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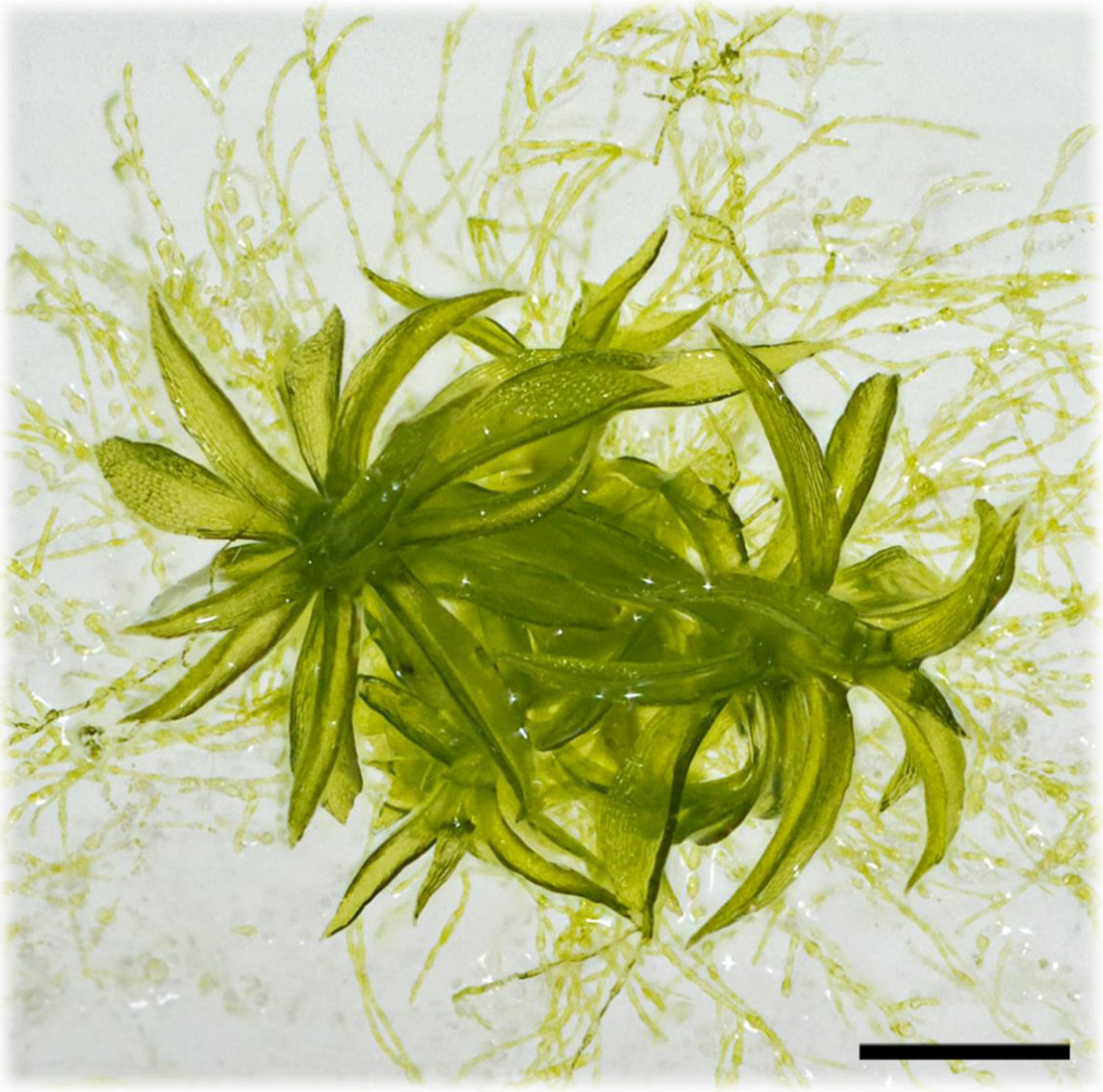
Physcomitrella patens and heavy metal stress

From the Impact of Metals on Growth and Morphology via Uptake towards Intracellular Localization and Detection of Stress Induced Reactive Oxygen Species



Habitus of *Physcomitrella patens* gametophyte consisting of the leafy gametophore and the filamentous protonema; scale bar: 1 mm

VELUT PHOENIX EX CINERE SURGO

Danksagung

Ich möchte der Universität Wien für die Vergabe einer einjährigen Finanzierung dem "Forschungsstipendium2010" danken. Dies hat den Start meines PhD erst ermöglicht. Des Weiteren gilt mein Dank auch den weiteren Förderinstitutionen wie dem Siegfried Ludwig-Fonds des Landes Niederösterreich, der Hochschuljubiläumsstiftung der Stadt Wien sowie der Republik Österreich.

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1. Zusammenfassung

1. Zusammenfassung

Erhöhter Metalleintrag durch menschliche Aktivitäten ist ein weltweites Umweltproblem. Einmal im Substrat belasten diese Metalle den ansonsten fruchtbaren Boden auf Dauer. Metallstandorte wie z.B. Bergbauhalden unterliegen außerdem verstärkter Erosion, wodurch Schwermetalle abgetragen oder ins Grundwasser gelangen können. Studien über Toleranz und Eignung spezieller Pflanzenarten für Biomonitoring und Phytoremediation sind daher von zunehmender Wichtigkeit. Obwohl viele Metalle essentielle Nährstoffe sind, wirken sie im Überschuss giftig. Andere Metalle sind nicht essentiell und wirken immer toxisch. Metalle kommen natürlicherweise in der Erdkruste vor; der Metallgehalt in Böden und anderen Substraten unterliegt jedoch starken Schwankungen. Pflanzen haben daher Metallaufnahme- und Homöostasemechanismen entwickelt. Trotzdem können nur wenige spezialisierte Arten die toxischen Bedingungen an metallreichen Standorten ertragen, sodass die weniger kompetitiven Bryophyten in diesen ökologischen Nischen zu gedeihen vermögen. Dies führte sogar zu der Annahme, dass Moose generell metalltolerant seien.

Zusätzlich zum Metallstress sind Pflanzen an Metallstandorten vielen verschiedenen weiteren Stressfaktoren ausgesetzt. Dies erschwert Aussagen zur spezifischen Auswirkung des jeweiligen Faktors. Daher wurde in dieser Doktorarbeit das Modellmoos *Physcomitrella patens* unter kontrollierten Umweltbedingungen gezogen. Wir verwendeten Zellen des filamentösen Protonema und des beblätterten Gametophors um die Auswirkungen der Metalle Kupfer, Zink und Kadmium zu untersuchen. Da diese Metallkationen nie ohne zugehörige Anionen vorkommen, wurde der Einfluss von Chloriden, Sulfaten und Ethylendiaminteraessigsäuresalzen (EDTA) ebenfalls untersucht.

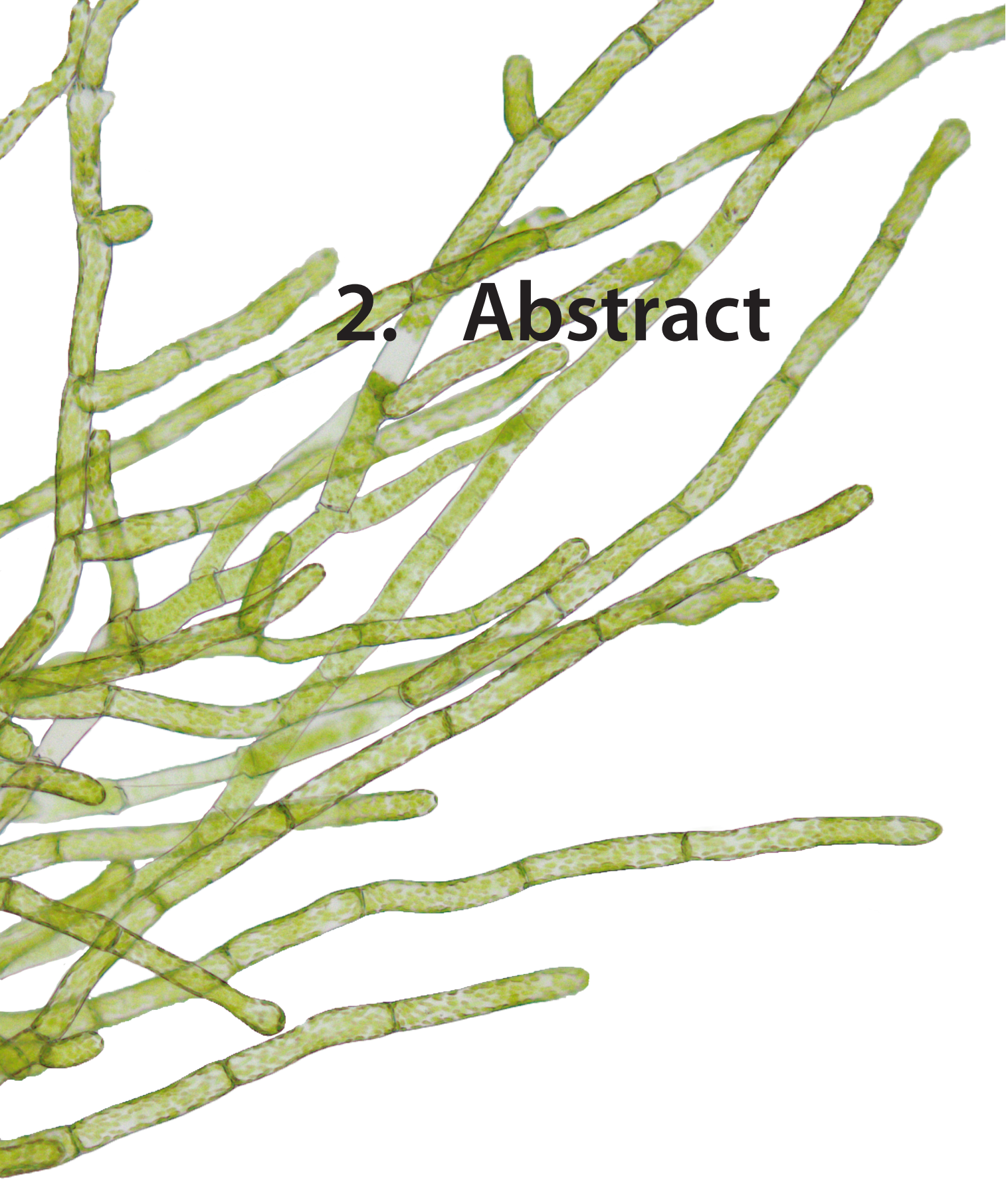
Die Metalltoleranz von *P. patens* wurde zytologisch ermittelt und zeigte absteigende Giftigkeit in der Reihenfolge „Kadmium > Kupfer > Zink". Des Weiteren verhielt sich die Giftigkeit in Bezug auf die Anionen wie „Chloride = Sulfate > EDTA". Modellierung der Metallspeziation mittels Visual MINTEQ zeigte auf, dass die freien Metallionen der entscheidende Einzelfaktor für die Giftwirkung sind. Trotz der hohen Wasserlöslichkeit von EDTA-Komplexen zeigen diese die niedrigsten Konzentrationen an den freien Cu^{2+}- und Zn^{2+}- Ionen und sind folglich am wenigsten giftig.

Die Metallaufnahme der Protonemata und der Gametophoren von *P. patens* wurde im Rasterelektronenmikroskop in Kombination mit energiedispersiver Röntgenspektroskopie gemessen. Hier beobachteten wir Unterschiede in der Aufnahme von Kupfer und Zink, sowie wiederum eine starke Anionenabhängigkeit. Obwohl in höheren Metall-EDTA-Konzentrationen die Aufnahmeraten nicht ganz der Berechnung entsprachen, ist auch die Metallaufnahme klar vom Gehalt an freien Metallionen im Medium abhängig.

Im Gegensatz zum Wachstum des filamentösen Protonema, konnte das Wachstum des beblätterten Gametophor bereits bei niedrigeren Metallkonzentrationen negativ beeinflusst sein. Dies erlaubte die Entwicklung eines Verhältnismodells von Gametophor zu Protonema (G : P) in Abhängigkeit des jeweiligen Metallstresses. Wieder erklärte der Gehalt an freien Metallionen die Wachstumshemmung besser als die gesamte Metallkonzentration im Medium. Das Stressniveau der Zellen wurde mittels Fluoreszenzfärbung von stressinduzierten reaktiven Sauerstoffspezies visualisiert. In den metallexponierten Zellen wurde ein erhöhter H_2O_2 Gehalt festgestellt, welcher ein feineres Bild der Metallauswirkung als die Toleranztests lieferte.

Die Toleranztests zeigten generell weder eine spezielle Sensitivität noch eine besonders hohe Toleranz von *P. patens*, sondern eine stark anionabhängige Metalltoleranz. Die freie Metallionenkonzentration lieferte überlegene Erklärungen zu den Effekten von Schwermetallen in allen Bereichen, angefangen von der Metallaufnahme einzelner Zellen bis zu Veränderungen im Wachstumsverhalten ganzer Moospflanzen.

2. Abstract



2. Abstract

Increased metal deposition by human activities is a worldwide problem, since these metals can permanently pollute otherwise fertile soils. On one hand, many metals are essential nutrients for life but on the other hand, toxic in excess concentrations. Other metals are non-essential and always toxic. Metals occur naturally in soils and other substrates in varying amounts; consequently plants developed metal uptake and homeostasis mechanisms. Nonetheless, metal rich sites such as spoil heaps are populated only by a few specialized plant species that are able to tolerate toxic condition of the soil. Those loosely covered sites allow less competitive bryophytes to thrive in this highly specific niches which led to the general assumption that mosses are generally metal tolerant. However, mine spoil heaps are subject to increased erosion and may leak heavy metals into ground and surface waters, thereby threatening people and environment. As a consequence, studies on plant tolerance and suitability of plants for biomonitoring and phytoremediation are gaining importance.

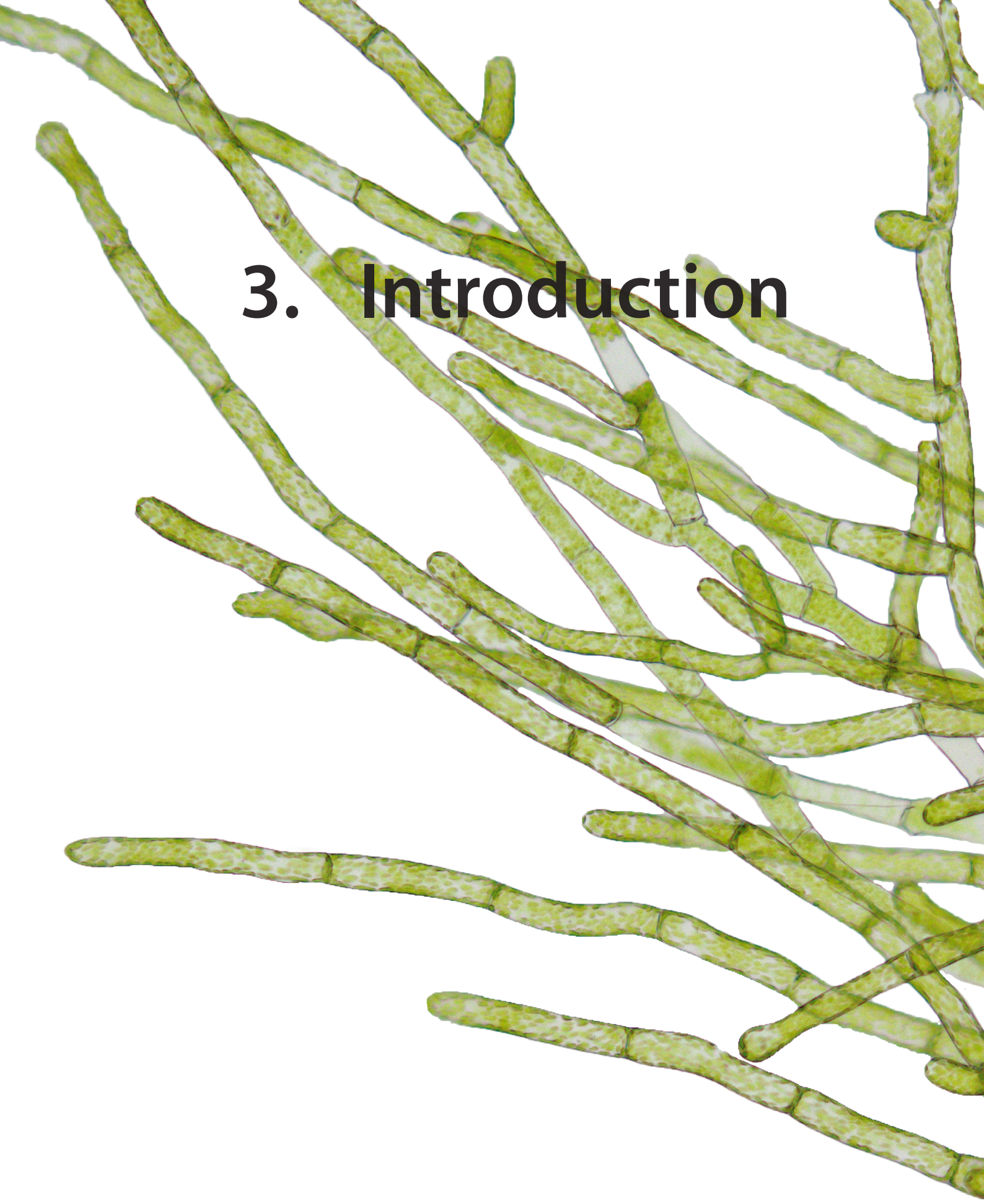
Plants on such metal sites are exposed to many different stress factors in addition to metal stress complicating statements on the respective effects. In this PhD thesis, the moss model *Physcomitrella patens* (Funariaceae) was grown under controlled environmental conditions. We studied cells of filamentous protonemata and leafy gametophores to investigate the effects of three important metals: copper, zinc and cadmium. Since metal cations never occur without corresponding anions, the contribution of three different anions, i.e. chloride, sulfates, and ethylenediaminetetraacetic acid (EDTA), were considered as well.

