-, Pb-, and Cr-driven toxicity seems to be tolerated by reducing heavy metal access to the cytoplasm of the cells, as well as by stimulating different components of antioxidant defense in the cells of both species; however, this was more prominent in *T. barbuloides* than *P. squarrosa*.

Key words: *Pleurochaete squarrosa*, *Timmiella barbuloides*, lead, nickel, copper, chromium, antioxidant, oxidative stress, bioaccumulation

1. Introduction

Bryophytes provide important experimental tools and models to explain complex biological processes in plants. They are also passive accumulators of heavy metals and some radioactive isotopes. Morphological and genomic variations caused by high accumulation of heavy metals make them useful for modeling (Bassi et al., 1995) and biosensor studies (Uyar et al., 2009). Contrary to higher plants, bryophytes have a relatively high surface/volume ratio and a differentiated epidermal layer without cuticle; thus, they absorb heavy metals with their whole surface in higher amounts (Sun et al., 2009).

Nickel (Ni), lead (Pb), copper (Cu), and chromium (Cr) are heavy metals and their increasing concentrations in the atmosphere, water, or soil cause toxic effects on metabolism; thus, they inhibit growth and development of plants. Heavy metal-triggered generation of reactive oxygen species (ROS) such as superoxide radicals ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) in cells has been reported both in mosses and other plants (Dazy et al., 2009; Sytar et al., 2013). Oxidative stress caused by heavy metals induces damage to lipids, proteins, and nucleic acids of cells. Plants evolved and developed antioxidant defense mechanisms to protect cells against oxidative stress. Enzymatic components of the antioxidant defense system include superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), and the ascorbate-glutathione pathway enzymes ascorbate peroxidase (APX) and glutathione reductase (GR). In addition, an increasing number of nonenzymatic antioxidant compounds are being defined, such as ascorbate, glutathione, proline, carotenoids, and phenol compounds, with roles in scavenging ROS (Mittler, 2002).

The heavy metal accumulation capacity of mosses has made them important indicator organisms and thus numerous reports have been presented on the biomonitoring of heavy metal pollutants using mosses (Sun et al., 2009; Basile et al., 2013). However, studies on oxidative stress level and antioxidant response mechanisms of mosses in relation to heavy metal bioaccumulation are very limited. There are only a few research studies published on the antioxidant defense responses of different bryophyte species (Dazy et al., 2009; Sun et al., 2011),

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providing limited information about the species and metal-specific models of defense in this lower plant group.

In this study, we aimed to evaluate the differences in bioaccumulation of Pb, Ni, Cu, and Cr and their oxidative stress impacts in the common bryophyte species *Pleurochaete squarrosa* and *Timmiella barbuloides*, as well as the antioxidant defense response patterns of these species.

2. Materials and methods

2.1. Moss material and heavy metal treatment

Pleurochaete squarrosa (Brid.) Lindb. and *Timmiella barbuloides* (Brid.) Moenk. are widespread moss species in Anatolia. Samples were collected from the Kepez region in Aydın, Turkey. The area was not affected by heavy metal pollution. Collected moss samples were transferred to the laboratory using polyethylene bags. Samples were cleaned under a stereomicroscope and washed with tap water to remove epiphytes, dust, and soil particles. Samples were then rinsed with distilled water in order to eliminate ions carried by the mosses or the tap water. After removal of excess water, moss samples were used in the experiment.

The top parts of fresh mosses were placed into bottle beakers containing 250 mL of solutions of 1 mM nickel chloride (Ni), lead acetate (Pb), copper sulfate (Cu), or potassium dichromate (Cr). Plants without heavy metal treatment served as controls. The specimens were cultured in a growth chamber at 24 ± 1 °C and 70% relative humidity with a 16-h light/8-h dark (40 μmol m^{-2} s^{-1}) photoperiod for 48 h. The experiment was run in triplicate per metal and species.

2.2. Accumulation of heavy metals

Oven-dried moss samples were wet-digested in a mixture of HNO_3 and $HClO_4$ (4:1, v/v), and the concentrations of Ni, Pb, Cu, and Cr were analyzed by Varian Atomic Absorption Spectrophotometer (model AA 220/FS) (Kacar and İnal, 2008).

2.3. Dry weight and pigment content

Fresh weights of harvested mosses were measured. Dry weights of samples were recorded after drying them out at 70 °C for 48 h. Moss pigments were extracted as described by Welburn (1994). Fresh moss samples (0.15 g) were immersed in dimethyl sulfoxide (DMSO) in the presence of polyvinylpolypyrrolidone (PVPP) for 60 min at 65 °C in the dark. After cooling to room temperature, extracts were diluted with DMSO (1:1 v/v) and filtrated. The absorption spectra of the pigments were measured using a spectrophotometer. Pigment concentration was calculated using Welburn's equations (Welburn, 1994).

2.4. H_2O_2 content

The H_2O_2 content of mosses was measured according to the procedure described by Patterson et al. (1984). H_2O_2 was extracted from samples (0.5 g) with 5% trichloroacetic acid (TCA) and activated charcoal. After centrifugation (30 min, 15,000 × *g*) the supernatant was filtered and neutralized with an ammonia solution (NH_4OH) (pH 8.4). A colorimetric reagent (equal mixture of 4-(2-pyridylazo) resorcinol (0.6 mM) and potassium-titanium oxalate (0.6 mM)) was added to the sample and to catalase-containing blank tubes, and both were incubated for 60 min at 45 °C. The absorbance of the sample was measured at 508 nm against the blank at room temperature. H_2O_2 content was calculated by using a standard curve prepared with known concentrations of H_2O_2.

2.5. Lipid peroxidation

Lipid peroxidation was measured by the content of total thiobarbituric acid-reactive substances (TBARS), as described by Cakmak and Horst (1991). Moss samples (0.15 g) were homogenized in 1% (w/v) TCA. The homogenate was centrifuged at 10,000 × *g* for 20 min, and then 1.5 mL of 20% (w/v) TCA containing 0.5% (w/v) thiobarbituric acid (TBA) solution was added to a 0.5-mL aliquot of supernatant. The mixture was heated in a boiling water bath for 30 min and then cooled on ice. The absorbance of the supernatant was measured at 532 nm. The malondialdehyde extinction coefficient (0.156 μM^{-1} cm^{-1}) was used for calculations.

