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Biomonitoring heavy metal contaminations by moss visible parameters

Yang-Er Chen[a,1], Jun-Mei Cui[a,1], Jin-Chuan Yang[a], Zhong-Wei Zhang[b], Ming Yuan[a], Chun Song[c], Hui Yang[d], Han-Mei Liu[a], Chang-Quan Wang[b], Huai-Yu Zhang[a], Xian-Yin Zeng[a], Shu Yuan[b,*]

[a] College of Life Sciences, Sichuan Agricultural University, Ya'an 625014, China
[b] College of Resources Science and Technology, Sichuan Agricultural University, Chengdu 611130, China
[c] College of Environment, Sichuan Agricultural University, Chengdu 611130, China
[d] College of Agronomy, Sichuan Agricultural University, Chengdu 611130, China

HIGHLIGHTS

- Moss anthocyanin, chlorosis pattern and chlorophyll fluorescence images can roughly reflect metal species groups and concentrations in aquatic media.
- Enzymatic and non-enzymatic anti-oxidative abilities and photosynthetic protein contents of *E. eustegium* are higher than those of *T. taxirameum*.
- The study provides new ideas to monitor water heavy metals rapidly and non-invasively in a large-scale.

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ABSTRACT

Traditional sampling for heavy metal monitoring is a time-consuming and inconvenient method, which also does not indicate contaminants non-invasively and instantaneously. Moss is sensitive to heavy metals and is therefore considered a pollution indicator. However, it is unknown what kind physiological parameters can indicate metal contaminations quickly and non-invasively. Here, we systematically examined the effects of six heavy metals on physiological parameters and photosynthetic activities of two moss species grown in aquatic media or moist soil surface. We suggest that a phenotype with anthocyanin accumulation pattern and chlorosis pattern and two chlorophyll fluorescence parameters with their images can roughly reflect metal species groups, concentrations and differences between the two moss species. In other words, metal contaminations could be roughly estimated visually using the naked eye. Enzymatic and non-enzymatic anti-oxidative abilities and photosynthetic protein contents of *Eurhynchium eustegium* were higher than those of *Taxiphyllum taxirameum*, indicating their differential metal tolerance. Neither anti-oxidative abilities nor photosynthetic proteins were found to be ideal indicators. This study provides new ideas to monitor heavy metals rapidly and non-invasively in water or on wetland and moist soil surface.

1. Introduction

In recent decades, with an increase in city development and industry, heavy metals and their compounds have been widely used as important materials of many industries. Soluble salts of some heavy metals cause serious pollution in the atmosphere, water, and soil. Therefore, it is necessary to monitor metal pollution constantly and to investigate pollutant toxicology widely. At present, the most widely-used atmosphere pollution bioindicators in the world are mosses and lichens. Some mosses have also been used to investigate water pollution [1]. In the late 1960s Ruhling and Tyler used mosses for surveying heavy metal deposition [2,3] and since then terrestrial mosses as pollution biomonitors have been widely used in different countries and regions [4–7]. Mosses obtain most of their nutrients directly from the soil and water surface and from atmospheric deposition, which are then distributed throughout the entire moss due to the lack of true roots and a true vascular system [8]. However, the physiological parameters that could quickly and accurately reflect metal contamination is still not clear. Traditional

* Corresponding author. Tel.: +86 28 86291325.
E-mail address: roundtree318@hotmail.com (S. Yuan).
[1] These authors contributed equally to this work.

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water sampling is a time-consuming and inconvenient method that does not indicate the contaminant *in situ* instantaneously. In this study, we examined the effects of Cu, Zn, Pb, Cr, Cd, and Hg on physiological parameters and photosynthetic activities of two moss species *Taxiphyllum taxirameum* (a common scale-leaf moss) and *Eurhynchium eustegium* (a common acutifoliate moss) grown in aquatic media or moist soil surface. Statistical analysis of the data suggests that chlorophyll (chlorosis pattern) and anthocyanin levels and chlorophyll fluorescence parameters F_v/F_m and Φ_{PSII} may roughly indicate the metal species groups and concentrations as well as differences between the two moss species. In other words, species group and concentration of the metal contaminant may be roughly estimated visually by using the naked eye.

2. Materials and methods

2.1. Plant materials and treatments

T. taxirameum (Mitt.) Fleisch and *E. eustegium* (Besch.) Dix. were collected on some green and moist area at Sichuan Agricultural University, Ya'an, PR China and brought back to laboratory. *T. taxirameum* and *E. eustegium* were thoroughly washed with tap water and double-distilled water for 3 times. Then *T. taxirameum* was cultured in distilled sterile modified Mohr medium (KNO_3 100 mg, $CaCl_2{\cdot}4H_2O$ 10 mg, $MgSO_4$ 10 mg, KH_2PO_4 136 mg and $FeSO_4$ 0.4 mg to 1000 mL distilled water, pH 7.5) [9] for three days in lab before the metal stress to make them adjust to indoor environment (16/8 h photoperiod at 100 μmol of photons $m^{-2}\,s^{-1}$, 25 ± 1 °C). Metal stresses applied by adding 0 (control), 10, 25 or 50 μM $CuSO_4{\cdot}5H_2O$, $ZnSO_4{\cdot}7H_2O$, $Pb(NO_3)_2$, K_2CrO_4, $CdCl_2{\cdot}2.5H_2O$, $HgCl_2$ to the Mohr solution. Solutions were replaced every two days. The mosses were cultured or stressed for 30 days.