Metal tolerance evaluated by cytoplasmic tests for *P. patens* showed decreasing toxicity in the order "cadmium > copper > zinc". Furthermore, toxicity decreased in the order "chloride = sulfate > EDTA". Modelling metal speciation with Visual MINTEQ revealed that free metal ions were the most important single factor for metal toxicity. Despite their high water solubility, EDTA-chelates showed the lowest free metal ion concentrations (for Cu^{2+} and Zn^{2+}) and therefore the lowest toxicity.

Metal uptake in *P. patens* protonemata and gametophores was analyzed by X-ray microanalysis in a scanning electron microscope. Again, we observed differences in the uptake of copper and zinc and a strong influence of the anion. Free metal concentration clearly influenced metal uptake as well, though in high metal-EDTA concentrations uptake rates were higher than predicted by the estimated free metal ion concentration of the model.

Our investigations showed that growth of leafy gametophores was stronger affected by lower metal concentrations than the growth of the filamentous protonemata allowing for the development of a ratio model of gametophore to protonema (G : P). Once more, the amount of free metal cations explained growth inhibition better than total metal concentration. The stress level of the cells was visualized by fluorescent staining of stress induced reactive oxygen species. In metal treated cells, we observed increased levels of H_2O_2 providing a more detailed insight than the tolerance tests.

In conclusion, tolerance tests of *P. patens* revealed neither special sensitivity nor high tolerance towards the tested metals per se but tolerance was strongly anion dependent. Free metal ion concentrations provided superior explanations on the effects of heavy metals on all levels, from the metal uptake of single cells to changes in growth patterns in tissues and whole plants.



3. Introduction

3. Introduction

3.1 Why study heavy metal stress?

Metals are essential elements for life. Some metals such as potassium, calcium, magnesium and iron are macronutrients and therefore essential for cell metabolism, just like the micronutrients manganese, zinc, copper and molybdenum. In photosynthetic organisms, the macronutrient magnesium is located in the center of chlorophylls thereby forming the key complex for photosynthesis, the major biological energy source on earth. In all organisms, many physiological functions depend on metal containing enzymes (Hansch and Mendel, 2009). However, not all naturally occurring metals have a physiological function and also the rule that only the dose makes the poison applies, hence all metals are toxic above certain threshold concentrations[1].

The present work is focused on the micronutrients copper and zinc and the non-essential cadmium. Copper and zinc are both needed in the catalytic center of the copper-zinc superoxide dismutase, an important enzyme to regulate reactive oxygen species (ROS): it catalyzes the dismutation of the superoxide radical into molecular oxygen or hydrogen peroxide. Zinc alone is part of over 70 enzymes (P. Sitte, 1998; Yruela, 2013). Additionally to copper and zinc, we investigated the effects of the non-essential cadmium as it is highly mobile within the cells and its toxicity is well studied (Carginale et al., 2004; Chopra and Kumra, 1988; Lepp and Roberts, 1977; Prasad, 1995; Volland et al., 2013). All three investigated metals are considered to be heavy metals. This term is discussed as there are different specifications and some "heavy metals" are classified as metalloids as well. Still, even though plants do not differentiate by density, the most commonly used definition in plant sciences is by weight: heavy metals have a specific weight > 5 g cm^{-3} (Gupta et al., 2013; Rascio and Navari-Izzo, 2011) – hence the name "heavy metal".

Metals are important nutrients for plants but deficiency of those key elements can result in changes or severe ramifications of the metabolism leading to cell damage and ultimately to cell death (Apel and Hirt, 2004; Tewari et al., 2013).

3.2 Ecological Background

Metals are part of many different minerals which occur in varying concentrations within the earth's crust. Fueled by weathering and erosion metals are incorporated according to those variations into the pedosphere consequently resulting in varying, and usually relatively low, substrate concentrations. However, general soil properties, pH and redox potential significantly alter the metal availability for plants (Marschner and Marschner, 1995). As a consequence, total substrate content of metals might differ considerably from the plant available fraction. Iron is one of the most abundant ele-

1 *Alle Ding' sind Gift und nichts ohn' Gift; allein die Dosis macht, das ein Ding' kein Gift ist; lat. dosis sola venenum facit; Theophrast Paracelsus: Werke. Bd. 2, Darmstadt 1965, S. 508-513. Permalink: http://www.zeno.org/nid/20009261362*

ments in the earth's crust, but plants may still suffer from iron deficiency on calciferous sites due to the low availability. Both, nutrient deficient sites (White and Zasoski, 1999) as well as sites with excess metal content occur naturally (Baumbach and Schubert, 2008). Plants have specialized and adapted to these conditions but investigations of the respective tolerance mechanisms are difficult due to the many factors that need to be considered.

Man-made metal pollution started with mining and ore processing thousands of years ago. Already in Roman times, lead pollution was considerable. Metal pollution in Europe peaked again in the middle ages and with the start of industrialization (Brännvall et al., 1999; Renberg et al., 2000). New man-made metal polluted habitats were formed. Those habitats can vary widely from dry to rather humid conditions and in their metal compositions (Adlassnig et al., 2013a). As a consequence, research about mining and metal pollution became of paramount importance. It has also a long tradition at the University of Vienna e.g. Biebl (1947). Also at the Core Facility Cell Imaging and Ultrastructure Research (formerly Department of Cell Physiology and Scientific Film, Institute of Plant Physiology), Url (1956) investigated the toxicity of metals and tolerance of plants towards metals in different metal solutions. Nowadays – with the exceptions of some hot spots – lead and metal pollution by atmospheric deposition is generally declining across Europe, (Harmens et al., 2015). However, research on the ecological impact of elevated metal concentrations is still of uttermost importance as already deposited metal contaminates the soil for centuries (Adlassnig et al., 2012) and other regions with a booming mining industry show increased metal pollution. This affects not only emerging markets like China or Peru, but also e.g. the Appalachian mountain region (USA) is affected by mountain top mining (Lindberg et al., 2011; Palmer et al., 2010). In all these locations, metal pollution threatens the water and food production. Thus, the new mining waste sites and spoil heaps have generated increased interest on phytostabilization and phytoremediation, and research on metal sites was intensified in search for suitable plants (Ernst, 1996; Korzeniowska and Stanislawska-Glubiak, 2015; Lone et al., 2008; Pastor et al., 2015). However, the mix of different elements in the substrate, the varying availability for plants and different microclimatic factors require selective research on one specific stress factor.

3.3 Cormophyta and metal stress

Cormophyta are characterized by a high degree of morphological differentiation and therefore possess a wide range of mechanisms to cope with high metal content in the soil. However, most plants are sensitive to elevated metal levels and can be classified as metal sensitive in contrast to metal tolerant species. Those tolerant plant species endure elevated metal concentrations in the soil but are usually specialized towards a certain metal and react sensitive towards another. Baker (1981) characterized a metal tolerance system and introduced the terms "excluder-", "accumulator-" and "hyperaccumulator-plants" by grouping plants according to their respective abilities to cope with excess metal concentrations in the substrate. In this process, the roots play a key role: they actively increase or decrease metal uptake or even chelate unwanted metal ions in the rhizosphere via root exudates (Marschner, 1995). Further research identified mycorrhiza (plant associated fungi) as the

first and most important line of defense for metal tolerance (Bothe et al., 2010; Marschener, 1998; Turnau et al., 2010). Suitable plants from metal sites where investigated and besides excluder- and accumulator-plants for phytostabilization, the main focus was on hyperaccumulator plants with their potential use for phytomining. Further, studies concentrated on root associated bacterial and fungal partners in order to test their role in metal tolerance (Leyval et al., 1997; Wernitznig et al., 2014).

At high doses, metals affect the metabolism by interfering with cellular enzymes and generating free radicals, hence metal homeostasis is of uttermost importance for plant cells (Prasad and Freitas, 2003). Plants have complex systems for metal tolerance to prevent these intracellular stress inducing effects. One strategy is the formation of metal binding ligands e.g. cysteine rich polypeptides such as phytochelatin (PC) and metallothionein (MT). These proteins bind the metal cations and assist in homeostasis (Cobbett and Goldsbrough, 2002; Hall, 2002). Another mechanism – often combined with the previously described one - is the sequestration of metals into the vacuole by various metal transporters thereby maintaining optimal metal concentrations in the cytoplasm (Gupta et al., 2013; Migeon et al., 2010).

3.4 Mosses and metal stress

Mosses belong to the bryophytes and are cryptogams with general differences to the cormophyta. After spore germination mosses form filamentous primary protonemata. Later, the (leafy) gametophore is originating from these filaments. Spores, protonemata and gametophores form the gametophyte and are developed in the dominant haploid phase of the moss life cycle. Besides the protonemata, other filaments, so called rhizoids, can be formed from the gametophore. The rhizoids are used for surface adhesion as mosses do not possess a root system. Unlike true roots, rhizoids lack barriers like an endodermis or exodermis. Mycorrhiza partners for bryophytes were found in rhizoids of certain liverworts e.g. Lepidoziaceae but not in mosses (Pressel et al., 2014). Therefore, metal protection by a fungal shield that yields enhanced tolerance, as was reported for higher plants by Schutzendubel and Polle (2002), does not apply either. In addition, moss leaves are often built from only one cell layer. They take up nutrients with their whole gametophyte surface as they mostly lack a protective cuticle. As a consequence, it seems impossible for mosses to avoid metal uptake from the substrate or atmospheric deposition. This led to the assumption that the metal content of mosses would reflect their exposition to metals and therefore resulted in their widespread use for bioindication and biomonitoring (Frahm, 1998; Spagnuolo et al., 2011). However, mosses are such a diverse group of plants that it appears difficult to make general assumptions that can hold every species.

There are several mosses that occur on metal rich sites (Adlassnig et al., 2013b) and show a remarkable tolerance towards heavy metals e.g. *Pohlia drummondii*, *Mielichhoferia elongata* and *Scopelophila cataractae*; the latter two even seem to be specialized in copper rich habitats (Martensson and Berggren, 1954; Shaw, 1993; Shaw and Owens, 1995). Although considerable effort has been made by several groups (Frahm, 2001; Shaw, 1990; Tyler, 1990), the reasons for this tolerance are still not fully explained.

As reviewed by Tyler (1990), metal tolerance in mosses can be caused by the immobilization of heavy metal cations due to negative charges of the cell wall. Therefore, it can be expected that mosses occurring on metal rich substrates possess increased cation exchange capacity. On the other hand, some response mechanisms to heavy metal stress in mosses may be similar to higher plants; the occurrence of glutathione (GSH) and MT seem common for all mosses (Gupta et al., 2013). However, the role of PC which is of major importance for metal defense in higher plants, seems to be species dependent since PCs were reported to be missing in bryophytes (Bleuel et al., 2011; Bruns et al., 1999; Bruns et al., 2001)but have been recently found in *Sphagnum pallustre* (Petraglia et al., 2014).

3.5 *The moss* Physcomitrella patens

Physcomitrella patens (Hedw.) [syn.: *Aphanorrhegma patens* (Hedw.) Lindb.] belongs to the family of Funariaceae, in the order of Funerales of the class of Bryopsida. It is common throughout Europe and its occurrence ranges from Ireland to Russia within the temperate zone. It grows approximately 5 mm high and prefers silted or argillaceous soils e.g. dry fallen river banks but can also occur in wet meadows (Frahm and Frey, 2004; Nebel et al., 2001). The moss does not naturally occur on metal rich sites and is therefore considered as sensitive to metals. Research on *P. patens* intensified due to its highly efficient homologous recombination known only of few multicellular organisms (Schaefer and Zrÿd, 1997). Furthermore, its great suitability for microscopy purposes and the haploid genome of the gametophyte render this plant a prime candidate for systems biology approaches. It is fully sequenced (Rensing et al., 2008), has become a model organism of rising importance (Cove et al., 2006; Cove and Knight, 1993; Lang et al., 2008; Strotbek et al., 2013; Widiez et al., 2014) and was used for drought and salt tolerance studies. While those mostly molecular investigations on stress and defense mechanisms confirmed a high drought and salt tolerance, actual ecological studies on long term metal stress are missing. Frahm (2001) postulated a general high tolerance of bryophytes towards metals. However, little is actually known about *P. patens* in long term tolerance experiments to copper, zinc and cadmium and these data are especially interesting as genome analyses revealed that *P. patens* lacks the gene for the PC synthase which is considered important for metal tolerance (Kopriva et al., 2007).

3.6 *Aims of the study*

The papers presented in this thesis study the reaction of *P. patens* towards exposure to copper, zinc and cadmium in above-optimum concentrations. We aimed to determine the tolerance of *P. patens* towards the used metals, analyze possible changes in growth and morphology and investigate if its metal uptake behavior in different tissues is influenced by the metal anion, more specifically by the corresponding free metal ion availability.

First, we tested *P. patens* for its tolerance towards the metals copper, zinc and cadmium in short term (48 h) and long term (5 weeks) experiments. Long term experiments require media with suf-

ficient nutrients to support plant growth. However, these nutrients may influence metal availability as many metals tend to form poorly soluble salts. Therefore, the actual content of free metal cations which are available to the moss plant needed to be estimated. We modelled the actual ion concentrations using the free chemical equilibrium model software MINTEQ V. 3.0 (Gustafsson, 2010). Additionally, Kaho (1933) reported different toxicity levels of metals influenced by their anions, so we tested copper and zinc linked to three different anions (chloride, sulfate, EDTA) to determine a possible difference in toxicity.

Having shown that the metal tolerance of *P. patens* depends both on the element and its chemical speciation, the question if it will show a general response to all three different metals and anions remained. Mosses can grow as filamentous protonemata and as leafy gametophores, thus, we tested the influence of metal exposure on the growth of those two different tissues. Possible changes were documented as a ratio of gametophore to protonema (G : P ratio) of the total gametophyte in *P. patens*. Furthermore, we analyzed these two different tissues of the moss with regard to their metal uptake and total metal content. The protonema is in more direct contact with the substrate and as cylindroid filaments, it has a larger surface area for metal uptake. Therefore, possible differences in metal content of the two tissues needed to be investigated. Further, we questioned if the uptake of copper is different to the amount of zinc and if the uptake is linear to the added concentrations. These experiments were performed using energy dispersive X-ray spectroscopy (EDX) on a scanning electron microscope (SEM).

Finally, the localization of the metals remained unclear, as metals could either be bound to the cell wall or be absorbed to the living cytoplasm. To answer this question on the cellular level, a confocal laser scanning microscope and fluorescent heavy metal tracer dyes were used. Additionally, we hypothesized that metal stress increased the amount of ROS in the cells, which was tested by specific ROS markers. A possible increase should be related to the metal concentration, but also depend on the metal itself or the respective anion.

3.7 Hypotheses

The aims of this work combined with a thorough literature research lead to the following hypotheses that were tested in the frame of this thesis:

- *P. patens* is not occurring on metal rich sites but since all bryophytes are postulated to be metal tolerant, we expect *P. patens* to have similar tolerance as mosses from metal sites. However, various forms of stress induce ROS, supposedly also leading to elevated ROS levels in heavy metal stressed moss cells. Hence, *P. patens* will show differences in ROS levels if sensitive, when compared to *Mielichhoferia elongata* and *Pohlia drummondii* which occur on metal contaminated sites.

- In their role as bioindicators mosses are supposed to accumulate heavy metals in a gradient manner over time. This assumption, however, is poorly tested and needed to be confirmed under controlled growth conditions.

- We expect a general reduction of growth, growth rate and morphology, regardless of a proposed elevated tolerance towards heavy metals in first experiments, if metal stress is applied. Here, mosses can react by increased protonema formation. As a consequence, the G : P ratio is a useful tool to estimate stress levels in mosses.

- The solubility of a metal, its extractability, and its bioavailability for mosses are not necessarily equal and as complexed metals are supposedly not available to mosses, we expect the metal complexing ligand of the metal cation to be the most important single factor determining its toxicity.

