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Research article

Limited accumulation of copper in heavy metal adapted mosses

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ABSTRACT

Copper is an essential micronutrient but has toxic effects at high concentrations. Bryophytes are remarkably tolerant to elevated levels of copper but we wondered if this tolerance might be species dependent. Therefore, in three moss species, *Physcomitrella patens*, *Mielichhoferia elongata* and *Pohlia drummondii*, the accumulation of copper was compared with semiquantitative SEM-EDX analyses after six weeks of cultivation on copper containing media. We investigated the role of the copper-linked anion and applied copper as $CuCl_2$, $CuSO_4$ and CuEDTA, respectively. Line scans along the growth axis of moss gametophores allowed for a detailed analysis of copper detection from the base towards the tip. Mosses originating from metal-containing habitats (i.e. *M. elongata* and *P. drummondii*) revealed a lower accumulation of copper when compared to the non-adapted *P. patens*. CuEDTA had a shielding effect in all three species and copper levels differed greatly from $CuCl_2$ or $CuSO_4$. The detection of reactive oxygen species (ROS), H_2O_2 and O_2^-, was further used to indicate stress levels in the gametophore stems. ROS staining was increased along the whole stem and the tip in the non-adapted species *P. patens* whereas the tolerant species *M. elongata* and *P. drummondii* generally showed less staining located mainly at the base of the stem. We discuss the relation between metal accumulation and ROS production using indicator dyes in the three moss species. As moss gametophores are very delicate structures, ROS staining provide an excellent alternative to spectrophotometric analyses to estimate stress levels.

1. Introduction

Copper is an essential micronutrient for plants. Amongst other functions, it is part of enzymes involved in electron transfer chains like plastocyanin in chloroplasts or cytochrome c oxidase in mitochondria, and it is involved in the reduction of reactive oxygen species (ROS) like superoxide dismutase (SOD) (Abreu and Cabelli, 2010). Lack of copper therefore leads to reduced photosynthetic activity (Baszynski et al., 1988) and inhibition of the ROS defense system (Tanaka et al., 1995). On the other hand, excess copper is also toxic for plants due to its binding to various enzymes thereby changing or blocking the original function of the enzyme (Baron et al., 1995; Yruela, 2005). This causes the production and accumulation of ROS which in turn damage membranes and lead to the destruction of the redox potential (Sharma, 2009). Both, shortage and surplus of copper have negative effects on plants, thus it is evident that plants have developed sophisticated homeostasis mechanisms to balance the uptake and intracellular amount of copper.

In contrast to cormophytes, mosses are small, non-vascular plants that mostly lack a cuticle as well as roots which could function as a barrier to exclude undesired metals in the substrate. Mosses are believed to take up water and nutrients over the whole surface. However, when grown on metal containing media, we found copper, zinc or cadmium in the leafy parts of the gametophore suggesting a transport from the base towards the tip of the plantlets (Sassmann et al., 2015b). Many studies use sequential elution techniques to locate metals at the tissue level (Brown and Brumelis, 1996; Perez-Llamazares et al., 2011; Vazquez et al., 1999) mostly focusing on the tip regions. Detailed information on the distribution from the base to the tip, however, is often missing. Therefore, we performed elemental analyses in the scanning electron microscope (SEM) along the entire growth axis of the moss. This allowed for a detailed detection of copper accumulation

Abbreviations: CEC, Cation exchange capacity; DAB, 3,3′-Diaminobenzidine; EDTA, Ethylendiaminetetraacetic acid; EDX, Energy-dispersive X-ray spectrometer; NBT, Nitroblue tetrazoliumchloride; ROS, Reactive oxygen species; SEM, Scanning electron microscope; SOD, Superoxide dismutase.

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including the effect of the anions SO_4^{2-}, Cl^- or ethylendiaminetetraacetic acid (EDTA) linked to the metal. Furthermore, the localization of elemental copper along the stems of moss gametophores was correlated to the production of ROS after staining with 3,3′-diaminobenzidine (DAB) and nitroblue tetrazoliumchloride (NBT) to detect H_2O_2 and O_2^-, respectively, on the cellular level (Jabs et al., 1996; Thordal-Christensen et al., 1997). Higher levels of ROS can be provoked in the plant either by abiotic stress factors like high irradiation or by biotic factors like pathogens, where the ROS is involved in the pathogen defense system (Apel and Hirt, 2004). The correlation between the accumulation of copper with the production of ROS gives the possibility to differentiate the "sensitivity" of different copper adapted moss species towards differently linked copper.

Cellular tolerance tests showed that bryophytes generally withstand high metal concentrations (Frahm, 2001; Url, 1956) although the reasons remained unclear. We hypothesize that tolerance levels are species dependent and caused by the copper-linked anions and the different uptake capacities of mosses. To test this hypothesis we compared the sensitive species *Physcomitrella patens* to the tolerant species *Miehlichhoferia elongata* and *Pohlia drummondii* (Shaw, 1990; Shaw and Schneider, 1995; Tyler, 1990).