2.6. Nonenzymatic antioxidants

2.6.1. Proline content

Endogenous free proline content was estimated according to the modified acid ninhydrin method of Bates et al. (1973). Moss samples were homogenized with 3% (w/v) sulfosalicylic acid and a 2-mL aliquot was mixed with 2 mL of acetic acid and 2 mL of ninhydrin reagent. The reaction mixture was incubated in a boiling water bath for 1 h and then treated with toluene. The absorbance of the collected toluene phase was read at 518 nm using toluene as a blank.

2.6.2. Ascorbate content

Ascorbate content was measured by the method of Kampfenkel et al. (1995). Moss samples (0.1 g) were homogenized with 6% TCA and then centrifuged at 15,000 × *g* for 5 min at 4 °C. The supernatant (0.2 mL) was mixed with 0.2 mL of 10 mM DTT (1,4-dithiothreitol) and 0.6 mL of 0.2 M potassium phosphate buffer (pH 7.4). The mixture was incubated at 42 °C for 15 min. The reaction was stopped by adding 0.5% (w/v) *N*-ethylmaleimide. The reaction mixture containing 0.2 mL of extract, 0.2 M potassium phosphate buffer (pH 7.4), 10% TCA, 0.2 mL of H_2O, 42% H_3PO_4, 4% dipyridyl, and 3% $FeCl_3$ was incubated at 42 °C for 15 min. The absorbance value of the final colored solution was recorded at 525 nm. Total ascorbate content was calculated from a standard curve plotted with its known concentration.

2.6.3. Glutathione content

Total glutathione content was determined by the method of Griffith (1980). Samples were ground in a mortar with 5% sulfosalicylic acid in 0.1 M potassium phosphate buffer (pH 7.6) containing 5 mM ethylenediaminetetraacetic acid (EDTA). The extract was centrifuged at 14,000 × *g* for 30 min. Supernatant aliquots were mixed with a reaction mixture containing 3 mM NADPH, 6 mM 5,5'-dithiobis-2-nitrobenzoic acid, and 0.5 M potassium phosphate buffer (pH 7.6). After stirring and equilibration at 30 °C for 5 min, 4 EU of glutathione reductase was added and the absorbance was measured at 412 nm. Oxidized glutathione (GSSG) content was measured by treating the supernatant with 5 mL of 2-vinylpyridine to mask reduced glutathione (GSH). The sample was then partitioned with diethyl ether and the water phase containing GSSG was used in the same reaction mixture described above. Tubes were stirred and equilibrated at 30 °C for 5 min. After adding 4 EU of glutathione reductase the absorbance was monitored at 412 nm. GSH content was calculated as the difference between total glutathione and GSSG. GSH was estimated based on a glutathione standard graph.

2.7. Antioxidant enzyme activities

2.7.1. Enzyme extraction

Fresh moss samples were homogenized in an ice-cooled mortar with polyvinylpyrrolidone (2%, w/v) and 100 mM potassium phosphate buffer (pH 7.8) containing 2 mM EDTA, 10% (w/v) glycerol, and 1 mM phenylmethanesulfonyl fluoride. The homogenate was centrifuged at 12,000 × *g* for 30 min at 4 °C; the supernatant was used for protein assay and enzyme determination. The total protein content in enzyme extracts was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

2.7.2. Superoxide dismutase (EC 1.15.1.1)

SOD activity was assayed by the method of Beauchamp and Fridovich (1971). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 33 µM NBT, 10 mM L-methionine, 0.66 mM EDTA, and 0.0033 mM riboflavin. Absorbance was recorded at 560 nm and the nonirradiated reaction mixture served as a control. One unit of enzyme defined the inhibition of 50% of the reaction.

2.7.3. Catalase (EC 1.11.1.6)

The activity of CAT was assayed by monitoring the consumption of H_2O_2 at 240 nm according to the method of Bergmeyer (1970). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 30% (w/v) H_2O_2. Decrease of mixture absorbance was recorded for 3 min.

2.7.4. Peroxidase (EC 1.11.1.7)

POX activity was assayed by measuring the oxidation of 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Herzog and Fahimi, 1973). Three milliliters of assay mixture contained DAB solution including 0.4 mM DAB, 50% (w/v) gelatin and 150 mM sodium phosphate-citrate buffer (pH 4.4), 50 µL of enzyme extract, and 3 mM H_2O_2. The increase in absorbance was recorded at 465 nm for 3 min.

2.7.5. Ascorbate peroxidase (EC 1.11.1.11)

APX activity was measured spectrophotometrically by following the oxidation of ascorbate (Gonzalez et al., 1998). The assay mixture (3 mL) consisted of 50 mM potassium phosphate buffer containing 1 mM EDTA (pH 7.8), 0.03% H_2O_2, 30 mM ascorbate, and 0.1 mL of enzyme extract. Ascorbate oxidation was determined by absorbance decrease at 290 nm.

2.7.6. Glutathione reductase (EC 1.6.4.2)

GR activity was assayed by monitoring the oxidation of NADPH according to the method described by Carlberg and Mannervik (1985). The reaction mixture (3 mL) contained 60 mM potassium phosphate buffer (pH 7.4), 1 mM oxidized glutathione (GSSG), 0.1 mM NADPH, and 0.2 mL of enzyme extract. The oxidation of NADPH was measured by absorbance decrease at 340 nm.

2.8. Antiradical activity

Antiradical activity, i.e. free radical scavenging capacity, was determined by 2,2'-diphenyl-1-picrylhydrazyl radical ($DPPH^{\cdot}$) test (Brand-Williams et al., 1995) with some modifications according to a previous study (Yildiz-Aktas et al., 2009). A volume of 0.05 mL of ethanolic (80% v/v) extract and 1.95 mL of DPPH solution in methanol (6×10^{-5} M) were mixed, and the decrease in absorbance was followed at 515 nm by a spectrophotometer. Trolox, a water-soluble analog of vitamin E, was used as a reference standard.

2.9. Statistical analysis

The results were compared by one-way ANOVA using SPSS 16. The least significant difference test was used for multiple comparisons of data at the $P < 0.01$ and $P < 0.05$ levels.