- There is a limit of uptake capacity, resulting in a saturation curve, regardless of the offered metal concentrations. As copper is considered more toxic to mosses than zinc, *P. patens* will endure lower copper concentrations and use its homeostasis mechanisms to avoid uptake of copper rather than zinc, resulting in lower overall concentration of copper in the organism.

- As a non-metal adapted plant, *P. patens* cannot segregate metals after the uptake and will therefore store them in a concentration dependent manner. Neither additional accumulation nor segregation will occur over time.

- *P. patens* showed augmented growth of the protonemata under metal stress. Protonema cells are structurally and physiologically different to the leafy gametophore, suggesting differences in metal uptake. We postulate higher metal tolerance, and significantly higher metal concentrations in the protonemata.

- Growth reduction and morphological changes are better explained by metal uptake than by metal exposition. As chelated metals are likely to be unavailable for *P. patens*, we postulate that uptake itself is therefore best explained by the concentrations of free metal cations in the media.

- In spite of the high cation exchange capacity of moss cell walls, not all metal ions are adsorbed to the apoplast but some enter the living cytoplasm. Hence, sequestration to specific compartments serves as a second line of defense.

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4. Publications

4. Publications

A. Comparing copper resistance in two bryophytes: Mielichhoferia elongata *Hornsch. versus* Physcomitrella patens *Hedw.*

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SHORT COMMUNICATION

Comparing copper resistance in two bryophytes: *Mielichhoferia elongata* Hornsch. versus *Physcomitrella patens* Hedw.

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Abstract The bryophyte *Mielichhoferia elongata* is known to occur on copper-rich substrate, but the exact resistance level remained to be determined by in vitro experiments. Here, we tested its copper tolerance in graded copper solutions and compared the results to the moss *Physcomitrella patens* that is not known to inhabit heavy metal sites. Our results confirm the survival of *M. elongata* in classical resistance experiments of up to 10 mM Cu-ethylenediaminetetraacetic acid (EDTA) solution. Interestingly, *P. patens* is equally resistant. Cultured on copper-enriched agar plates for over 5 weeks, *P. patens* survived even higher copper levels of up to 100 mM Cu-EDTA and an increment of growth was detected on all concentrations tested. Obviously, *P. patens* is able to withstand harmfully high levels of copper in both solution and substrate. In this short communication, we give a detailed description of the growth rates and discuss the results in comparison to other moss species and heavy metals.

Keywords Bryophytes · Copper resistance · Heavy metal resistance · *Mielichhoferia elongata* Hornsch. · *Physcomitrella patens* Hedw.

Introduction

Copper is an important micronutrient for plants but toxic if applied at high doses. Copper tolerance is species-dependent and some metallophytes show copper levels of over 1,000 ppm dry weight (Shaw 1990; Clemens 2001). The moss *Mielichhoferia elongata* belongs to the small group of such specialists and occurs on substrates with high copper concentrations. It has been termed a "copper moss" (Martensson and Berggren 1954) and used as a geobotanical indicator plant to mineralization (Url 1956; Brooks 1971). However, a remarkable tolerance to metal pollution and an exceptionally high accumulation of heavy metals from the soil is postulated for all bryophytes (Frahm 2001). In the present work, we tested this hypothesis on *Physcomitrella patens* that is not known for its heavy metal tolerance and compared the results to the copper moss *M. elongata*.

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Material and methods

P. patens Hedw. was chosen due to its role as a model organism (Cove and Knight 1993; Reski 1999) and the first sterile plants were a kind gift by R. Reski (Freiburg, Germany). *M. elongata* Hornsch. was collected from a former copper mine in Salzburg, Austria and taken into tissue culture.

Both mosses, *P. patens* and *M. elongata*, were cultivated on sterile agar plates. The medium was prepared after Benecke (1903) and modified according to Gang et al. (2003). It contained 200 mg/L NH_4NO_3, 100 mg/L $MgSO_4 \cdot 7H_2O$, 400 mg/L KH_2PO_4, and 100 mg/L $CaCl_2 \cdot 2H_2O$; the pH was adjusted to 5.8. Agar (0.8%; VWR, Leuven, Belgium) was added and the mixture autoclaved before casting into sterile plastic Petri dishes. For copper enrichment, we used concentrations of 100 µM, 1 mM, 10 mM, and 100 mM Cu-ethylenediaminetetraacetic acid (EDTA) as well as 100 µM $CuCl_2$ added to the

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medium. The control experiments were done on agar plates without copper enrichment. Both mosses grew at 24°C on a 14-h day/10-h night regime. The average light intensity was 48.08 µM s^{-1} m^{-2}. The plantlets were photographed weekly over a period of 5 weeks. The increase or decrease in growth was calculated in percent of the starting point using planimetry area measurement. Under stress conditions, *P. patens* formed protonemata rather than plantlets. Two-dimensional area measurements alone would, therefore, lead to distorted calculations and widely differ from the actual biomass accumulation. To account for this phenomenon, we subtracted 10%, 20%, or 40% of the increment of growth depending on the amount of protonemata built (>25%, 25–50%, or <50%, respectively). A minimum of five plates was chosen from each copper concentration and nine plants from each plate were used for the measurements, giving a total of at least 45 measurements per concentration. For the control, 83 measurements from plates without copper enrichment were averaged. The mean values and standard error were calculated and plotted in a graph (Excel, Microsoft).

For resistance experiments, the leaflets or whole gametophytes of *P. patens* and *M. elongata* from control plates were submersed in graded Cu-EDTA solutions for 48 h. Subsequent plasmolysis in 0.8 M mannitol, followed by deplasmolysis reflected the viability of the cells. Additionally to Cu-EDTA, *P. patens* was also exposed to 100 µM $CuCl_2$ in classical resistance experiments.

Macrographs were taken with a Canon EOS 20D digital camera and a 28- to 80-mm zoom lens.

For microscopic documentation, we used an Olympus BX41 microscope with the objectives ×10 (N.A. 0.85), ×20 (N.A. 0.40), ×40 (N.A. 0.64), and ×60 oil (N.A. 1.25) and an attached digital camera (Color View III, Soft Imaging Systems, Olympus). The images were loaded into the computer using the CellD (Olympus) software.

Results

Resistance tests were performed by submersion of gametophytes in gradient Cu-EDTA and $CuCl_2$ solutions for 48 h followed by plasmolysis in 0.8 M mannitol. Only living cells are able to plasmolyse (Fig. 1). *M. elongata* is known for its high copper tolerance, and in the resistance experiments, we can confirm that it survives in solutions of up to 10 mM Cu-EDTA. Surprisingly, the "sensitive" *P. patens* showed a Cu-EDTA tolerance up to the same high level (Table 1). *P. patens* was also tested in gradual $CuCl_2$ solutions where we detected a much lower tolerance of only up to 1 µM. We refer this fact to the more harmful chloride compared to EDTA and to the cumulative effect of copper plus chloride. Our preliminary tests with Zn-EDTA and $ZnCl_2$ gave similar results (data not shown). Further experiments will show why chloride ions in micromolar concentrations have such a negative effect on mosses.

On copper-enriched agar plates (100 µM, 1 mM, 10 mM, and 100 mM Cu-EDTA or 100 µM $CuCl_2$, respectively), all investigated plants of *P. patens* showed a constant increment of growth over a period of 5 weeks (Fig. 2). Unexpectedly, survival of the plants on Cu-EDTA plates up to 100 mM is even higher than predicted by the classical resistance experiment (see above). The plants exhibit a perfectly linear increase on control plates without copper (Fig. 2). Compared to the control, only the highest Cu-EDTA concentration of 100 mM and, again, $CuCl_2$ (100 µM) showed damaging effects and reduced growth. $CuCl_2$ had immediate harmful effects lasting for the whole period observed, whereas the plants on 100 mM Cu-EDTA started off like the control until week 2 and only the long-term exposure resulted in significantly reduced growth. Both $CuCl_2$ (100 µM) and 100 mM Cu-EDTA resulted in mean values of 400% augmentation after 5 weeks; control plants reached 500% within this time. Lower Cu-EDTA concentrations in the agar (100 µM, 1 mM, and 10 mM) resulted in similar growth compared to the control. The two lowest Cu-EDTA concentrations of 100 µM and 1 mM showed best growth, followed by the next higher concentration of 10 mM. At these three copper concentrations, the mosses showed a higher variation in growth than on control medium: beneficial effects at the start of the experiment, slightly harmful effects during weeks 3 and 4, and acceleration of growth at week 5 (at least for 1 mM and 100 µM).

After the second week, the biomass of all samples was at least doubled and, within 5 weeks, even highly stressed plants grew 400% compared to the start (Fig. 2).

Discussion

Our results show that the "sensitive" *P. patens* tolerates Cu-EDTA concentrations up to the same and even higher levels as the known copper moss *M. elongata*. The submersion of the plants and total contact with the heavy metal solution during the classical resistance experiments is apparently more harmful than growth on metal-enriched plates. Although atomic absorption measurements of the media showed high availability of copper, the solid medium absorbed a certain percentage compared to the solution. Together, the submersion and higher availability of copper within the solution could cause the difference in resistance on solid and liquid media.

Long-term experiments on graded, low Cu-EDTA levels even show a slightly positive effect and elevated growth rates when compared to controls without copper. However, there is no significant difference to the control plates. In

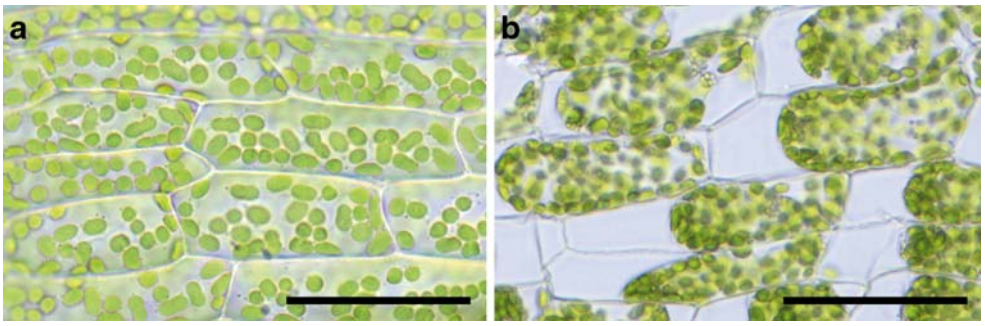

Fig. 1 Classical resistance experiment: plasmolysis as a means to analyze the viability of *P. patens* cells after exposure to heavy metal solution. **a** Gametophyte cells. **b** Vital parts of the leaf show plasmolysed cells. After 48 h in 10 mM Cu-EDTA, immediate transfer of the cells into 0.8 M mannitol reflects vitality; only living cells show plasmolysis (as in **b**) and deplasmolysis; in this concentration, 50% of the gametophyte cells were dead. Bar=50 µm

consonance with Frahm (2001), these data support the high resistance of bryophytes to abiotic stress factors in general and, as shown here, to copper in particular. Genetic variation within populations as well as differentiation between populations might be a strategy of mosses for copper and zinc tolerance (Shaw 1988). Furthermore, adaptation to metal-contaminated soils seems to occur relatively rapid in response to strong selective pressures, as shown in the moss *Ceratodon purpureus* (Jules and Shaw 1994).

Already, Url (1956) showed the high $CuSO_4$ tolerance (up to 500 mM) of *M. elongata* in resistance experiments and reported that mosses from nonmetal-polluted habitats, i.e., *Mnium affine* or *Madotheca platyphylla*, survived up to exceptionally high concentrations of over 500 mM $CuSO_4$. However, those mosses displayed "dead zones" (Iljin 1935) at lower concentrations (between 500 µM and 50 mM) where no living cells could be observed. The author suggested the formation of heavy metal precipitates on the plasma membrane as a protective layer for the living cell. However, other species like *Funaria hygrometrica*, *Mnium undulatum*, or *Hookeria lucens* reacted very sensitively to $CuSO_4$ exposure and died already at low concentrations of 5 µM (Url 1956). *P. patens*, as shown in the present experiment, tolerated the high copper levels without showing "dead zones" in both experimental setups: in resistance experiments of 48-h direct exposure to liquid Cu-EDTA and in long-term growth experiments on Cu-EDTA-enriched agar plates. Detoxification of excessive copper by exclusion or deposition at internal storage sites could be the reason for the high tolerance of *P. patens* and investigations are underway to define the putative intracellular distribution of copper in this bryophyte.

In flowering plants, phytochelatins have been shown to play a key role in metal homeostasis and the final disposal of heavy metals in the vacuole (Cobbett 2000; Krämer and Clemens 2005). Phytochelatins are heavy-metal-binding peptide ligands that are structurally related to glutathione. However, *P. patens* lacks phytochelatins, suggesting different mechanisms of metal detoxification (Rother et al. 2006). The authors give a comprehensive study of Cd^{2+}-mediated effects on transcript, enzyme, and metabolite levels in *P. patens* and detected changes in the assimilatory sulfate reduction pathway and in glutathione biosynthesis. To which extent those mechanisms are involved in copper-mediated stress remains to be determined.

Table 1 Summary of resistance experiments

	Concentration of solutions										
	1M	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	C
Physcomitrella patens											
Cu-EDTA	–	+/–	+/–	+	+	+	+	+	+	+	+
$CuCl_2$	–	–	–	–	–	–	+/–	+	+	+	+
Mielichhoferia elongata											
Cu-EDTA	–	–	+/–	+/–	+	+	+	+	+	+	+

P. patens survives up to the same Cu-EDTA levels as *M. elongata*. $CuCl_2$ has more harmful effects than Cu-EDTA. Dead cells are marked with a minus sign, living cells with a plus sign, and 50% surviving cells within the gametophyte with a plus/minus sign

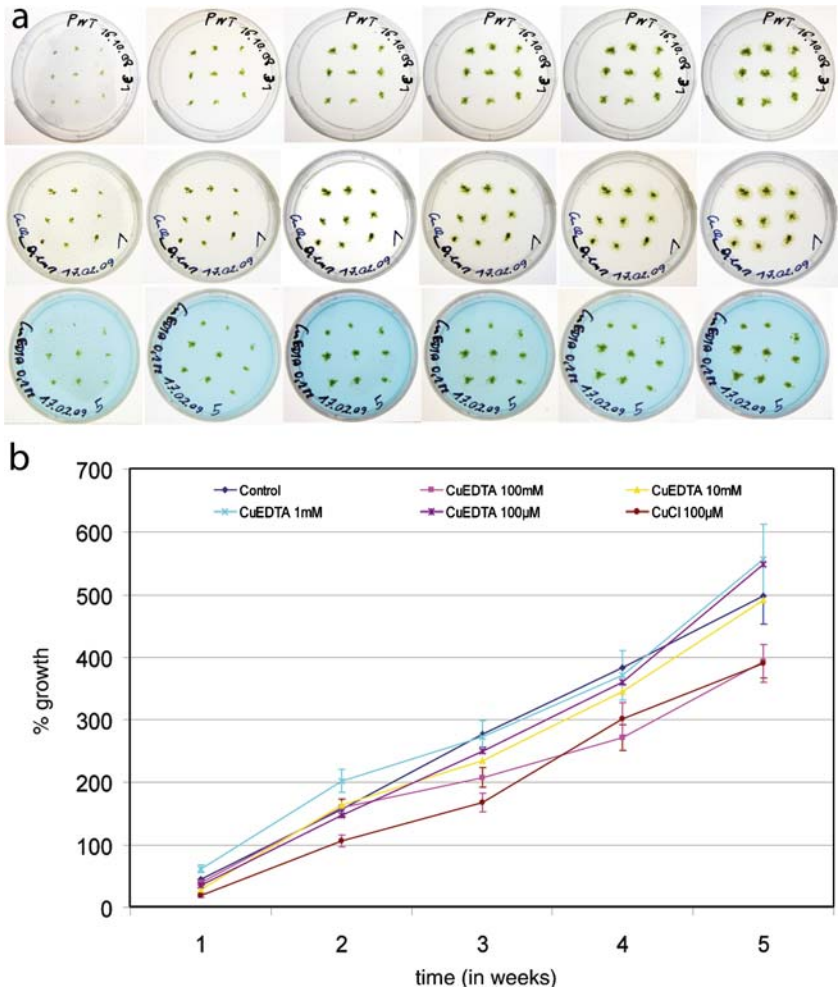

Fig. 2 In vitro experiment on gradient copper plates for a period of 5 weeks. **a** Addition of biomass in tissue culture of *P. patens* on three experimental sets. *Top row* control plants on medium without heavy metals. *Middle row* plants on medium containing 100 µM $CuCl_2$. *Bottom row* plants on medium containing 100 mM Cu-EDTA. The plates are photographed weekly from the time of subculture (*left* to *right*). Diameter of Petri dishes=6 cm. **b** Graphic depiction of growth on copper-containing (Cu-EDTA and $CuCl_2$) and control media. Relative to the starting point, *P. patens* plantlets gained biomass on all concentrations; 100 µM $CuCl_2$ and 100 mM Cu-EDTA have the most harmful effects, 10 mM Cu-EDTA is very similar to the control, and the lowest Cu-EDTA concentrations of 1 mM and 100 µM have positive growth effects. Mean values and standard error of at least 45 measurements per concentration

Early studies of Kaho (1933) and Url (1956) suggest that the application of heavy metal salts is least harmful to the cells when applied as sulfates rather than chlorides or nitrates. In the natural habitat, the metals appear as sulfides, copper- and iron-sulfates as well as copper-containing calcium carbonate (Günther 2006). In preliminary experiments using low concentrations, $CuSO_4$ showed to be more harmful than Cu-EDTA and *P. patens* survived only up to 100 µM $CuSO_4$ (data not shown). Here, we used Cu-EDTA because, at high concentrations, the $CuSO_4$ could not be dissolved properly at the pH used. In addition, metal-chelating agents like EDTA have been widely used in phytoremediation to enhance metal uptake and accumulation in plants (Alkorta et al. 2004).