2. Material and methods

2.1. Cultivation

Plant species were carefully chosen to meet the required range of copper tolerance. The three moss species used were (1) *Physcomitrella patens* (Hedw.) Bruch & Schimp. [Funariaceae], as a copper-sensitive species. It is a pioneer species on wet habitats. Furthermore, it is also used as a model organism in molecular sciences. The used *P. patens* wild type was kindly supplied by the Reski-group, University of Freiburg, Germany. This species was compared to two copper-tolerant species (2) *Mielichhoferia elongata* (Hoppe & Hornsch.) Nees & Hornsch. [Bryaceae] occurring mainly on copper enriched areas – e.g. former mining sites – and is therefore known as copper tolerant species (Shaw, 1990) as well as (3) *Pohlia drummondii* (Müll. Hal.) A.L. Andrews [Bryaceae] growing originally on wet sands but also occurs on heavy metal containing sites. The species *M. elongata* and *P. drummondii* were collected on the Schwarzwand in Salzburg, Austria, from a former copper mine. After surface sterilization, the mosses have been subcultured in the lab over a period of several years. For the experiments of the present work, all three species were taken from these sterile cultures.

Mosses were grown on Benecke-Media (Benecke, 1903) modified by Gang et al. (2003) at 21 °C, (±2; light/dark, 14/10) under sterile conditions to minimize other stress factors than the heavy metal stress. The media were supplemented with 0.1 mM $CuCl_2$, 0.1 mM $CuSO_4$ or 10 mM CuEDTA for the copper treatments. These concentrations caused beginning growth deficiencies in preliminary studies with *P. patens*. Repeatedly, the gametophores of all three species were collected and analyzed for element composition and the production of reactive oxygen species, after six weeks.

2.2. Element analysis by energy-dispersive X-ray spectroscopy (EDX)

The analysis of the copper content was performed by a scanning electron microscope (SEM, Phillips XL 20) coupled with an energy-dispersive X-ray spectrometer (EDX, EDAX with Genesis software). For the measurements, gametophores were cut closely above the media, washed thoroughly in distilled water and dried at 70 °C for 48 h. Dry samples were mounted on an aluminum stub using double-sticky carbon tape and coated with carbon (Leica EM MED 020).

Measurements were obtained at 30 keV to get the spectrum of all detectable elements in the moss samples (C, O, Na, Mg, P, S, Cl, K, Ca, Fe and Cu) and were taken, both in the top and in the bottom region of the stem, carefully avoiding the leaves. The penetration depth of the electron beam with at least 5% electron energy was calculated at ~30 μm by a Monte Carlo simulation using CASINO software version 2.4.8.1 (Drouin et al., 2007). The mean density of 0.5 g cm^3-1 and major element composition of the stems were used after an estimation of dry moss leaf density and major element composition as described in Sassmann et al. (2015b). Microscopic determination of dried moss stems showed a diameter of 50–80 μm for all three moss species.

For each region, the measurements were repeated on at least eight spots which were selected randomly from a minimum of three different gametophores. The weight percent (wt%) of copper was calculated according to all measured elements. Additionally, line scans along the entire stem of the moss allowed to visualize the continuing change of the copper content from base to tip.

2.3. Staining of reactive oxygen species (ROS)

We have used the occurrence of ROS, i.e. O_2^- and H_2O_2, to indicate stress in the different moss regions according to the protocol of Kumar et al. (2014). Nitroblue tetrazoliumchloride (NBT) was applied, which produced a blue formazan complex upon reaction with O_2^-. Staining was performed after (Jabs et al., 1996). Single gametophores were submersed in 50 mM potassium phosphate buffer (KH_2PO_4, pH 6.4) with 10 mM sodium acid (NaN_3) and 0.1% NBT. To promote dye infiltration and exhaust the air of the gametophore low vacuum was applied for 10 min. Subsequently, the samples were stored at room temperature for 6 h.

Staining of H_2O_2 was performed using 3,3′-diaminobenzidin (DAB). After interaction with the endogen peroxidase it forms a brown complex which is associated with H_2O_2. Gametophores were incubated in 0.1% DAB in 10 mM MES buffer (2-(*N*-morpholino) ethanesulfonic acid; pH 7.2) and also infiltrated under vacuum for 10 min as described above. Afterwards, samples were stored in the dark at room temperature for 12 h.

Both staining protocols required the removal of chlorophyll and other native pigments of the mosses by boiling in a mixture of ethanol, glycerol and acetic acid (3/1/1, v/v/v) in order to reveal the dye precipitations. Imaging of the gametophores was performed in a stereomicroscope (Olympus CX41). For both staining methods, between 3 and 9 gametophores of each treatment were used depending on their availability in the different copper treatments. The amount of tissue stained was expressed as a percentage of the stem area using GSA Image Analysis software (GSA, Rostock); complete staining of a whole stem would therefore result in 100% staining. This allows for the identification of ROS production sites along the stem and gives the possibility to compare the stress levels between the species as the percentage of the stained area.

2.4. Statistics

For statistical analyses, the program Statistica 7.1 (Statsoft) was applied. Data were tested for normal distribution and homogeneity in variance by Shapiro-Wilk-test and Levene-test. As data were not always normally distributed, they were analyzed using a Kruskal-Wallis-Test with a *post-hoc* comparison of the middle ranks. Significant differences of p-values are marked with * ($p < 0.05$).