3. Results

3.1. Bioaccumulation of heavy metals

Samples of *P. squarrosa* and *T. barbuloides* receiving short-term heavy metal treatment showed higher total concentrations of Ni, Pb, Cu, and Cr compared to control samples (Table). The magnitude of Ni and Pb metal bioaccumulation was found to be higher than Cu and Cr bioaccumulation in both moss species. The highest Ni accumulation was measured in the *T. barbuloides* samples; it reached 3253.33 ppm in the specimens (263 times increase). In *P. squarrosa* samples the Ni level increased by 3900% (39 times) of its control level, which is about half of the metal accumulation in the *T. barbuloides* samples.

Table. Bioaccumulation of Ni, Pb, Cu, and Cr metals in moss species *P. squarrosa* and *T. barbuloides* after 48 h of exposure. Values represent mean ± SE (n = 3).

Treatments	Concentration of metals in *Pleurochaete squarrosa* (ppm)		Concentration of metals in *Timmiella barbuloides* (ppm)	
	Control	Metal	Control	Metal
Ni	38.2 ± 7.4	1558.86 ± 78.2**	12.3 ± 1.4	3253.33 ± 67.43**
Pb	70.4 ± 10.99	3312.01 ± 63.5**	60.2 ± 6.46	3193.33 ± 56.9**
Cu	21.6 ± 3.3	795.2 ± 25.26**	8.4 ± 1.04	398.8 ± 25.73**
Cr	22.4 ± 5.13	558.4 ± 13.2**	8.2 ± 1.46	286.4 ± 16.7**

**: Significant difference at $P < 0.01$.

The two moss species accumulated almost the same level of Pb; the increase in Pb level was calculated at about 46 and 52 times higher in *P. squarrosa* and *T. barbuloides*, respectively, compared to those of the controls. Copper and chromium accumulated to a lesser degree than Ni and Pb. Cu and Cr accumulation ratios were different between the species; *T. barbuloides* had higher concentrations of these heavy metals than *P. squarrosa* (Table).

3.2. Dry weight

Dry weight is a commonly used parameter to reveal the toxic effect of heavy metals on plant growth, being related to synthesis processes of the cells. Dry weights were found to be similar in the control samples of both species. Ni, Pb, Cu, and Cr treatment did not cause any species-specific difference in dry weight between the two moss species (Figure 1). Dry weights of moss species increased significantly ($P < 0.01$) with Ni, Pb, and Cr treatment by 27%, 21%, and 29% for *P. squarrosa* and by 34%, 48%, and 29% for *T. barbuloides*, respectively. However, Cu treatment caused a significant decrease by 25% for *P. squarrosa* and by 22% for *T. barbuloides* compared with the control samples.

3.3. Pigment content

The effects of heavy metal treatments on total chlorophyll (Chl) content, chlorophyll *a*/*b* (Chl *a*/*b*) ratio, and carotenoids (Car) content of *P. squarrosa* and *T. barbuloides* are shown in Figure 2. The basic level of photosynthetic pigments was similar in control samples of both species. In *T. barbuloides*, total Chl content remained unchanged relative to the control in Pb, Cu, and Cr treatments, but Ni treatment caused a significant increase. In contrast to *T. barbuloides*, total Chl content of *P. squarrosa* was decreased significantly by Ni, Pb, and Cu treatment; however, Cr-treated samples displayed Chl levels similar to those of the control. Cu and Cr treatment caused a significant decrease in the Chl *a*/*b* ratio by 75% and 28%, respectively,

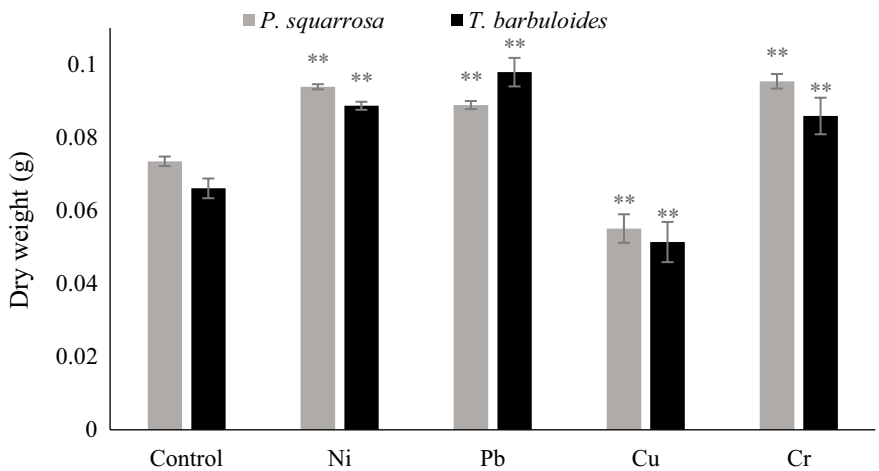

Figure 1. Dry biomass of moss species *P. squarrosa* and *T. barbuloides* exposed to Ni, Pb, Cu, and Cr metals for 48 h. Values represent mean ± SE (n = 3). **: Significant difference at $P < 0.01$.