P. patens is fully sequenced and has become a model organism. It will be interesting to see further outcomes on heavy metal homeostasis at the molecular and genetic level. To broaden the view, ecological aspects and genetic variation have to be taken into account and we hope to see further interesting outcomes by future comparison of mosses from natural habitats with *P. patens*. Up to now, there are no reports of *P. patens* growing naturally on heavy-metal-enriched sites. However, a similar study on *F. hygrometrica* Hedw., the same family as *Physcomitrella*, compared the population of mosses from a mining site and an unpolluted site (Basile et al. 1994). When grown on lead- and zinc-spiked media, the authors found only minimal effects on the population originating from the mining site. By contrast, the population from the unpolluted site showed severe structural and morphogenetic alterations. The authors suggest cell wall and vacuolar compartmentation of lead and zinc to account for higher tolerance in the adapted samples. It remains to be determined if this is also the case for copper.

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B. Free metal ion availability is a major factor for tolerance and growth in Physcomitrella patens

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Free metal ion availability is a major factor for tolerance and growth in *Physcomitrella patens*

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ABSTRACT

Metal rich sites are populated by only a few specialized vascular plant species allowing less competitive bryophytes to inhabit these ecological niches. On such sites, many different stress factors may interact with the plants and foreclose statements on the individual effects of a single factor, therefore the means for this tolerance are difficult to investigate. This study uses the cultivation of the moss *Physcomitrella patens* on solid media under controlled environmental conditions in order to study the growth inhibiting effects of copper, zinc and cadmium and the contribution of corresponding anions as chlorides, sulfates and ethylenediaminetetraacetic acid, respectively. Availability of the metals to plants was estimated both by water extraction with subsequent measurement by inductively coupled plasma mass spectroscopy and by modeling metal speciation using Visual MINTEQ a free chemical equilibrium model software.

A decreased ratio of gametophyte to protonema growth (G:P) was observed as a first reaction to even very low metal levels; G:P measurements can therefore be used as sensitive stress indicators in *P. patens*. Though all metals caused inhibiting effects with all anions, ethylenediaminetetraacetic acid chelates showed up to three orders of magnitude less toxic. Using Visual MINTEQ, we modeled the water solubility of zinc almost perfectly but achieved less accurate results for copper. For both metals, water solubility was rather under- than overestimated, indicating that adsorption to the agar played only a minor role in controlling metal solubility. Free metal cations were useful to explain growth inhibition which could not be fully explained by total metal concentration, calculated or experimentally determined water solubility. Especially the low toxicity of ethylenediaminetetraacetic acid chelates could be explained satisfactory by shielding of the metal ions.

1. Introduction

Some metal rich habitats occur naturally in Central Europe, e.g. Harzvorland or Galgenberg (Baumbach and Schubert, 2008), but artificial metal sites have been constantly gaining importance. Due to continuous mining activities, environmental pollution from industrial activities and inherited waste sites, elements such as copper, zinc or cadmium are entering the biological cycle. Increased concentrations of such elements result in serious ecological effects including diminishing plant growth and diversity (Ernst, 1974). Most contaminated habitats contain a mixture of metals in varying concentrations within the substrate with a poorly defined speciation. Additionally, the environmental conditions may vary widely from wet (Adlassnig et al., 2013) to rather dry habitats (Adlassnig et al., 2011). Therefore, a clear statement on the effects of each metal is impossible in the field, and growth experiments under controlled conditions on graded substrates are necessary to identify the metal specific effects. To further complicate the matter, the application of metals under defined conditions requires the use of counter-ions. However, the anion may influence the toxicity of the metal, an effect described already by Kaho (1933). So far, only few studies aimed to investigate this phenomenon in mosses. It is known, however, that *Physcomitrella patens* [Funariaceae] tolerates 1000 times higher concentrations of Cu-EDTA than of $CuCl_2$ (Lang and Wernitznig, 2011; Sassmann et al., 2010).

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Table 1
Tolerance test of *P. patens* grown on solid heavy metal enriched media under sterile culture conditions showing survival (after 5 weeks) in Cu-, Zn-EDTA up to 100 mM, while sulfate and chloride metal salts proved more toxic (**+** = surviving, – = not surviving, **o** = not tested; C: control).

Media	Concentration in growth medium									
	100 mM	10 mM	5 mM	1 mM	100 μM	10 μM	5 μM	1 μM	0.1 μM	C
Cu-EDTA	+	+	+	+	+	o	o	o	o	+
Zn-EDTA	+	+	+	+	+	o	o	o	o	+
$ZnCl_2$	–	–	+	+	+	o	o	o	o	+
$ZnSO_4$	–	–	–	+	+	o	o	o	o	+
$CuCl_2$	o	–	–	–	+	o	o	o	o	+
$CuSO_4$	o	–	–	–	+	o	o	o	o	+
$CdCl_2$	o	o	o	–	–	–	+	+	+	+

In contrast to flowering plants, mosses do not possess a root system and absorb ions through the entire surface; exclusion mechanisms such as a cuticle or a Casparian strip are missing (Lorch, 1931). For this reason they are widely used as bioindicators for atmospheric depositions (Frahm, 1998; Spagnuolo et al., 2011; Sun et al., 2011; Uyar et al., 2009). But – like all other organisms – mosses possess homeostatic mechanisms to maintain suitable concentrations of metal ions in different cellular compartments and to minimize damage from exposure to increased metal concentrations (Krämer and Clemens, 2006). If these mechanisms for detoxification are overburdening, mosses show different symptoms than vascular plants. Promoted protonemal growth, reduced formation of leafy gametophytes and decreased sexual reproduction under extreme copper stress were reported in species like *Scopelophila cataractae* (Nomura and Hasezawa, 2011).

Some mosses grow specifically in metal habitats (Jules and Shaw, 1994; Saukel, 1980; Shaw, 1990), and also for *P. patens*, although not occurring on metal-rich habitats, an astonishingly high tolerance towards EDTA chelates was reported (Sassmann et al., 2010). This raises the question of specific metabolic or anatomical adaptation mechanisms and/or substrate metal availability. The present study aims to explore the visible morphological and growth influencing effects in relation to metal availability and metal toxicity in the model moss *P. patens* and tests the following hypotheses:

1. The solubility of a metal, its extractability and its bioavailability for mosses are not necessarily equal.
2. The metal complexing ligand of the metal cation is the most important single factor determining its toxicity.
3. The gametophyte/protonema ratio (G:P-ratio) is a useful tool to estimate stress in mosses.

To test these hypotheses, we analyzed the survival and growth of *P. patens* on solid media spiked with copper, zinc and cadmium separately, in graded concentrations and linked to sulfate (SO_4^{2-}), chlorate (Cl^-) or chelated by ethylenediaminetetraacetic acid ($EDTA^{4-}$), respectively. Thus, anion and metal specific results as well as concentration-dependent developmental data were obtained.

2. Material and methods

2.1. Plant material and cultivation

Physcomitrella patens (Hedw.) [syn.: *Aphanorrhegma patens* (Hedw.) Lindb.], Funariaceae, a model organism to explore plant physiology (Cove and Knight, 1993; Lang et al., 2008; Rensing et al., 2008), was used for all studies (Fig. 1a). The great suitability for microscopy purposes and the haploid genome of the gametophyte render this plant a prime candidate for systems biology approaches. *P. patens* gametophytes were grown under aseptic culture conditions on solid Benecke medium (see below) at 21 °C on a 14 h day/10 h night regime over a period of 5 weeks. The average light intensity was 48.08 μM s^{-1} m^{-2}.

The haploid moss spore germinates and forms protonemata consisting of single cell filaments with occasional branches. It further develops into the leafy moss plant (Vidali and Bezanilla, 2012). In the following, we distinguish between the filamentous protonemata (Fig. 1b; referred to as "protonema") and the leafy gametophyte *sensu stricto* (Fig. 1c; referred to as "gametophyte"), well aware that both structures belong to the gametophyte *sensu lato*. The short lived diploid sporophyte phase did not occur during the experiments described here.

2.2. Metal application and tolerance

In preliminary tests, the metal tolerance of *P. patens* was estimated by placing leaves in graded metal solutions ranging from 10^{-9} M to 1 M for 48 h according to Url (1956). On solid media, tolerance towards metals was defined by the ability of *P. patens* to survive and form new biomass (protonema and/or gametophyte) on metal enriched growth media over a total time of 5 weeks.

The culture media were based on Benecke medium containing 200 mg l^{-1} NH_4NO_3, 100 mg l^{-1} $MgSO_4{\cdot}7H_2O$, 400 mg l^{-1} KH_2PO_4 and 100 mg l^{-1} $CaCl_2{\cdot}2H_2O$, solidified with 0.8% agar (VWR, Prolab®) at a pH of 5.8 according to Gang et al. (2003). The pH changed to pH 5.41–5.84 during sterilization at 121 °C over 20 min. A water loss of the media of 6.5 ± 0.5% was taken into account.

For cultivation on metal spiked agar plates, solutions of ethylenediaminetetraacetic acid copper (II) disodium salt hydrate (Cu-EDTA), $CuCl_2$, $CuSO_4$ (applied as pentahydrate), ethylenediaminetetraacetic acid zinc disodium salt hydrate (Zn-EDTA), $ZnCl_2$ and $ZnSO_4$ (applied as heptahydrate) were added to the media ingredients. Furthermore, as non-essential metal cadmium was applied as $CdCl_2$ in considerably lower concentrations due to the preceeding tolerance test in liquid media, any possible toxicity of the chloride can be excluded. Final concentrations are given in Table 1.

Final metal concentrations in solid media, pH values and the number of petri dishes used for each respective concentration are summarized in (Sup. 1). For control, *P. patens* was grown on Benecke medium without any supplements.

2.3. Determination of metal content

For the extraction of metals, bi-distilled water was used. 20 g of each medium was extracted with 80 ml water while gentle shaking for 24 h. The supernatant was filtered through paper (Whatman 925 HM free); and the metal content was determined by inductively coupled plasma mass spectroscopy (ICP-MS; GBC OptiMass 9500 ICP OA TOF MS Spectrometer).

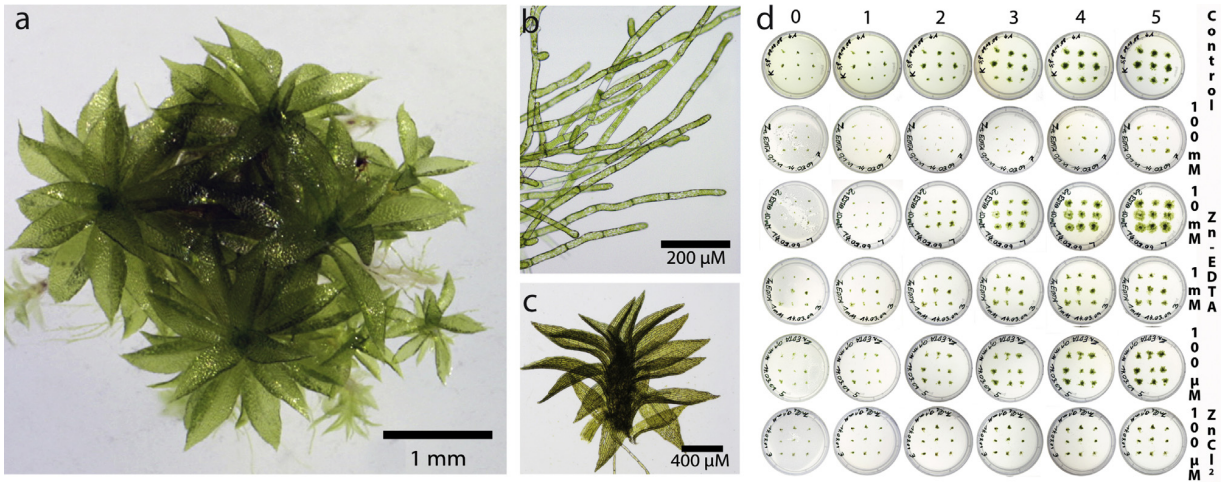

Fig. 1. (a)–(c) *P. patens* gametophyte *senso lato* and (d) cultivation method. (a) Leafy moss plants on a control plate with few protonemal filaments. (b) Protonema filaments. (c) Isolated leafy gametophyte *sensu stricto* of *P. patens*. (d) Cultivation on solid medium petri dishes from week 0 to 5 (Control, Zn-EDTA 100 mM, 10 mM, 1 mM, 0.1 mM and $ZnCl_2$ 100 µM as an example). Note the varying increase of biomass from the time of inoculation (week 0) to week 5.

2.4. Solution state modeling

Visual MINTEQ V3.0 (MINTEQ) was chosen to model the solution state equilibria (Gustafsson, 2010). The concentrations of all ionic media components were taken as input and the equilibrium state concentrations of all chemical species in the solution were calculated using the included default MINTEQ databases. Each simulation was iterated 2000 times and was not terminated if the charge balance exceeded 30%. Activity correction was done by the Debye–Hückel method with a Davies *b* parameter of 0.3. Oversaturated solids were allowed to precipitate each time a mineral precipitated or dissolved. The pH was fixed according to the pH of the media, ionic strengths were automatically calculated.

2.5. Growth analyses

Nine plantlets of *P. patens* were grown in each Petri dish over a period of 5 weeks. Images of plantlets were taken weekly, starting at the day of inoculation and ending at week 5 (Fig. 1d). Biomass formation was measured in square centimetres covered by either protonemata or gametophytes. The measured raw data were transformed to a percentage value in relation to the inoculated biomass at the starting point which was defined as 100% and growth rate was calculated according to:

$$\text{Growth Rate}(t_0 t_x) = \left(\frac{A(t_x)}{A(t_0)}\right)^{\frac{1}{x}} - 1$$

$A(t_0)$ Size at inoculation
$A(t_x)$ Size after x weeks

A mix of leafy gametophyte and filamentous protonemata was used for inoculation. The unavoidable heterogeneity levelled out during the 5 weeks of the experiment. The initial G:P-ratio is shown in Sup. 7. Thus, the starting values for protonemata and gametophytes sum up to 100% in week 0. After inoculation, plantlets developed protonemata and leafy gametophytes to a variable extent. Both values were recorded separately during the growth period. This G:P-ratio was calculated as the ratio of areas overgrown by protonemata and gametophytes, respectively.

For tolerance tests and documentation, we used a Leica DM6000CS. Macrographs of the Petri dishes including a scale were taken with a Canon EOS 20D digital camera and a 28–80 mm zoom lens. Analysis and identification of gametophyte and protonema tissue, respectively, were performed using GSA Image Analysis Software (GSA, Rostock).

2.6. Statistics

Samples were tested for normality (Kolmogorov–Smirnoff-test) and homogeneity of variances (Leveenes's-test). Differences in treatments and time points were investigated by analyses of variances (Anova, Students *T*-test and paired *T*-test). G:P-ratios were not normally distributed, thus, the Wilcoxon and Mann–Whitney tests were applied. Values are given in the arithmetic mean ± standard error. Significant differences to control data are labelled with: $^{***}P<0.001$, $^{**}P<0.01$ and $^{*}P<0.05$. All statistic tests were performed using IBM SPSS Statistics software V.20.

3. Results

3.1. Tolerance tests

P. patens, although normally occurring on unpolluted soil, is able to grow on solid media containing up to 100 mM of Zn- and Cu-EDTA. $ZnCl_2$ was tolerated up to 5 mM and $ZnSO_4$ up to 1 mM. $CuCl_2$ and $CuSO_4$ had severe toxic effects in concentrations of 1 mM; plantlets could still survive up to 4 weeks but no gametophyte growth was observed ($CuSO_4$ 1 mM). In contrast to copper and zinc, *P. patens* tolerated cadmium as $CdCl_2$ only up to 5 µM. Therefore, metal toxicity seemed not only dependent on the metal but also the anions SO_4^{2-} and Cl^- proved to be more harmful, emphasizing the importance of the metal anion and availability in the present study (Table 1).