For soil treatments, mosses were cultured on metal-contaminated soil for 30 days. The soil was were collected at Sichuan Agricultural University, Ya'an, PR China with high organic matter concentration (about 4%) and fine texture (about 20% sand) with high clay content (more than 60%). Soil pH was about 6.2. For each treatment, the appropriate amount of metal ions (0, 10, 25 or 50 μM Cu, Zn, Pb, Cr, Cd or Hg kg^{-1} soil) was dissolved in 1 L of double-distilled water and sprayed on the soil which was thinly layered. To ensure uniformity of metal distribution, the soil was subsequently mixed and the procedure repeated several times until all the solution had been applied. Moss plants were planted into plastic pots filled with 1 kg of air-dried soil. Mosses were watered every day to maintain soil moisture at approximately 80–100% of soil field water capacity.

2.2. Inductively coupled plasma mass spectroscopy (ICP-MS) analysis

The moss samples were immersed in a solution that contained 1 mM EDTA for 2 h and then thoroughly rinsed with distilled water. The samples were oven-dried at 75 °C for 48 h. The dried moss tissues were ground and digested in concentrated nitric acid for 2–3 h at room temperature. The samples were then boiled for 1–2 h until they were completely digested [10]. After adding 4 mL of Millipore-filtered de-ionized water and a brief centrifugation, the contents of Cu, Zn, Pb, Cr, Cd and Hg were determined using ICP-MS (Optimal 2100DV, PerkinElmer Instruments, Waltham, MA, USA). For metal content determination, five individual mosses for each treatment were measured.

2.3. Pigment determination

Chlorophyll (Chl) contents were determined by using the equations of Lichtenthaler and Wellburn [11]. Chl *a* ($mg\,g^{-1}$) = [12.21 × A_{663} − 2.81 × A_{646}] × Volume (80% acetone; mL)/[1000 × Weight (tissue; g)]. Chl *b* ($mg\,g^{-1}$) = [20.13 × A_{646} − 5.03 × A_{663}] × V/(1000 × W). Total Chls ($mg\,g^{-1}$) = [17.32 × A_{646} + 7.18 × A_{663}] × V/(1000 × W).

For anthocyanin assay, mosses were ground with a plastic pestle in a 1.5 mL microcentrifuge tube in 300 μl (or μL, I do not know which one is right) of 1% HCl in methanol. The samples were diluted with 200 μl H_2O and centrifuged for 3 min at 14,000 *g*. The supernatant was recovered, 500 μl chloroform added, and the samples vortexed and centrifuged for 2 min at 14,000 *g*. The upper aqueous phase was removed to a clean tube and 300 μl of 1% HCl in methanol and 200 μl H_2O added. Absorbance at 657 nm and 530 nm was measured with a spectrophotometer. Anthocyanin content was calculated from A_{530} corrected for the background A_{657} [12].

2.4. Chlorophyll fluorescence visualization

Chlorophyll fluorescence images were obtained at room temperature using a modulated imaging fluorometer (the Imaging PAM M-Series Chlorophyll Fluorescence System, Heinz-Walz Instruments, Effeltrich, Germany) according to the instructions provided by the manufacturer. Values of F_0 (for minimum fluorescence yield) and F_m (for maximum fluorescence yield) were averaged to improve the signal-to-noise ratio. Image data acquired in each experiment were normalized to a false color scale. The maximum efficiency of photosystem II (PSII) photochemistry in the dark-adapted state (F_v/F_m); the quantum yield of PSII electron transport (Φ_{PSII}) and the non-photochemical quenching coefficient (NPQ) were visualized [13].

2.5. CO_2 assimilation measurement

Gas exchange was measured by an open system TPS-1 (PP systems, Hitchin, UK). Net photosynthetic rate (*P*n) was determined at CO_2 concentration of 360 μmol mol^{-1}, relative humidity of 80%, irradiance of 0–1600 μmol $m^{-2}\,s^{-1}$ and temperature of 25 °C [14].

2.6. Water content, electrolyte leakage, malonaldehyde and total protein content determination

Relative water content (RWC) was measured according to Havaux et al. [15], which was determined as the ratio of [(fresh mass − dry mass)/(water-saturated mass − dry mass)] × 100. Electrolyte leakage was measured with a conductivity meter. After measuring the conductivity, the samples were boiled for 15 min to achieve 100% electrolyte leakage. Lipid peroxides were estimated by measuring the malonaldehyde (MDA). Approximately 0.2 g of moss thallus were homogenized by the addition of 5 mL 5% trichloroacetic acid (TCA) in ice bath. Equal volumes of supernatant and 0.5% thiobarbituric acid (TBA) in 20% TCA solution were added into a new tube. This mixture was incubated at 98 °C for 40 min, and then centrifuged at 8000 *g* for 5 min. The supernatant was subjected to analysis with a spectrophotometer [16]. Total soluble protein was also assayed as described by Lowry's method [17]. Fresh mosses (0.5 g) were homogenized with 5 ml Na–Phosphate buffer (pH 7.2) and then centrifuged at 4 °C for 10 min (3000 rpm). Supernatants were used for the analysis of soluble protein by a UV spectrophotometer.

2.7. Qualitative and quantitative measurement of reactive oxygen species (ROS)

Superoxide and H_2O_2 levels were visually detected with nitro blue tetrazolium (NBT) and 3,3-diaminobenzidine (DAB), respectively, as described previously [18] with some modifications. Moss thallus were excised at the base with a razor blade and immersed