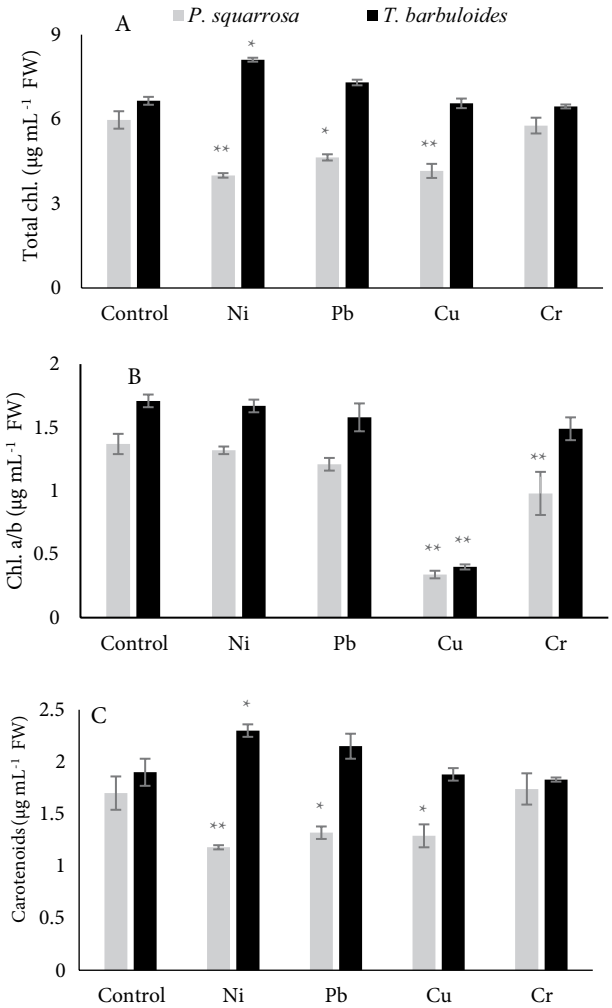

Figure 2. Total chlorophyll (Chl) content (A), chlorophyll *a*/*b* (Chl *a*/*b*) ratio (B), and carotenoid (Car) contents (C) of moss species *P. squarrosa* and *T. barbuloides* exposed to Ni, Pb, Cu, and Cr metals for 48 h. Values represent mean ± SE (n = 3). ** Significant difference at $P < 0.01$; *: at $P < 0.05$.

relative to the control in *P. squarrosa*, while only the Cu treatment decreased the ratio (76%) in *T. barbuloides*. Ni, Pb, and Cu treatment decreased the carotenoids content of *P. squarrosa* by 30%, 22%, and 24%, respectively, but only the Ni treatment affected the Car content of *T. barbuloides* samples, causing a 21% increase relative to the control.

3.4. H_2O_2 content

Heavy metal-induced generation of ROS has been reported in various plant species (Sytar et al., 2013). Hydrogen peroxide content is the most common indicator of oxidative stress, being the most stable form of ROS. The extent of oxidative stress in both moss species was revealed by measuring H_2O_2 accumulation that was induced by Cu and Cr treatments in both moss species, but by Ni only in *P. squarrosa* (Figure 3A). In *P. squarrosa*, Ni, Cu, and Cr significantly enhanced H_2O_2 by about 3, 5, and 4 times, respectively, compared with the controls. H_2O_2 accumulation in *T. barbuloides* increased by about 43% and 35%, respectively, in response to Cu and Cr exposure, relative to the control.

3.5. Lipid peroxidation level

ROS-induced membrane hazard level was determined by measuring MDA content in moss species, as shown in Figure 3B. According to the data obtained, lipid peroxidation levels of both moss species remained similar to those of controls with Ni, Pb, and Cr treatment. However, Cu induced membrane hazard with 155% and 146% enhancements of MDA levels in *P. squarrosa* and *T. barbuloides*, respectively.

3.6. Nonenzymatic antioxidant molecules

3.6.1. Proline content

The amino acid proline is commonly known as an osmolyte, but recently its antiradical and antioxidant features were presented in several reports (reviewed by Ashraf and Foolad, 2007). Based on these reports, the endogenous proline contribution to heavy metal response was measured in moss species. Proline content showed both species- and metal-specific variation (Figure 4A). The basal level of proline in *P. squarrosa* was twice higher than that in *T. barbuloides*. The proline content remained at control levels with Ni and Pb exposure, whereas Cu and Cr exposure caused a significant decrease by 82% and 36%, respectively, relative to the control in *P. squarrosa*. In contrast to *P. squarrosa*, *T. barbuloides* positively responded to Ni and Pb treatment, showing 81% and 57% enhancement in proline level, respectively. Exposure to Cu and Cr did not affect the proline level in *T. barbuloides*.

3.6.2. Ascorbate content

Ascorbate is a powerful antioxidant that quenches free radicals. Because of its higher affinity to even low concentrations of H_2O_2, it has an important role in detoxification mechanisms, particularly in chloroplasts (Mittler, 2002). The constitutive level of ascorbate was found to be similar in *P. squarrosa and T. barbuloides*. Ni and Pb exposure decreased the level of this antioxidant in both moss species (Figure 4B). However, Cu and Cr treatment induced a dramatic increase in ascorbate content that was enhanced by 397% and 360% in *P. squarrosa* and by 348% and 179% in *T. barbuloides*, respectively, compared to the controls. The enhancement rate was more prominent in *P. squarrosa* than in *T. barbuloides* with Cr exposure.

3.6.3. Glutathione content

The ratio of GSH to GSSG is an important parameter for determining the cellular redox status of cells. Glutathione contribution to the antioxidant metabolism of moss species under heavy metal treatment is represented by the GSH/GSSG ratio in Figure 4C. The ratio was higher

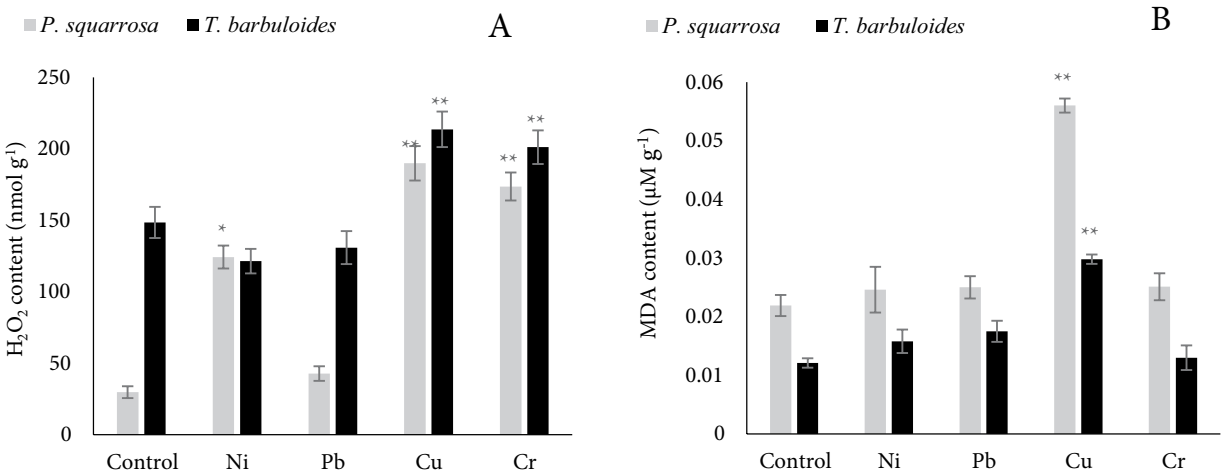

Figure 3. Hydrogen peroxide (H_2O_2) (A) and malondialdehyde (MDA) (B) content of moss species *P. squarrosa* and *T. barbuloides* exposed to Ni, Pb, Cu, and Cr metals for 48 h. Values represent mean ± SE (n = 3). **: Significant difference at $P < 0.01$; *: at $P < 0.05$.

in *P. squarrosa* than in *T. barbuloides* control samples. However, metal treatment caused a significant decrease in the GSH/GSSG ratio in *P. squarrosa* and *T. barbuloides*, with an exception in Cu-treated *T. barbuloides* samples that showed a 59% increase in the ratio.