3.2. Growth analyses

The areal biomass of the leafy gametophyte of *P. patens* was heavily decreased by the addition of $ZnCl_2$ (1 and 5 mM), $ZnSO_4$ 1 mm, Zn-EDTA 100 mM, $CuCl_2$ 0.1 mM, $CuSO_4$ (0.1 and 1 mM) and $CdCl_2$ 5 µM. (Fig. 2) On one hand, Cu-EDTA and Zn-EDTA at all concentrations had no significant effect on the growth of the leafy gametophyte, with the exception of Zn-EDTA 100 mM. On the other

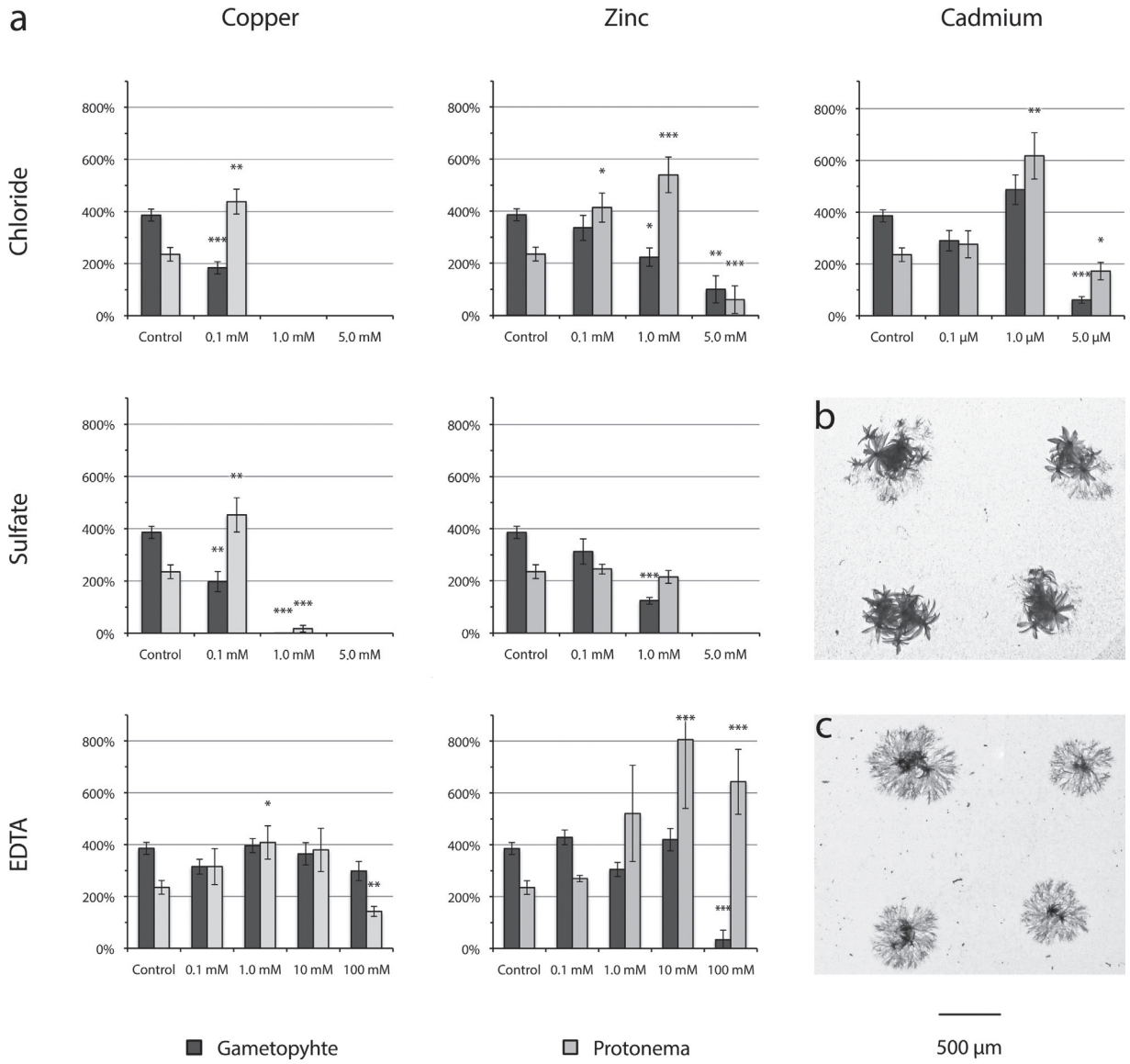

Fig. 2. Gametophyte and protonema growth of *P. patens* after 5 weeks in sterile culture. (a) The influence on growth after supplementing copper, zinc and cadmium as chloride (row 1), as sulfate (row 2) and EDTA (row 3) in their respective concentrations and comparison to control. (b) Gametophyte development on control medium. (c) Prominent protonema development on Zn-EDTA 100 mM (scale bar: 500 µM).

hand, however, the metals added as ionic salts clearly influenced the growth, with toxic and inhibiting effects on plantlets grown on $CdCl_2$ 5 µM over 5 weeks. In these samples, the biomass decreased to $61.2 \pm 10.3\%^{***}$ of the starting value after 5 weeks and hardly any new gametophyte biomass was formed. Other concentrations e.g. $CuSO_4 \cdot 1$ mM resulted in severe and significant growth effects, and leafy gametophytes were not able to survive 4 weeks of metal exposure.

The significant effect of the added metals on gametophyte growth can be summarized as follows (biomass decreases from left to right; non-significant differences to the control are not listed):

Copper: Control > $CuSO_4$ 0.1 mM = $CuCl_2$ 0.1 mM > $CuSO_4$ 1 mM
Zinc: Control > $ZnCl_2$ 1 mM = $ZnSO_4$ 1 mM = $ZnCl_2$ 5 mM;
$ZnCl_2$ 1 mM = $ZnSO_4$ 1 mM > Zn-EDTA 100 mM = $ZnCl_2$ 5 mM
Cadmium: Control > $CdCl_2$ 5 µM

In the leafy gametophyte, metals showed either negative or not significant effects. In the protonema, a more complex reaction was found. Contrary to most gametophytes, protonemata showed a general growth retention after inoculation (week 0). A clear lag phase could be observed in 12 of 20 media including control (Sup. 2). The amount of protonema development changed compared to the control; *P. patens* formed significantly more protonema when exposed to heavy metals.

The changes in protonema growth can be described as follows (biomass decreases from left to right; non-significant differences to the control are not listed):

Copper: $CuSO_4$ 0.1 mM = $CuCl_2$ 0.1 mM = Cu-EDTA 1 mM > Control > Cu-EDTA 100 mM > $CuSO_4$ 1 mM
Zinc: Zn-EDTA 10 mM = Zn-EDTA 100 mM = $ZnCl_2$ 1 mM;
Zn-EDTA 10 mM > $ZnCl_2$ 0.1 mM;
Zn-EDTA 100 mM = $ZnCl_2$ 1 mM = $ZnCl_2$ 0.1 mM > Control > $ZnCl_2$ 5 mM
Cadmium: $CdCl_2$ 1 µM > Control > $CdCl_2$ 5 µM

3.2.1. Growth rate

We considered a 50% reduction of growth rate as a clearly damaging effect of the added metals, analyzed separately for gametophyte and protonema. A ≥50% reduction was observed for $CdCl_2$ 5 μM, $ZnCl_2$ 1 mM, $ZnCl_2$ 5 mM, Zn-EDTA 100 mM, $ZnSO_4$ 1 mM and $CuSO_4$ 1 mM. In $CdCl_2$ 1 μM, Cu-EDTA 1 and 10 mM, both gametophyte and protonema showed an increased growth rate compared to control in at least the first week. None of the treatments resulted in 50% protonema growth rate reduction. The control showed the maximum growth rate of the gametophyte in the first week. Total biomass increased in a linear manner, thus the relative growth rate continuously decreased in the following weeks. Under metal influence, protonema usually showed a lag phase of 1 week followed by peaking growth rate (Sup. 3).

3.2.2. G:P-ratio

We hypothesized that metals did not only influence total biomass accumulation but shifted biomass production towards protonema development as a response to metal stress. As an indicator of this effect, we summarized this gametophyte–protonema ratio as G:P-ratio (Fig. 3). Control samples starting from 85% gametophyte and 15% protonema shifted to 66% gametophyte and 34% protonema over the growth period of 5 weeks.

This shift was drastically increased by plantlets grown on metal spiked media. With the exception of Cu-EDTA 100 mM and $ZnSO_4$ 5 mM, all tested concentrations showed the shift towards protonema production. Furthermore, this shift was strengthened by rising metal concentrations. While increased protonema in zinc-plates correlated with the extractable and the total zinc content, the correlation with the total copper was negative to protonema growth. This is in contrast to the positive correlation of the observed protonema shift to the calculated free Cu^{2+}. The uppermost extremes of the shift towards protonema development were Zn-EDTA 10 mM ($39.12/60.88 \pm 2.41^{***}$), Zn-EDTA 100 mM ($5.67/94.33 \pm 1.27^{***}$), $CdCl_2$ 5 μM ($28.31/71.69 \pm 2.01^{***}$) and $CuSO_4$ 1 mM ($2.94/97.06 \pm 1.44^{***}$; week 4), showing a strong to almost complete shift towards protonema. Furthermore, a significant shift towards increasing protonema was observed for $CdCl_2$ 0.1 μM, $CdCl_2$ 1 μM, Zn-EDTA 1 mM, $ZnSO_4$, $ZnCl_2$, $CuCl_2$ and $CuSO_4$ 100 μM (Fig. 3).

3.2.3. Metal availability

Like nutrients, metals need to be available to the plant to influence its metabolism. To determine the availability and retention of metals in the media, we compared total added metal concentrations with experimentally determined metals extracted from the substratum with water, and total soluble and free metal ions as calculated with the modeling program MINTEQ for estimation of equilibrium state concentrations (Fig. 4).

Solubility data confirmed that ionic salts of copper and zinc differed in their water extractability. While $ZnSO_4$ and $ZnCl_2$ showed a high solubility of zinc (68% and 56% respectively; both at 0.1 mM), copper was far less soluble ($CuSO_4$ 0.1 mM: 17%; $CuCl_2$ 0.1 mM: 21%). Lower metal concentrations resulted in an increased portion of solubility metal ions, both for copper and zinc and for Cl^- and SO_4^{2-} (Fig. 4).

Only a fraction of all soluble metal ions occurred as free cations (Fig. 4). In the case of $ZnSO_4$ and $ZnCl_2$, calculations of free Zn^{2+} resemble quite closely the values of extractability. A bigger difference was calculated between total soluble copper and free Cu^{2+} in the case of $CuSO_4$ and $CuCl_2$. The situation of cadmium was similar to zinc (cadmium data not shown). In all these setups, most soluble metals where coordinated by HPO_4^{2-}, NO_3^- or HSO_4^- – i.e., by constituents of the nutrient medium. In the case of EDTA chelates, however, soluble EDTA complexes counted for 99% whereas free metal concentrations where less than 0.28% of the calculated soluble metals (Fig. 4).

If growth is compared to the originally added, total metal concentrations (Total Metal), negative correlations of protonema growth with copper concentrations and of gametophyte growth with zinc concentrations are found. Interestingly, gametophyte growth correlated positively with copper concentrations and protonema growth with rising zinc concentrations (Fig. 5a and b). Those correlations did not account for solubility, possible precipitations or other interactions with the media nor the chelation of the added metals.

A comparison of water extractability and plant growth showed that all zinc concentrations correlated well with the reduced growth of the gametophyte (-0.37^{**}) and increased protonemal growth (0.4^{**}; Fig. 5d). Calculated zinc solubility showed very similar correlations with plant growth as measured zinc extractability. Concerning copper, the difference between calculated and measured solubility was much larger; our calculations indicate the precipitation of copper mainly as $Cu_3(PO_4)_2$ (Fig. 4). Here, significant correlations between plant growth and solubility where only found for calculated but not for measured soluble copper, in both gametophyte and protonema (Fig. 5c and d). Free metal ions (Cu^{2+}; Zn^{2+}; Cd^{2+}) were highly negatively correlated with gametophyte growth (-0.53^{**}; -0.46^{**}; -0.57^{**}). Protonema growth also correlated negatively with increasing free Zn^{2+} concentrations (-0.27^{*}; Fig. 5h). However, protonema growth showed no significant correlation with free Cu^{2+} (Fig. 5g) and free Cd^{2+} (data not shown).

In summary, rising total metal concentrations in the media led to rising free metal cations. A general reduction of relative metal availability depending on the metal concentration and anion was found as well. The difference of plant available metal as free metal ions is smaller than what the added media concentrations would suggest.

4. Discussion

4.1. Excessive metals affect growth and G:P-ratio

In *P. patens* two principal reactions towards metal stress were observed in all treatments: reduced biomass formation and a shift to protonema formation. Growth reduction is a widespread reaction towards excess metal concentrations but may differ extremely according to the bryophyte species, the element and its application. (reviewed by Glime, 2007; Tyler, 1990; Chopra and Kumra, 1988). In our experiments, the growth reduction was strongly related to the used anion (Fig. 2). However, within each test series, copper proved to be more toxic than zinc, and cadmium toxicity drastically exceeded both. However, cadmium caused basically the same effects as zinc and copper at much higher concentrations; it can be concluded that the observed effects are due to the toxicity of the metal and not of the anion. The comparatively low toxicity of zinc seems to be a common feature in bryophytes whereas for copper and cadmium a similar toxicity has been frequently reported (reviewed by Tyler, 1990). Thus, *P. patens* seems to exhibit a surpassing tolerance to copper as already reported earlier (Sassmann et al., 2010).

We determined the ratio of gametophyte to protonema growth for all metal concentrations and found highly significant changes of the G:P-ratio (Fig. 3). The increase in protonema corresponded to rising metal addition in 17 of 20 treatments (Sup. 7). The increased percentage of protonema may be due to an inhibition of bud formation by the metals, as observed in *Timmiella anomala* (Kapur and Chopra, 1989). Low concentrations of metals did not negatively affect but even stimulated the growth of the protonema and growth stopped only near the tolerance limit. The promotion of protonema

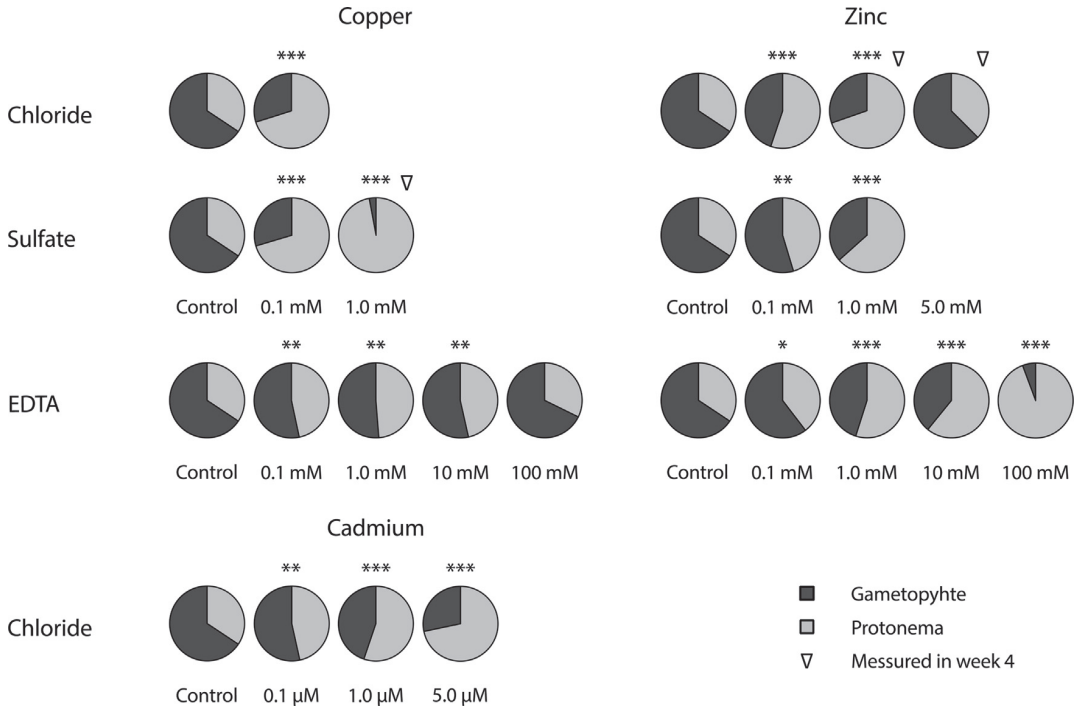

Fig. 3. Pie charts showing the amount of gametophyte (dark grey) and protonema (light grey) on solid media containing copper, zinc and cadmium displayed as a G:P-ratio. Apart from Cu-EDTA 100 mM, all other concentrations show a shift from leafy gametophyte to protonema after 5 weeks (for more detail see Sup. 6).

development up to a certain stress level may be a general feature in bryophytes (Shaw, 1990) and a similar stimulating effect of low metal concentrations was observed for *Ceratodon purpureus* protonema (Jules and Shaw, 1994). An increased protonema growth at high copper concentrations is also found in the highly specialized copper moss *S. cataractae*, where copper is accumulated in cell wall pectins of protonemata (Konno et al., 2010; Nomura and Hasezawa, 2011). Remarkably and contrary to *P. patens*, in those studies no promoted protonemal growth was found for other transition metals (e.g. Zn^{2+}) in *S. cataractae*.

The stimulating effect of sub-toxic metal concentrations can be explained in the case of the essential micronutrients zinc and copper since plants possess homeostatic mechanisms for these elements (Krämer, 2005; Sinclair and Kraemer, 2012). The non-essential cadmium, however, showed the same effect, though at a much lower concentration, both in the present study and in *Marchantia polymorpha* (Lepp and Roberts, 1977). These authors suggest that low concentrations of cadmium may interact with the micronutrient zinc and thereby increase respiration.

Though EDTA chelated metals proved to be rather harmless for *P. patens*, the G:P-ratio was reduced even if total growth was not affected. Here, a possible effect of the EDTA anion at concentrations of 100 mM has to be considered as well: calcium readily forms EDTA chelates and has shown to be essential for bud formation in the moss *Stereophyllum radiculosum* (Olarinmoye et al., 1981). Thus, our results show that the G:P-ratio is a useful tool to determine stress in *P. patens*, especially at stress levels where growth rates are not yet significantly reduced. Similar observations in other species suggest that this tool may be used in other bryophytes as well.

4.2. Metal toxicity is crucially influenced by the anion

Early studies on metal toxicity in plants already observed an influence of the anion, which, however, were not the same for each metal (Kaho, 1933): in *Brassica oleracea* e.g. zinc toxicity decreased in the order $Br^- > Cl^- > NO_3^- > C_2H_3O_2^- > SO_4^{2-}$, cadmium toxicity on the other hand decreased in the order $NO_3^- > Cl^- = C_2H_3O_2^- > Br^- > SO_4^{2-}$. Nonetheless, numerous studies still used combinations of e.g. $CuSO_4$ and $ZnCl_2$ when quantifying cation toxicity (Shaw, 1988; Brune et al., 1995).

In *P. patens*, growth inhibition of metals added as sulfate and chloride did not differ significantly but metals added as EDTA chelates were tolerated in approximately 1000 × higher concentrations. This is in good accordance with data on gemmae of *M. polymorpha* surviving Cu-EDTA concentrations 500 times higher compared to ionic salts (Coombes and Lepp, 1974). Thus, toxicity for *P. patens* on Benecke growth media can be summarized as $Cd > Cu > Zn$ and $SO_4^{2-} = Cl^- > EDTA^{4-}$, which correspond well with data of other moss species (Coombes and Lepp, 1974; Tyler, 1990), and with data on short term tolerance in *P. patens* (Table 1).

The EDTA anion is of specific interest as it does not only balance charges but also chelates the cation thereby increasing its solubility. Therefore, EDTA and other chelating agents are used to enhance metal solubility, e.g. in fertilizers or hydroponic nutrient solutions. At physiological concentrations, the volume yield did not differ between fertilization using Cu- and Zn-EDTA and ionic salts, respectively, as reported for lettuce by Kozik et al. (2012). The use of EDTA in bioremediation is controversially discussed as well, because it mobilizes metals (Sun et al., 2001) and therefore may drastically increase the leakage into the ground water. The increase of metal solubility is widely acknowledged but the uptake into plants is sometimes enhanced (Evangelou et al., 2007) and sometimes inhibited (Custos et al., 2014).

4.3. Metal extractability in the media is controlled by precipitation rather than adsorption

Overall solubility of zinc was slightly but highly significantly (paired *T*-test) higher than predicted by modeling with MINTEQ, though the observed and the calculated values correlated very

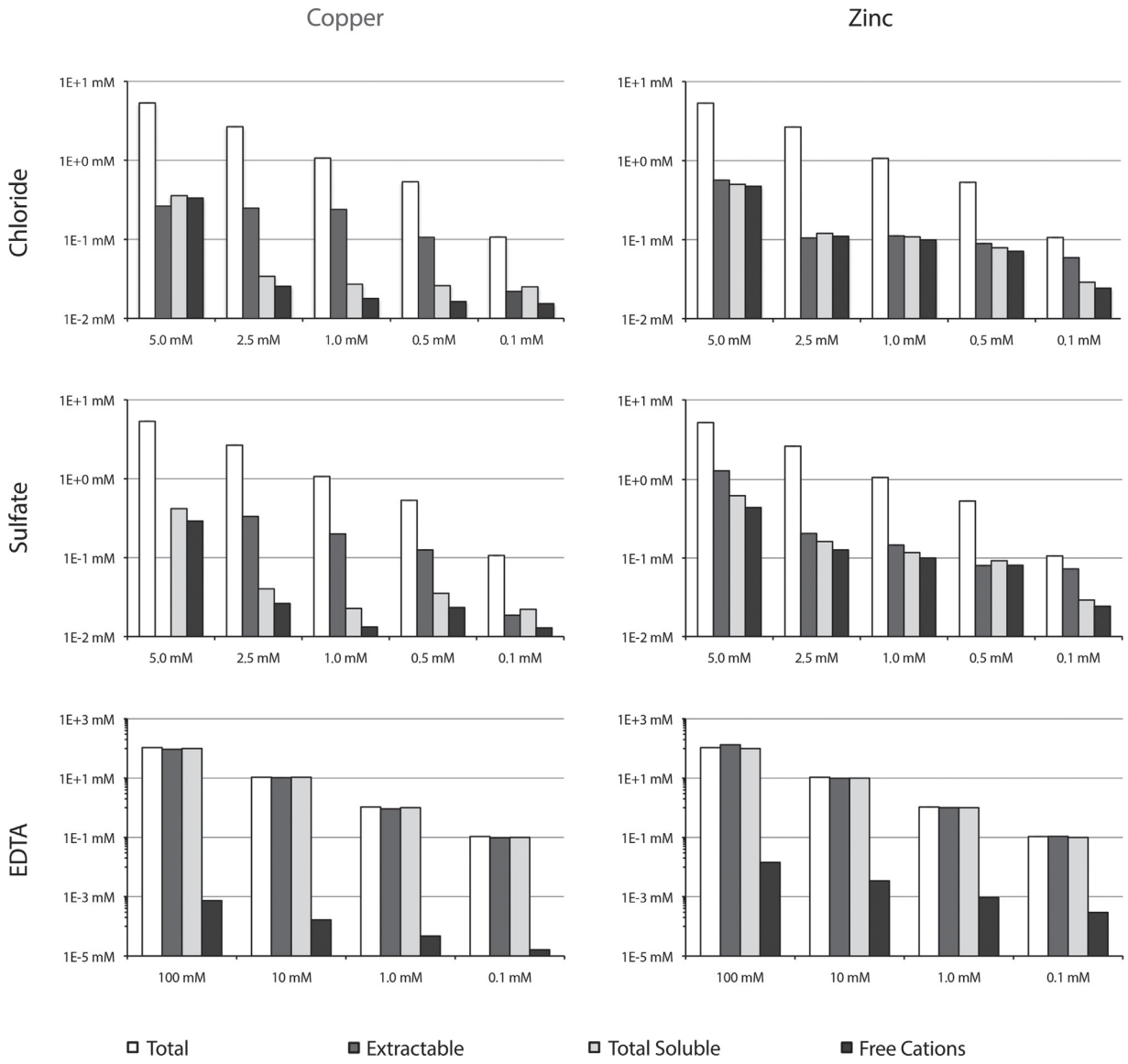

Fig. 4. Comparison of total added copper and zinc, respectively, to growth media (Total), its measured water extractability after 24 h (Extractable), the calculated total soluble metal ions (Total Soluble) as well as the calculated free Cu^{2+} and Zn^{2+} ions (Free Cations).

well ($R^2 = 0.997^{**}$). In the case of copper, MINTEQ drastically and highly significantly underestimated the water solubility, and the correlation between observed and calculated values was poorer ($R^2 = 0.353^{**}$). This difference can be explained by the addition of a surplus of water during the extraction process which dissolved some precipitates that had been insoluble in the medium. According to the model, most of the loss can be explained by precipitation of $Cu_3(PO_4)_2$ and $Zn_3(PO_4)_2{\cdot}4H_2O$, respectively, caused by the interaction with the nutrient solution where phosphates are indispensable for long term experiments. However, sufficient amounts of metals stay soluble in order to cause toxic effects. The calculation of the solubility enables the correction for the influence of the phosphate, thus overcoming the problems pointed out by Twiss et al. (2001).

Agar has been reported to immobilise metals as well. Gupta et al. (2008) found strong adsorption of manganese and cobalt to a 10% agar–agar matrix (85% after 30 min) but at concentrations comparable to our media (0.8%), adsorption proofed to be negligible. Lazaro et al. (2003) reported the binding of up to 25% of the offered nickel to agar plates; however, 17% were explained by diffusion into the agar matrix, and not by adsorption. Pandey et al. (2009), used lead and agarose and found that more than 50% of the added lead were to be adsorbed by the 1–3% agarose gel. Lead is extraordinarily strongly bound by algal cell walls (Yu et al., 1999) and for experiments using this metal, another substrate than agar should be used.

In our study total solubility (Fig. 4) of the metals was 5–23% for copper and 4–68% for zinc ionic salts, the correlation with calculated solubilities indicates that precipitation is more relevant than adsorption by agar in nutrient media which is in good accordance with the low metal adsorption of highly diluted agar. For EDTA chelates, virtually complete solubility of the metals was observed, showing that $EDTA^{4-}$ obviously prevented both precipitation and adsorption.

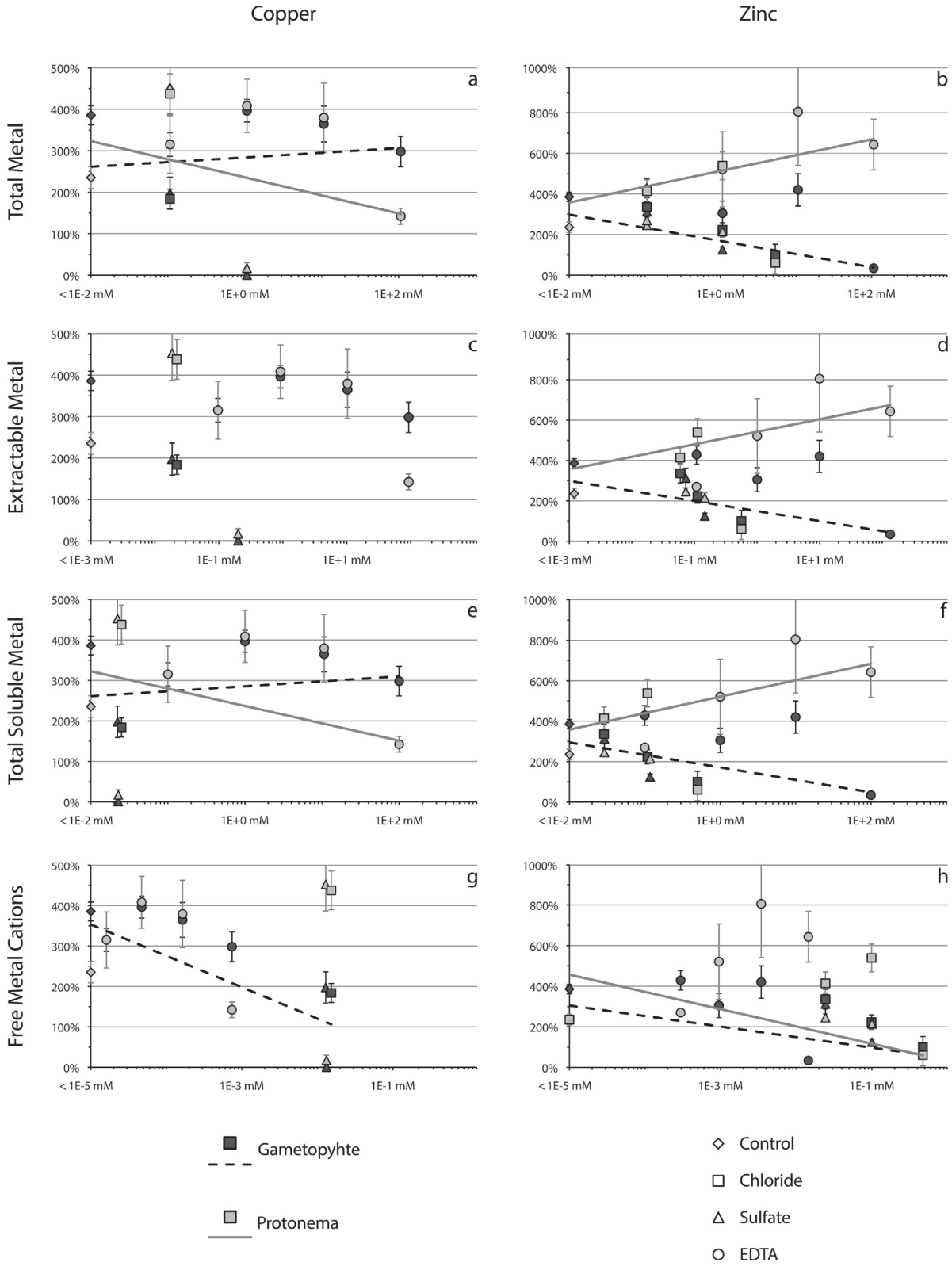

Fig. 5. Copper (a, c, e and g) and zinc (b, d, f and h) concentrations (Total Metal, Extractable Metal, Total Soluble Metal and Free Metal Cations) in correlation to gametophyte and protonema growth. *X*-axes show the logarithmic concentration of the metal fraction mentioned on the left; *Y*-axes show the biomass after 5 weeks. (a)–(f) show similar correlation of metal content to plant growth: more metal caused less protonema production in increasing copper concentrations (a, e), except for c where no significant correlation could be found, and protonema production correlated positively with increasing zinc concentrations (b, d, f), gametophyte growth behaved contrarily. (g)–(h) Plant growth is negatively correlated to free metal cations for both protonema and gametophyte.

4.4. Free metal cations are most relevant for availability

The anions used in this study, SO_4^{2-}, Cl^- and $EDTA^{4-}$, do not exhibit significant plant toxicity by themselves at the concentrations used. Therefore, we test the hypothesis that anions influence the toxicity of metals by forming complexes of different strengths. Our results (Fig. 5) show that plant growth correlates only poorly with total, experimentally extracted or calculated total soluble metals. Frequently, even improved growth was found at higher total, extractable or calculated soluble metal concentrations, especially in the case of EDTA chelated metals. A clear correlation, however, was found between calculated free metal ions and growth inhibition. The low toxicity of EDTA chelated metals is therefore easily explained by the negligible concentration of free metal cations in EDTA solutions. This observation is in good accordance with data on organisms other than mosses reviewed by Twiss et al. (2001). This review confirmed that total metal concentrations in aqueous solutions are not a good indicator of "bioavailability".

These results question the widespread use of water or neutral salt extracts of metal rich soils in order to estimate metal availability (Meers et al., 2005; Narwal et al., 1999). Furthermore, the extremely low availability of EDTA chelates should be taken into consideration if EDTA is used in bioremediation activities. Though the addition of EDTA usually increases the solubility of metals in the soil and the uptake into plants (Evangelou et al., 2007), other authors report reduced uptake (Custos et al., 2014) or even found indications for metal deficiency, as in the hyperaccumulator *Noccaea caerulescens* (Syn: *Thlaspi caerulescens*) grown on Zn-EDTA (Gerstmann, 2010). Thus, the capability to break EDTA chelates seems to be species dependent and should be tested before the use of EDTA in bioremediation is considered for a certain habitat.

5. Conclusions

The results presented here allow the following statements on the hypotheses formulated above:

1. Neither the total nor the soluble metals allow for a good estimate of the toxicity. The calculated free metal cations, on the other hand, are in good accordance with the growth response of *P. patens*.
2. The anion crucially influences the toxicity of a metal, however, not by its own toxicity but by its ability to form chelates which do not make the metal available for the plant.
3. Metal stress leads to a reduced growth of the gametophyte and, at higher concentrations, also of the protonema. The G:P-ratio shows a significant decrease even at concentrations where total growth is not affected, yet. The G:P-ratio therefore serves as a sensitive indicator for metal stress in *P. patens*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.envexpbot.2014.08.010.

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C. *Zinc and Copper Uptake in* Physcomitrella patens*: Limitations and Effects on Growth and Morphology*

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Zinc and copper uptake in *Physcomitrella patens*: Limitations and effects on growth and morphology

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ABSTRACT

Bryophytes are well-studied model systems for the investigation of uptake mechanisms and pollutant tolerance of plants. The moss *Physcomitrella patens* is among the most intensively studied organisms with regard to physiological and cellular pathways; however, direct ecological studies on metal stress are rare in this species. We conducted growth experiments on copper and zinc containing media for five and ten weeks, respectively in order to investigate the element and anion dependent metal uptake by energy–dispersive X-ray spectroscopy on a scanning electron microscope. We compared the uptake of metals to the growth and morphology of leaves and protonemata of *P. patens*. The metal content of both tissues was strongly correlated with availability of free metal cations. Differences between copper and zinc uptake included the limitation of zinc uptake found after five weeks for both tissues. No further sequestration of metal occurred after week five. The content of up to 1.3 wt% zinc and 0.4 wt% copper in the dry biomass correlated negatively to leafy gametophyte growth and to cell length in both tissues. No such correlation to protonema growth or cell width was found. The shortening of protonema cells exposed to zinc did not necessarily lead to decreased protonemal growth. This indicates a compensation of the shorter cells by increased cell division of the protonema filaments.