3.7. Antioxidant enzyme activities

3.7.1. Superoxide dismutase activity (EC 1.15.1.1)

SOD is a key enzyme of antioxidant metabolism to scavenge superoxide radicals generated by environmental stresses and heavy metal stress (Mittler, 2002). SOD enzyme activity was maintained at control levels under Ni and Pb exposure in both species and under Cu treatment in *T. barbuloides* (Figure 5A). However, in *P. squarrosa*, Cu and Cr treatment induced an increase in SOD activity by 268% and 194%, respectively, relative to the control. In contrast to *P. squarrosa*, Cr treatment inhibited SOD activity by 52% compared to the control in *T. barbuloides*.

3.7.2. Catalase activity (EC 1.11.1.6)

CAT, together with POX and APX, converts H_2O_2 to water and oxygen. CAT activity in response to Ni, Pb, and Cr treatment did not change in either moss species (Figure 5B). However, Cu treatment caused a significant ($P < 0.01$) decrease in CAT activity that was more prominent in *P. squarrosa* (87%) than in *T. barbuloides* (79%) compared to the control.

3.7.3. Peroxidase activity (EC 1.11.1.7)

POX is another H_2O_2 scavenging enzyme. Its activity varied depending on the species and the metals applied (Figure 5C). POX activity increased slightly in response to all heavy metals in *P. squarrosa* compared to controls. *T. barbuloides* did not express any response to Pb, Ni, and Cu treatment, whereas its POX activity decreased (26%) in Cr-treated samples in comparison to the control.

3.7.4. Ascorbate peroxidase activity (EC 1.11.1.11)

APX is a H_2O_2-detoxifying enzyme of the ascorbate-glutathione cycle. In the present study, heavy metal treatment did not cause any change in the APX activity of *T. barbuloides*. The same trend was observed in *P. squarrosa*, except for Pb exposure (Figure 5D). APX activity significantly increased in Pb-treated *P. squarrosa* samples by 79% compared to the control.

3.7.5. Glutathione reductase activity (EC 1.6.4.2)

In this study, GR activity remained unchanged by all the heavy metal treatments in *P. squarrosa*. Similar behavior was observed in Cr-treated *T. barbuloides* samples; however, Ni, Pb, and Cu treatment enhanced the GR activity. The extent of enhancement was 67%, 61%, and 107%, respectively, relative to the control (Figure 5E).

3.8. Total antiradical activity

Trolox equivalent antiradical activity represents the antioxidant-antiradical capacity of cells against oxidative stress caused by various conditions. In this study, *P. squarrosa* antiradical activity was substantially decreased by Ni, Pb, Cu, and Cr exposure by 16%, 21%, 49%, and 43%, respectively, compared to controls. Similarly to *P. squarrosa*, the antiradical activity of *T. barbuloides* was significantly decreased by Cu and Cr treatment, but the extent of decline measured was half of that obtained from *P. squarrosa* (Figure 5F). In contrast to Cu- and Cr-treated samples, Ni and Pb treatment caused an increase in the antiradical activity of *T. barbuloides* by 61% and 41%, respectively, compared to controls.

4. Discussion

Tolerance to high heavy metal concentrations in the environment is provided by exclusion or metabolic

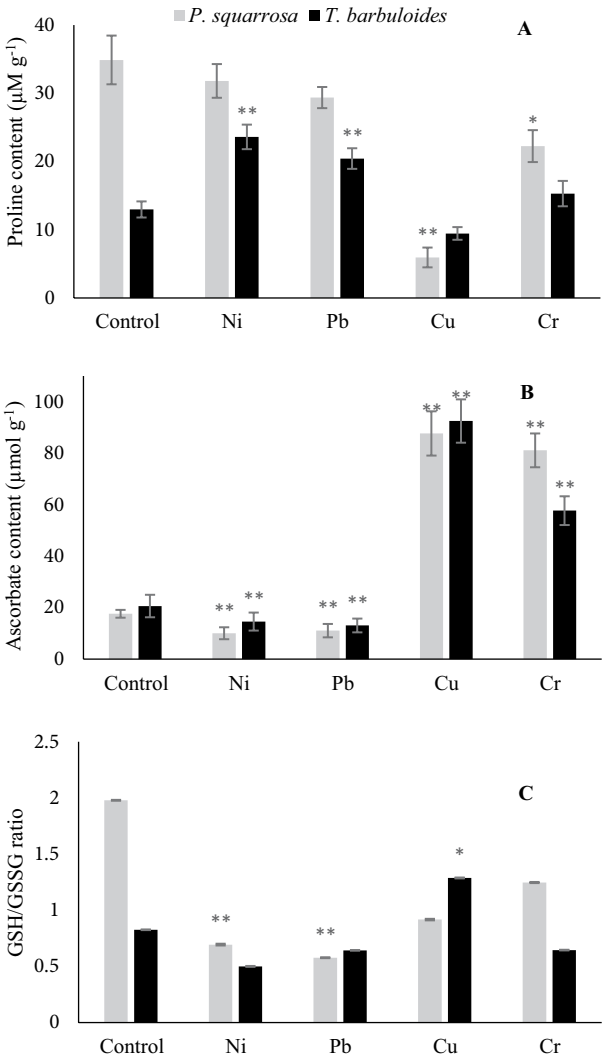

Figure 4. Proline contents (A), ascorbate contents (B), and reduced glutathione/oxidized glutathione (GSH/GSSG) ratio (C) of moss species *P. squarrosa* and *T. barbuloides* exposed to Ni, Pb, Cu, and Cr metals for 48 h. Values represent mean ± SE (n = 3). **: Significant difference at $P < 0.01$; *: at $P < 0.05$.

tolerance in plants (Singh et al., 2003). Detoxification and compartmentalization mechanisms of accumulated heavy metals consist of metal chelate complexes (phytochelatins and metallothioneins), or transport of heavy metals from the cytoplasm to cell walls or to the vacuole (Rascio and Navari-Izzo, 2011). Mosses are known as bioindicators for heavy metal pollution because of their high accumulation capacity of heavy metals (Shakya et al., 2008). For bryophytes, the tolerance mechanism operates by binding uptaken metal cations to the cell wall anionic exchange sites created by generally negatively charged polyuronic acids (Tyler, 1990; Chettri et al., 1998).