1. Introduction

Research on the impact of metals and bryophytes has come a long way together. Metal contaminated habitats have always been colonized by certain moss and liverwort species regardless of whether naturally occurring or man-made (Adlassnig et al., 2013; Baumbach and Schubert, 2008). Many studies investigated the toxicity of metals and the tolerance of plants but differences in tolerance are always dependent on plant strategy (Baker, 1981), species and metal. In bryophytes, Tyler (1990) reported decreasing toxicity for Hg > Cu > Cd > Pb > Zn, regarding zinc as the least harmful of the investigated metals.

Unlike flowering plants, mosses use rhizoids for surface adhesion and do not possess a root system for nutrient uptake or metal avoidance. Therefore, the avoidance via e.g., the casparian strip is impossible. In fact, mosses take up nutrients by surface adsorption—hence also toxic metals. This characteristic led to their widespread use as bioindicators and in biomonitoring of aerial metal deposition (Frahm, 1998; Spagnuolo et al., 2011; Uyar et al., 2009). However, suitable candidates for bioindication or biomonitoring need additional requirements. To enable estimates on environmental metal concentrations, the organism has to show a linear correlation in uptake and conservation of the level of metal-uptake. Therefore, the organism should not actively accumulate, avoid or segregate them.

To further complicate the matter, uptake of metals depends both on the plant and metal species, the availability of the metal in the substrate, and it further may be limited by a physiological maximum within the plant. Such an upper limit would lead to an underestimation of high metal concentrations in the environment (Boquete et al., 2011). Therefore, experiments under controlled conditions are necessary to investigate metal uptake behavior of a single species. In this study, we analyzed the metal uptake and its effects on cell morphology in the protonemata and leaves of the well-established model moss *Physcomitrella patens* (Cove and Knight, 1993). As *P. patens* naturally inhabits non-metal sites, knowledge about metal uptake in *P. patens* is limited. However, in a previous study on metal tolerance and its influence on growth, *P.*

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patens showed a remarkable tolerance toward zinc and copper. A shift to increased protonema formation could be observed suggesting that these cells could superiorly handle the metal induced stress (Sassmann et al., 2015). Growth reduction and increased protonema formation were best explained by free metal cation concentrations but questions remained concerning metal uptake and changes in metal content of this important model organism. Here, we aim to investigate the following hypotheses (H1 – H4):

- There is a limited uptake capacity, resulting in a saturation curve, regardless of the offered concentrations. As copper is considered more toxic to mosses than zinc, *P. patens* will endure lower copper concentrations and use its homeostasis mechanisms to avoid uptake of copper rather than zinc, resulting in lower overall concentration of copper in the organism (H1).
- As a non-metal adapted plant, *P. patens* cannot segregate metals after the uptake and will therefore store them in a concentration dependent manner. Neither accumulation nor segregation will occur over time (H2).
- *P. patens* showed augmented protonema growth under metal stress. Protonema cells are structurally different to the leafy gametophor, suggesting differences in metal uptake. We postulate higher metal tolerance, and significantly higher metal concentrations in the protonema tissue (H3).
- Growth reduction and morphological changes are best explained by metal uptake. As chelated metals are likely to be unavailable for *P. patens*, we postulate that uptake itself is therefore best explained by the concentrations of free metal cations in the media (H4).

To test these hypotheses, we analyzed the relative metal content using energy dispersive X-ray spectroscopy on a scanning electron microscope. This technique allowed to measure differences in the metal accumulation between protonema and leaf tissues. In addition, we investigated the influence of metal bioavailability and the effect of elevated metal levels. The data were compared to growth parameters and morphological changes (i.e., cell size and shape).

2. Material and methods

2.1. Plant material and cultivation

To investigate metal uptake behavior of a non-specialized moss, we chose *P. patens* (Hedw. [Lind.] *Funariaceae*; Fig. 1), a model organism to explore plant physiology (Cove, 2000; Reski and Cove, 2004). *P. patens* gametophytes are formed by protonemata (Fig. 1a) consisting of single cell filaments referred to as “protonema”, and the leafy moss plant (Fig. 1b,c; referred to as “gametophor”) well

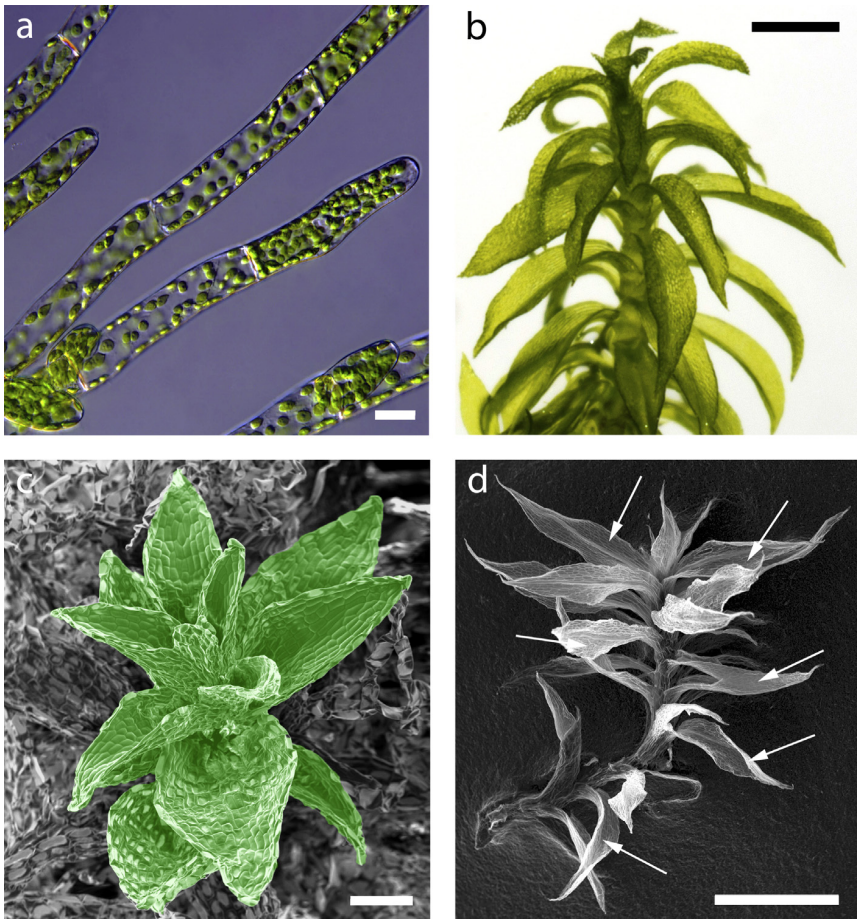

Fig. 1. (a) Differential interference contrast image of filamentous chloronema cells; (b) *P. patens* leafy gametophor; (c) colored SEM micrograph showing a young leafy gametophor (green); (d) example of SEM–EDX measurement sites on leaves (arrows); scale bar: *a* = 25 μM; *b,c* and *d* = 1 cm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Metal concentrations of media and leaf/protonema sample size. Measurements were performed after 5 weeks. [+]Additional measurements of leaves were taken after 10 weeks. [++]Effective added concentration due to the mean water loss of 6.5% during sterilization of the media, –, not tested, control measurements in leaves (80) and protonemata (31), in total 1634 measurements.

Media (mM)	Media[++] (mM)	$CuCl_2$	$CuSO_4$	$CuNa_2EDTA$	$ZnCl_2$	$ZnSO_4$	$ZnNa_2EDTA$
0.1	0.107	46[+]/28	30[+]/22	50[+]/20	83[+]/20	36[+]/20	58/16
0.5	0.534	16/–	–	–	34/26	–/22	–
1.0	1.065	20/–	–	59[+]/16	39[+]/33	32[+]/31	64/23
2.5	2.663	–	–	–	31/10	–	–
5.0	5.325	–	–	–	33/41	28/18	–
10.0	10.650	–	–	53[+]/19	–	–	90[+]/26
100.0	106.500	–	–	64/42	–	–	60/55

aware that both structures belong to the gametophyte (Vidali and Bezanilla, 2012).

Plants were grown in aseptic culture on Benecke media containing 0.8% agar at 19–21 °C under a 14 h day/10 h night regime over periods of five and ten weeks, respectively, for details compare Sassmann et al. (2010). Table 1 shows the offered metal concentrations and the respective anion (chloride, sulfate and ethylenediaminetetraacetic acid (EDTA)-chelates). The samples were grown on cellophane discs placed on the surface of the semisolid medium to facilitate harvest and prevent media adhesion. A maximum of 5 measurements per plantlet, at least 6 plants from different petri dishes and at least two experimental repetitions were performed to incorporate the biological variation of the moss plants, further details are shown in Table 1.

2.2. Morphological parameters

Morphological parameters were analyzed in a Leica DM 6000CS microscope by measuring cell lengths and widths. For leaf cells, we chose mid leaf cells apart from the mid rib due to their relative uniformity. The protonemata of *P. patens* consist of chloronemata and caulonemata which differ in lengths as well as in other parameters (Vidali and Bezanilla, 2012). Hence, we measured the chloronema cells as the protonemata were mostly composed of this cell type. Multiply branched cells were avoided and at least 100 cells per treatment were measured using Leica LAS AF software v.2.6.3 (Leica Microsystems, Germany). Images were processed and colored (Fig. 1c) using Adobe® Photoshop® CS4 extended and CS5 (Adobe Systems, USA).

Growth data had been obtained in a previous study using the same experimental setup (Sassmann et al., 2015). To investigate the impact of metal uptake on those morphological parameters, data on growth and metal availability from that study were correlated to metal content in tissue.

2.3. Analysis of metal uptake

Metal uptake was measured on an EDAX system (Ametek GmbH, Germany) connected to a Philips XL 20 scanning electron microscope (SEM) performing energy-dispersive X-ray microanalysis (EDX) (Echlin, 2011; Warley, 1997). This system allowed the detection of tissue specific changes in the element composition of *P. patens* protonema and gametophor (Fig. 1c,d; respectively). Prior to analysis, the plantlets were harvested carefully to avoid contamination by the growth media. To foreclose unbound metal cations and the adhesion of media, the samples were rinsed three times with *Aqua bidest*.

Fresh samples were mounted on 0.5" aluminum specimen stubs equipped with SEM-carbon foils (PELCO Tabs™ Carbon Conductive Tabs, Double Coated, Christine Gröpl, Austria). Subsequently, the mosses were air dried at 40 °C for 24 h and carbon coated with a 5–10 nm carbon layer (Leica, personal communication; Carbon coater EPA 101) to prevent surface charge.

Data collection, background subtraction and element specific spectra analyses were performed using the EDAX-Genesis software Version 5.11 (Ametek Material Analysis, USA). For deconvolution of the spectra, corrections for interference between elements were applied according to the software. Microanalyses were performed with the following constant SEM settings: acceleration voltage of 30 kV, working distance of 12 mm (sample to final lens); tilt 15°, take-off angle 27.21°, dead time ~30 percent and measurement time of 100 s (Lsec 100) for each analysis. Multiple measurements per plant were taken (Fig. 1d). Well aware that biological samples show an uneven surface and texture, all element analyses were performed at a magnification of 2500× for leaf and protonema cells with attention to their orientation. Thereby, a possible blur of the measurements could be minimized and was tested with no significance. Details of concentrations and amount of measurements are shown in Table 1.

The penetration depth of the electron beam was estimated by a Monte Carlo simulation using CASINO software version 2.4.8.1 (Drouin et al., 2007). A mean density of the cells of 0.4 g* cm^{-3} was estimated based on a mean density 1.05 g cm^{-3} for fresh moss cells (Schwuchow et al., 2000), a dry weight content of 7% determined experimentally and shrinkage of 70–80% determined microscopically. The major elemental composition of the samples was determined in a preliminary experiment; these elements were considered for the simulation.

The Monte Carlo simulation showed that electron energy was at 50% at a penetration depth of 10 μM and 5% at 35 μm depth. Excitation maxima for detectable X-rays is element dependent e.g., 15 μM for zinc (ZnK). Consequently, we avoided the measurement of tissue in direct contact to the underlying carbon carrier to avoid any possible bias.

As EDX is a semiquantitative method and heavy metal content is detected in relation to all other elements, only the following elements were measured: C, N, O, Na, Mg, Al, Si, P, S, Cl, Cd, K, Ca, Fe, Ni, Cu, and Zn. Since carbon is a major component of the cell wall which can vary due to metal exposure it is part of the measured elements. We are aware that a steady but minor part of the carbon originated from the carbon coating. All concentrations are shown as weight percent of the dry mass (wt%).

2.4. Statistics

Samples were tested for normality (Kolmogorov–Smirnoff-test, Skewness–Kurtosis test) and homogeneity of variances (Levee-nes's-test). Differences in treatments and time point were investigated by analyses of variances (Anova) and respective post-hoc tests (Tukey's-honest significant difference (HSD) for parametric data; Kruskal–Wallis-test for non-parametric data). As tests for normality revealed non normal distribution of data we performed an arcsinus transformation. Then the results of nonparametric *U*-test were confirmed by parametric tests (not shown) if normal distribution was given which was not always the case. *U*-test was used for all samples. Positive and negative

correlations of element uptake were examined by Spearman test (as data was non-normally distributed). If not specified otherwise, values are given in the arithmetic mean ± standard deviation (SD). Significant differences are labelled with: *** for $P < 0.001$, ** for $P < 0.01$ and * for $P < 0.05$. All statistic tests were performed using IBM SPSS Statistics software V.20.

3. Results

3.1. Metal uptake differed for zinc and copper

Using EDX, we measured copper and zinc content in *P. patens* leaves and protonemata grown on solid media. The copper content of leaves (Fig. 2a–c) ranged from 0.01 (±0.03%; Cu-EDTA 100 μM) to 0.36 (±0.25%; $CuCl_2$ 1 mM) and in the protonemata (Fig. 2a–c; checkered) from 0.07 (±0.04%; Cu-EDTA 1 mM) to 0.31 (±0.19; $CuCl_2$ 0.1 mM). Plants grown on media containing more than 1 mM $CuCl_2$ or $CuSO_4$ did not survive five weeks.

In mosses grown on zinc containing media, we measured a zinc content (Fig. 2d–f) of 0.02 (±0.03%; Zn-EDTA 1 mM) up to a content of 1.02 (±0.61%; $ZnSO_4$ 5 mM) in leaves and in the protonemata (Fig. 2d–f; checkered) 0.07 (±0.06: Zn-EDTA 100 μM) up to 1.35 (±1%; $ZnCl_2$ 5 mM). *P. patens* could not tolerate media concentrations above 5 mM $ZnCl_2$ or $ZnSO_4$ and stopped growing.

Fig. 2e clearly shows a plateau of the zinc content in both tissues of *P. patens*, regardless of the added $ZnCl_2$ concentration ranging from 0.5 to 5 mM. Mosses grown on $ZnSO_4$ did not show such a plateau but similar maximum zinc levels were found in leaves and protonemata at an added media concentration of 5 mM (Fig. 2d).

In general we measured remarkable differences of metal content depending on the metal cation. Plants of *P. patens* grown on copper containing media showed a lower copper uptake compared to the zinc in plants grown on zinc media with the same added anion concentrations. The only exception was $CuCl_2$ 100 μM which resulted in a higher metal content of the plants than $ZnCl_2$ 100 μM. Zinc could be detected up to ~1.3 wt% in leaves and protonemata, copper levels reached a peak at ~0.35 wt% for leaves and protonemata, respectively.

3.2. Reduced metal uptake of EDTA-chelates

The comparison between chlorides, sulfates and EDTA chelates showed a clear influence of the anion on metal uptake. Within one concentration, we found considerably higher levels of uptake for chlorides and sulfates compared to EDTA-chelates both for copper and zinc, and both for leaves and protonemata (Fig. 2a–f). EDTA

Table 2
Comparison of the different metal species tested. The mean metal uptake of *P. patens* shows significantly lower uptake in leaves and protonemata when testing chlorides and sulfates to the respective EDTA concentrations (Mann-Whitney-U-Test; * indicate significance). Even tenfold higher concentrations of Cu- and Zn-EDTA result in lower metal content in *P. patens* tissues when compared to chlorides or sulfates.