4.1. Accumulation of heavy metals

In this study, bryophyte species *P. squarrosa* and *T. barbuloides* showed a high accumulation capacity for short-term exposure to heavy metals. It was noticed that the variation in the extent of metal accumulation is unrelated to being an essential micronutrient or not. Among heavy metals, accumulation of micronutrient Ni and nonmicronutrient Pb exhibited higher levels than micronutrient Cu and nonmicronutrient Cr in both moss species. The nonspecific cation binding was confirmed by the accumulation levels of metals irrespective of being essential for plant metabolism in both moss species. The magnitude of heavy metal bioaccumulation was much higher in *T. barbuloides* than in *P. squarrosa*, which indicated species-specific variation for Ni, Pb, Cu, and Cr metals. The species-specific feature of heavy metal accumulation has been reported in mosses (Antreich et al., 2016) and other plants (Kabir, 2016).

4.2. Effect on dry weight

As a result of heavy metal treatment, the accumulated metals slightly increased the dry weights of the moss species, except for Cu (Figure 1). Deceleration and/or limitation of heavy metal movement into the cell by the cation binding capacity of moss cell walls (Tyler, 1990) may be the main reason for survival of mosses under high heavy metal conditions. Excess accumulation of Ni, Pb, and Cr showed no growth retardation in *P. squarrosa* and *T. barbuloides*; this could be explained by the heavy metal tolerance (Clemens, 2006) of these two species. However, Cu adversely affected the dry weight enhancement of both species. High redox active characteristics of Cu may result in significant toxicity for synthesis reactions. Cu-induced biomass retardation was reported in various studies of mosses (Dazy et al., 2009) and higher plants (Kachout et al., 2015).

4.3. Oxidative damage

Chl content, Chl *a/b* ratio, and Car content varied between species depending on the heavy metal. Cu-induced photosynthetic pigment loss was associated with observed dry weight loss for both moss species. Between the two moss species, *T. barbuloides* was affected only by Cu, but *P. squarrosa* was under the toxic influence of both Cu and Cr. These results can be attributed to the variability of the reactive potential of metals and species. Toxic effects of Cu and Cr on total chlorophyll were also registered for terrestrial moss *Taxiphyllum taxirameum* (Chen et al., 2015) and other mosses (Shakya et al., 2008). Chl and Car contents of *P. squarrosa* decreased slightly upon Ni and Pb exposure; similar data have also been reported in moss species *Hypnum plumaeforme* after 48 h of Ni and Pb exposure (Sun et al., 2009). The observed phenomenon was not reflected in the Chl *a/b* ratio, which is an indicator of heavy metal toxicity (Hartmut et al., 1990). Cu and Cr

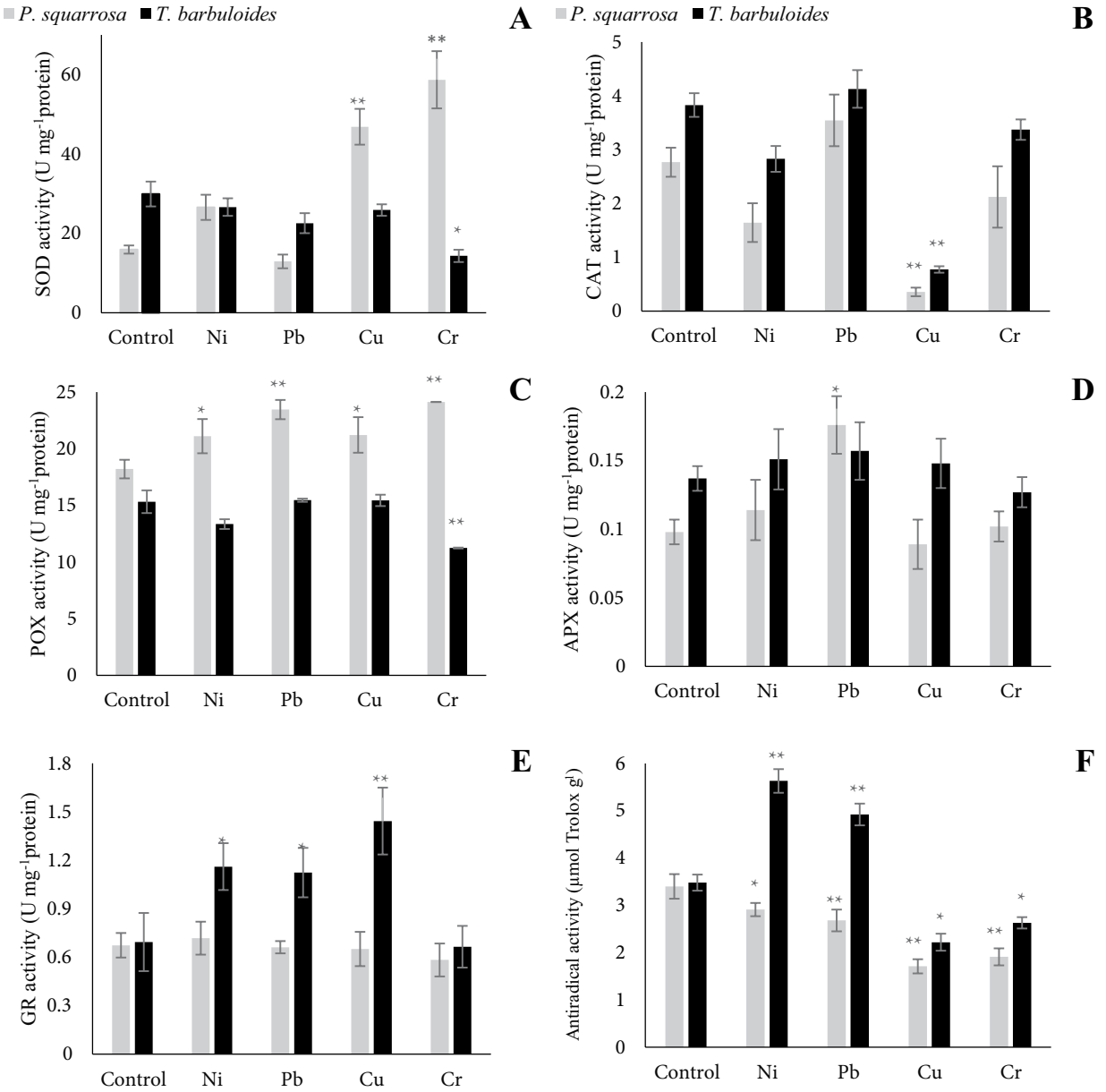

Figure 5. SOD (A), CAT (B), POX (C), APX (D), GR (E), and antiradical activities (F) of moss species *P. squarrosa* and *T. barbuloides* exposed to Ni, Pb, Cu, and Cr metals for 48 h. Values represent mean ± SE (n = 3). **: Significant difference at $P < 0.01$; *: at $P < 0.05$.

treatment led to the oxidation process of the Chl *a* methyl group (Chettri et al., 1998) to form Chl *b* in *P. squarrosa* and *T. barbuloides*. Detrimental effects of heavy metals on the content of photosynthetic pigments are based on the inhibition of pigment synthesis (Huang et al., 2013) and/or the oxidative damage to pigments that results in changes of the Chl *a/b* ratio. These results led us to suggest that both species are very sensitive to Cu, and *P. squarrosa* was also sensitive to Cr exposure. While *T. barbuloides* has higher Chl and Car contents, indicating higher tolerance to Ni exposure, *P. squarrosa* shows less pigment content in both Ni and Pb exposure compared to the control, which points to a lower tolerance of these metals. This is consistent with the data of Sun et al. (2009) from *H. plumaeforme*. Results of this study point out that the Chl *a/b* ratio instead of total chlorophyll content can provide a specific and efficient definition in biomonitoring of heavy metals for moss species.

Besides being a part of the light-harvesting complex of photosystems, carotenoid pigments act as a chemical

quencher of singlet oxygen (Edge and Truscott, 1999) and are defined as nonenzymatic antioxidant molecules (Kumar et al., 2012). Carotenoid content data corroborate the roles of these pigments in better protection of the photosynthetic apparatus and their larger contribution to antioxidant defense mechanisms (Kachout et al., 2015) against heavy metals in *T. barbuloides* than in *P. squarrosa*. Based on the results, it may be suggested that the Chl *a/b* ratio is a more efficient parameter for evaluating toxicity of heavy metals in moss species than total Chl and Car content.

The extent of stress caused by heavy metals on H_2O_2 content varied depending on the species and metals exposed. Cu and Cr exposure resulted in H_2O_2 accumulation in both moss species, and Ni induced H_2O_2 accumulation in *P. squarrosa* only. Heavy metal-triggered H_2O_2 accumulation was previously reported in bryophytes and higher plants (reviewed by Sytar et al., 2013). Cu-triggered H_2O_2 generation caused an increase in lipid peroxidation, which indicates damage in membrane integrity and toxicity of the metal (Schützendübel and Polle, 2002) for both species. Apart from Cu, Cr- and Ni-provoked H_2O_2 accumulation was not reflected in enhancement of lipid peroxidation products (MDA). This can confirm that a large portion of heavy metals accumulated in cell walls in both bryophyte species. This hypothesis was also supported by Sharma and Dubey (2005), who reported the association between Pb tolerance and the capacity of plant restriction of Pb in cell walls.

4.4. Nonenzymatic antioxidant molecules

Proline is reported as a component of stress signaling (Ashraf and Foolad, 2007), as well as a quencher of singlet oxygen and $OH^{\cdot}$ (Szabados and Savouré, 2010); its accumulation in response to heavy metal stress has been reported in bryophytes (Sun et al., 2011). Contrasting results were observed in which Cu and Cr exposure caused decrements of proline content in *P. squarrosa*, whereas the content was maintained in *T. barbuloides*. However, Ni and Pb exposure induced proline content only in *T. barbuloides*. These data support the idea that proline contributed to Ni and Pb metal tolerance in *T. barbuloides,* and that it has a role in overcoming heavy metal stress. Constitutionally higher content of proline in *P. squarrosa* decreased with heavy metal treatment. This indicates that sensitivity to metals in *P. squarrosa* may be caused by deprivation in proline synthesis, degradation, or excess use of proline.

Ascorbate has an effective role in ROS quenching, α-tocopherol regeneration, and ascorbate-glutathione pathways of antioxidant metabolism (Noctor and Foyer, 1998). Increase of ascorbate was induced in both moss species against ROS-generating metals Cu and Cr. Our data are in accordance with reports presenting Cu- and Cr-induced ascorbate increase in alleviation of oxidative stress for mosses (Choudhury and Panda, 2005). In response to Cu and Cr, the GSH/GSSG ratio exhibits a trend similar to ascorbate.

Glutathione is a peptide and plays a significant role in the redox balance of cells (Noctor and Foyer, 1998). Reduced glutathione may act as a scavenger of ROS, an electron source for ascorbate regeneration, and a metal-binding peptide (Hossain et al., 2012). Heavy metal treatment of both moss species led to GSH consumption to scavenge ROS and/or metal binding activity (Jozefczak et al., 2012); the data are very well correlated with the glutathione reductase enzyme activity of *P. squarrosa*, showing that it remained unchanged with heavy metal treatment. In the case of *T. barbuloides*, the reduced glutathione/oxidized glutathione ratio increased with Cu treatment only, whereas the GR activity of this species was induced by Ni, Pb, and Cu treatment. Data reveal that only Cu treatment induced enough GR activity to enhance GSH and to convert cellular redox status.

4.5. Enzymatic antioxidant molecules

SOD is a first barrier against oxidative stress. Both moss species maintained their SOD activity at control levels under Ni and Pb exposure, which was well correlated with data on stationary levels of H_2O_2 and lipid peroxidation. According to physicochemical properties, Ni and Pb are grouped among nonredox active metals, and they may indirectly cause oxidative stress in cells (Valko et al., 2005). This fact could be the reason for the effects of Ni and Pb on SOD activity, as well as for limiting their interaction with metabolic parts of cells in both species. Cu treatment induced SOD activity in *P. squarrosa*, but not in *T. barbuloides.* Increase in SOD activity was shown in moss species *Fontinalis antipyretica* under Cu exposure (Dazy et al., 2009) and in *Taxithelium nepalense* under Cr exposure (Choudhury and Panda, 2005). The opposite results of SOD activity in response to Cu and Cr between the two species could be due to possible differences in the redox status of cells.

CAT is an independent component of antioxidant metabolism for direct elimination of H_2O_2 without requiring any electron donor (Mittler, 2002). Although heavy metal ions behave like competitive inhibitors of the CAT enzyme, results of this study showed that Ni, Pb, and Cr exposure did not affect the activity of the enzyme in either moss species (Figure 5B). In contrast to our data, 100 μM Pb and Cr treatment of the moss *Taxithelium nepalense* (Choudhury and Panda, 2005) and Pb and Ni treatment of *Hypnum plumaeforme* (Sun et al., 2009) caused a decrease in CAT in short-term exposure. Therefore, this may suggest that CAT activity in response to Ni, Pb, and Cr is strongly based on species detoxification mechanisms and retention capacity of these metals in cell walls. However,

Cu is a redox active metal and a noncompetitive inhibitor of catalase and therefore it is the most effective metal to suppress CAT activity, causing oxidative stress in cells (Schützendübel and Polle, 2002). These results are well correlated with high H_2O_2 content, along with lipid peroxidation of Cu-treated samples of both species.

The H_2O_2-scavenging enzyme POX is particularly located in cell walls. In the present study, results of POX activity varied depending on species and metals. POX activity induced by toxic concentrations of Pb and Ni has been reported as a common response in various bryophyte species, such as *Hypnum plumaeforme*, *Thuidium cymbifolium*, and *Brachhythecium piligerum* (Sun et al., 2011). The induction of the POX enzyme was suggested as a potential biomarker in heavy metal toxicity. However, this hypothesis is in contradiction with our data that revealed that variations in enzyme activity depended on species. Toxic Cu induced POX activity in *P. squarrosa* but suppressed it in *T. barbuloides*. Cr-treated samples of *T. barbuloides* accumulated excess H_2O_2 in correlation with suppressed POX activity. Data are accordant with the results of declined POX activity in the bryophyte *Taxithelium nepalense* in 1 mM Cr (Choudhury and Panda, 2005) and *Taxiphyllum taxirameum* in 100 µM short-term Cr exposure (Chen et al., 2015). The discrepancy in POX activity in moss species indicates that different enzymatic (CAT, APX) and nonenzymatic ROS scavengers operate in the elimination of H_2O_2 from cells.

APX plays a major role in H_2O_2 detoxification in the ascorbate-glutathione cycle by using ascorbate as an electron donor. In our study, heavy metals did not induce APX activity in either species, except in Pb-treated *P. squarrosa*. Lead-provoked increase in APX and POX activity of the species may be responsible for low H_2O_2 levels, without the contribution of either the antioxidant enzymes or the nonenzymatic antioxidants that were measured in this study.

One of the important enzymes of the ascorbate-glutathione cycle, GR, contributes to the maintenance of cellular redox homeostasis (Thounaojam et al., 2012). Besides its role in the regeneration of oxidized glutathione and ascorbate by Halliwell–Asada reactions, the enzyme is important for metal chelation with reduced glutathione (Anjum et al., 2012). Enzyme activity showed variation depending on species and metals exposed. In this study, GR activity was at a steady level under Ni, Pb, Cu, and Cr treatment in *P. squarrosa*; the result is consistent with the declined GSH/GSSG ratio of the species. While GR activity was stimulated by Ni, Pb, and Cu exposure in *T. barbuloides*, its promotive role for a higher GSH/GSSG ratio and thereby a high reduced glutathione pool and ascorbate level was observed only in Cu-treated samples. Cu-induced GR activity was reported in *Talinum triangulare* (Kumar et al., 2012) and *Sesbania drummondii* (Israr et al., 2011).

The total antiradical capacity of moss samples represents a contribution of molecular components such as polyphenols to cellular detoxification mechanisms against heavy metal-induced oxidative stress. In this study, the antiradical activity varied depending on Fenton and non-Fenton metals, and on species. Antiradical activity decreased in response to all treated metals in *P. squarrosa*, but exposure to Fenton metals Cu and Cr (Valko, et al., 2005) caused much lower antiradical activity than did Ni and Pb. In contrast, *T. barbuloides* showed better tolerance to Ni and Pb with increasing antiradical activity, and less inhibition against Fenton metals.

The current study concluded that moss species *Pleurochaete squarrosa* and *Timmiella barbuloides* exhibit metal- and species-specific bioaccumulation and cellular responses to Ni, Pb, Cu, and Cr heavy metal exposure. Bioaccumulation of metals in both moss species differed and was independent of essential or nonessential nutrients. However, the obtained data point out that the non-Fenton metals Ni and Pb were accumulated more than Fenton metals Cu and Cr by the bryophyte species. The data collected on higher heavy metal accumulation, dry mass, Chl *a/b* ratio, and extent of oxidative stress parameters allow us to hypothesize that both moss species are tolerant to Ni and Pb exposure. The Ni, Pb, and Cr tolerance of these moss species can be attributed to the restriction of these metals in cell walls, in consistence with steady membrane integration of cells as indicated by the MDA level. Cu accumulation-driven toxicity was observed by a decrease in dry weight, Chl *a/b* ratio, and high MDA levels in both species. Cu-induced oxidative stress triggered different components of antioxidant metabolism in both moss species; however, *T. barbuloides* was more likely to activate antioxidant defense responses, showing a higher GSH/GSSG ratio and more GR activity than *P. squarrosa*. From the data, we may suggest that both *P. squarrosa* and *T. barbuloides* are sensitive to redox active Cu, and the induced antioxidant components of cells are not enough to overcome the phytotoxic effect of the metal.

Our data prove that the toxicity levels of environmentally monitored or exposed heavy metals in mosses can be simply and accurately estimated by measuring the dry weight, Chl *a/b* ratio, and lipid peroxidation product MDA of samples.

Acknowledgment
This study was supported by the Scientific Research Projects of Adnan Menderes University (Grant Number FEF11011).

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