Media	Leaves		Protonema	
	Mean (wt%)	SD	Mean (wt%)	SD
Cu-EDTA 100 μM	0.01	0.03	0.09	0.09
$CuCl_2$ 100 μM	0.11***	0.09	0.31***	0.19
$CuSO_4$ 100 μM	0.10***	0.03	0.26***	0.13
Cu-EDTA 10 mM	0.04	0.05	0.07	0.03
Zn-EDTA 100 μM	0.02	0.03	0.07	0.06
$ZnCl_2$ 100 μM	0.04***	0.04	0.17**	0.12
$ZnSO_4$ 100 μM	0.16***	0.13	0.18	0.08
Zn-EDTA 1 mM	0.02	0.03	0.12	0.06
$ZnCl_2$ 1 mM	0.78***	0.57	0.65***	0.69
$ZnSO_4$ 1 mM	0.38***	0.32	0.88***	0.40
Zn-EDTA 10 mM	0.09	0.08	0.60	1.34

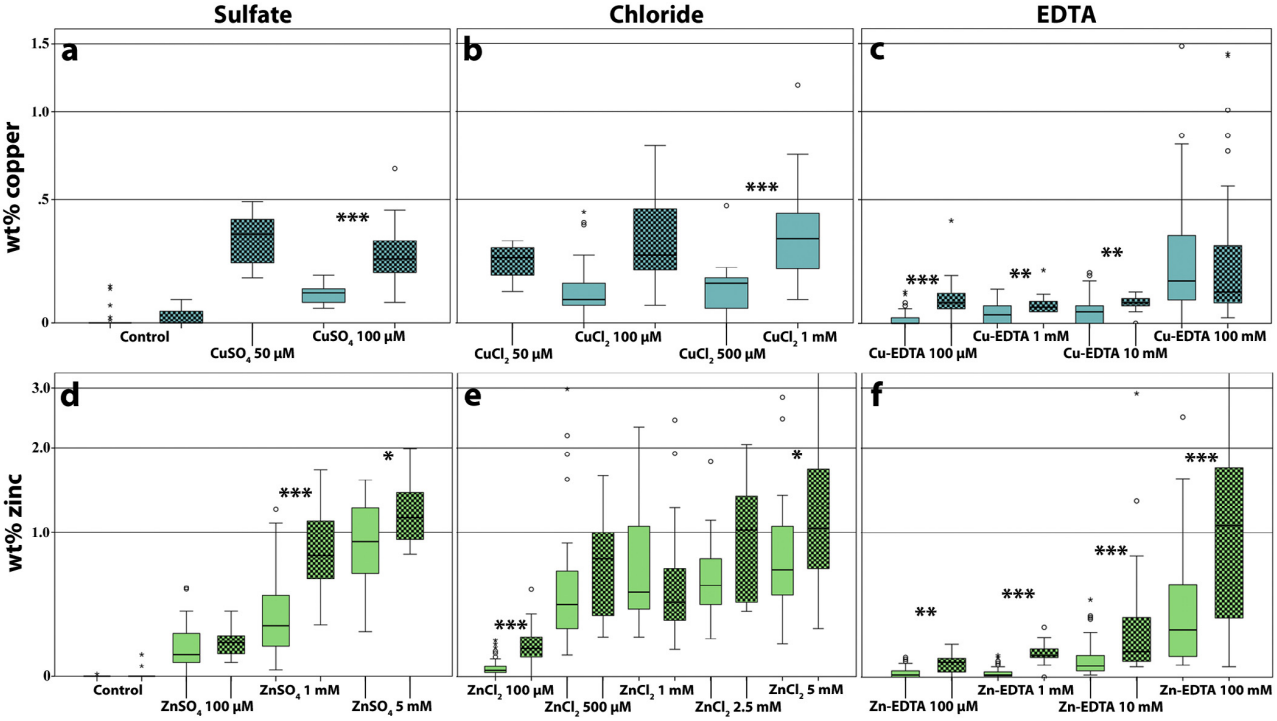

Fig. 2. Boxplots of zinc and copper uptake in *P. patens* leaves and protonemata (checkered); (a) copper content of control and of plants grown on $CuSO_4$; (b) copper content of mosses grown on $CuCl_2$; (c) detected copper content of plants originating from media containing Cu-EDTA; (d) zinc content of control and mosses from media containing $ZnSO_4$; (e) zinc content of *P. patens* grown on $ZnCl_2$ containing media, note the plateau; (f) uptake of the moss grown on Zn-EDTA containing media; wt% in a logarithmic scale; * indicates significant differences of leaves to protonemata. Note the different axis scaling for copper and zinc as the content differed widely for the two metals.

concentrations showed a significantly lower uptake. The metal content of leaves and protonemata grown on media with metal addition as chloride and sulfates could not be reached even by a tenfold EDTA-chelate concentration (Table 2; Supplementary Fig. 1).

3.3. Metal uptake increased over time

A possible sequestration of metals in *P. patens* was investigated by additional analyses of leaves from selected concentrations over a period of 10 weeks. Moss leaves from 100 μM $ZnCl_2$ and 10 mM Zn-EDTA media showed increased metal values after 10 weeks. However, leaves from media containing 1 mM $ZnCl_2$, 100 μM $ZnS0_4$ and 1 mM $ZnSO_4$ showed no significant differences after 10 weeks when compared to five weeks of metal exposure (Table 3).

Similar results were obtained for copper uptake. Plants from $CuCl_2$ and Cu-EDTA (100 μM) demonstrated a significant increase of copper content after 10 weeks. However, no significant increase of the copper content could be observed for samples from 1 mM Cu-EDTA and 10 mM Cu-EDTA plates. Interestingly, leaves from $CuSO_4$ (100 μM) showed a highly significant decline in copper content from 5 to 10 weeks. Apart from this exception, the prolonged metal exposure of 10 weeks showed a general increase in metal content on low media concentrations (Table 3; Supplementary Fig. 2).

Overall, our results indicated that metal segregation or sequestration and therefore a time dependant reduction of the metal content does not play an important role in *P. patens*.

3.4. Protonema cells contained more metal than leaf cells

When grown at the same concentrations, metal contents of protonema cells were higher than of leaf cells. This is particularly true for most of the lower sulfate, and chloride concentrations and generally for EDTA. The exceptions 100 μM $ZnSO_4$ and 100 mM Cu-EDTA showed the same metal content in leaves and protonemata (Table 4).

Plants from $ZnCl_2$ in concentrations above 500 μM did not show an increased metal content in protonemata when compared to leaf cells and exhibited high, but very similar values regardless of the added media concentrations. In general, most of the protonemata showed a higher metal content than leaves with the same differences for the copper or zinc content, respectively (see also Fig. 2).

3.5. Correlations of metal uptake to soluble cations and morphology

Metal content in tissue (EDX-data) was correlated to the added media concentrations and the calculated free metal ions (Cu^{2+} and Zn^{2+}). We did not find significant correlations between the metal content of the tissues and the media, neither for copper nor for zinc. However, the correlation of the metal content in leaves and protonemata to the calculated free metal ions was significant for both copper (Fig. 3a) and zinc (Fig. 3b).

Table 3
Display of the metal content in leaves after five and ten weeks of metal exposure and respective differences. Significance was tested with Mann-Whitney-U-Test and is indicated by *.

Media	5 weeks		10 weeks		*p*-value
	Mean (wt%)	SD	Mean (wt%)	SD	
Cu-EDTA 100 μM	0.01	0.03	0.03*	0.01	0.015
$CuCl_2$ 100 μM	0.11	0.09	0.16**	0.05	0.002
$CuSO_4$ 100 μM	0.10	0.03	0.05***	0.02	0.000
Cu-EDTA 1 mM	0.03	0.04	0.05	0.02	0.178
Cu-EDTA 10 mM	0.04	0.05	0.05	0.01	0.252
$ZnCl_2$ 100 μM	0.04	0.03	0.26***	0.10	0.000
$ZnSO_4$ 100 μM	0.16	0.13	0.11	0.05	0.681
$ZnCl_2$ 1 mM	0.78	0.04	0.48	0.19	0.227
$ZnSO_4$ 1 mM	0.38	0.32	0.37	0.10	0.171
Zn-EDTA 10 mM	0.09	0.08	0.31***	0.16	0.000

Table 4
Mean metal content of leaves and protonemata. A difference of metal content of the protonemata to metal content of leaves was tested for significance with Mann–Whitney-*U*-test (see * and *p*-values).

Media	Leaves		Protonema		Difference (wt%)	*p*-value
	Mean (wt%)	SD	Mean (wt%)	SD		
$CuCl_2$ 100 μM	0.111	0.09	0.307	0.19	0.20***	0
$CuSO_4$ 100 μM	0.101	0.03	0.255	0.13	0.15***	0
Cu-EDTA 100 μM	0.014	0.03	0.091	0.09	0.08***	0
Cu-EDTA 1 mM	0.035	0.04	0.066	0.04	0.03**	0.004
Cu-EDTA 10 mM	0.04	0.05	0.068	0.03	0.03**	0.003
Cu-EDTA 100 mM	0.28	0.40	0.32	0.52	0.03	0.47
$ZnCl_2$ 100 μM	0.042	0.04	0.171	0.12	0.13***	0
$ZnCl_2$ 500 μM	0.627	0.63	0.744	0.42	0.12	0.051
$ZnCl_2$ 1 mM	0.778	0.57	0.651	0.69	−0.13	0.105
$ZnCl_2$ 2.5 mM	0.629	0.33	1.053	0.60	0.42	0.052
$ZnCl_2$ 5 mM	0.934	0.90	1.351	1.00	0.42	0.066
$ZnSO_4$ 100 μM	0.155	0.13	0.182	0.08	0.03	0.058
$ZnSO_4$ 1 mM	0.38	0.32	0.883	0.40	0.50***	0
$ZnSO_4$ 5 mM	1.021	0.61	1.227	0.33	0.21*	0.019
Zn-EDTA 100 μM	0.023	0.03	0.073	0.06	0.05**	0.003
Zn-EDTA 1 mM	0.022	0.03	0.124	0.06	0.10***	0
Zn-EDTA 10 mM	0.086	0.08	0.6	1.34	0.51***	0

3.6. Metal exposure leads to shortening of the cells

The metal content of *P. patens* leaves correlated negatively with the growth of the leafy gametophyte in both metals. No such significant correlations could be found for the protonemata (Fig. 4a–b).

However, changes in cell morphology of *P. patens* were found in both tissues as a result of metal exposure. For copper treated plants our data showed a trend to form shorter cells in leaves and protonema filaments (Fig. 4c). Interestingly, no significant correlation to the respective metal content could be found in both. We observed the same trend of shorter cells in both tissues in zinc treatments. Here, the shortening of the cells correlated well with the tissue metal content (Fig. 4d). No such relationship occurred regarding the cell width (Supplementary Fig. 3a–b). As we compared the cell length and width only to the tissue metal content and not to total anion concentrations, we can exclude that differences were induced by the metal anion.

4. Discussion

In the present study we used a SEM-EDX system to differentiate the metal content of the leafy gametophor and the filamentous protonemata. SEM–EDX enabled us to determine metals at their original location. However, this technique measured both, metals bound to the cell wall as well as the intracellular metals. Due to constant exchange following stoichiometric binding properties and the possible influence on the mechanical properties of the walls (Fry et al., 2002) and therefore the influence on both, cell growth and nutrient uptake, we considered the EDX-measured uptake as physiologically relevant. This assumption is confirmed by our results pointing to a strong correlation of the total tissue metal content to growth and cell size. Furthermore, Büscher et al. (1990) stated that cation exchange properties of the cell wall do not protect mosses from toxic cations and Glime (2007) reviewed the cation exchange capacity which is explained by high concentrations of non-esterified pectins, mostly polyuronic acids,

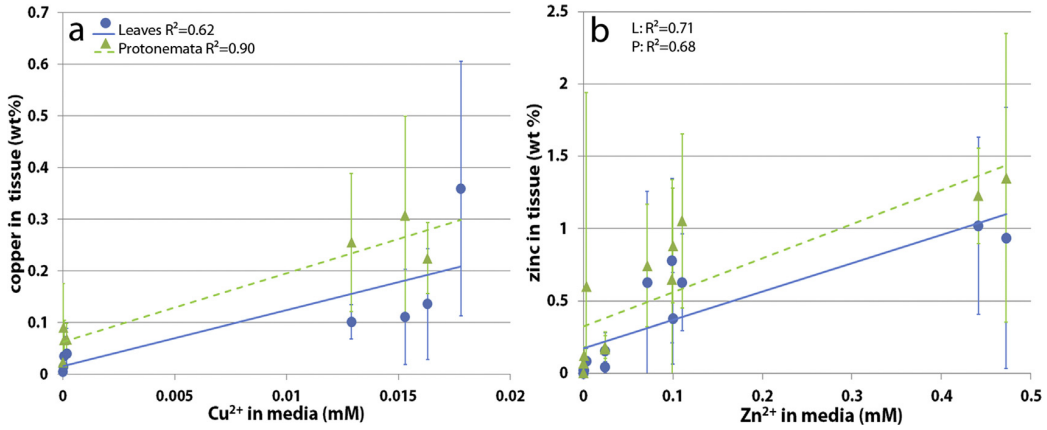

Fig. 3. (a) Correlation of the copper content in *P. patens* to the amount of Cu^{2+} in the media; (b) we found a strong and significant correlation for zinc to amount of free Zn^{2+} cations in the media. Correlation coefficients are shown only for significant correlations.

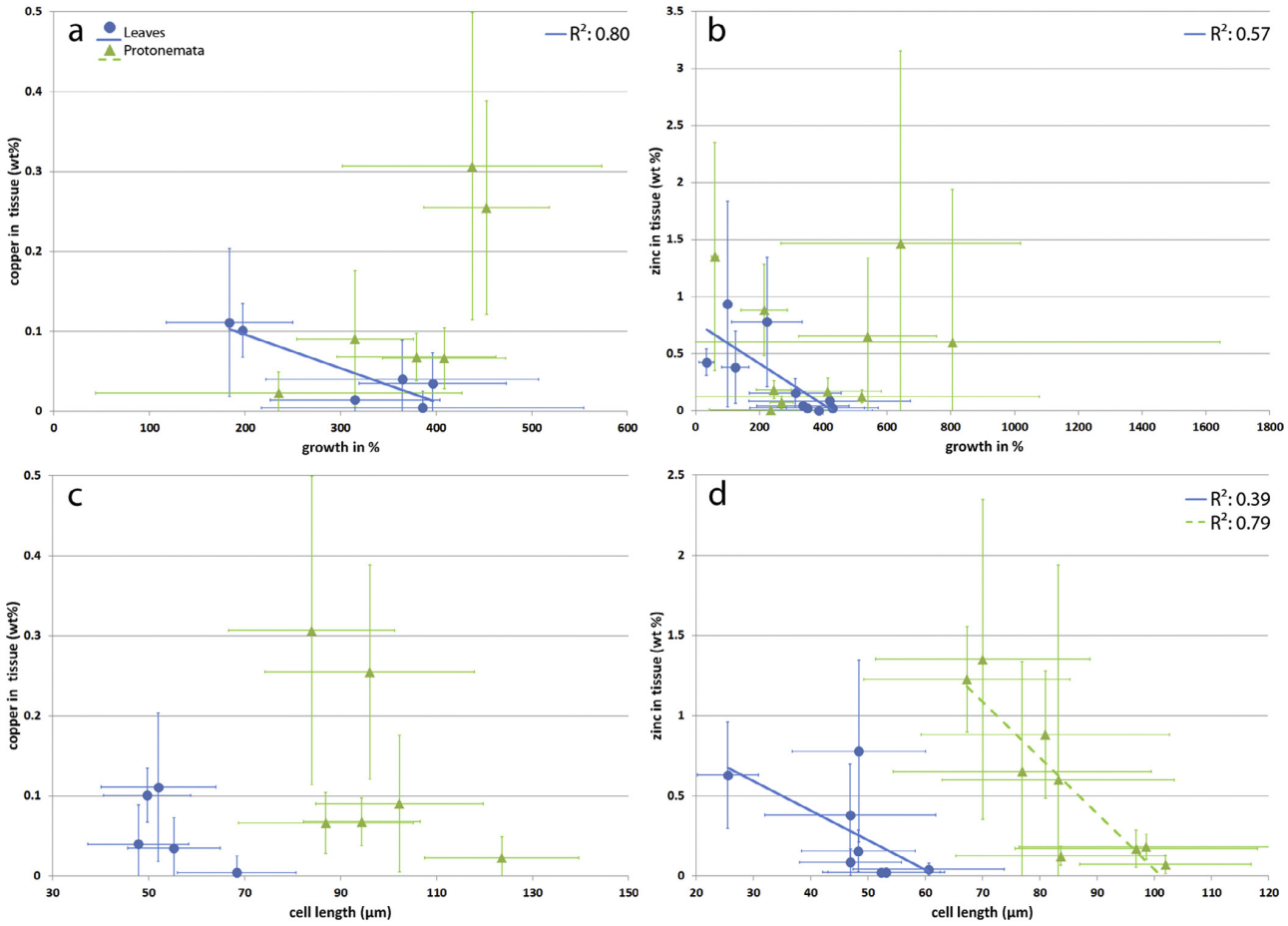

Fig. 4. Correlation of copper (a) and zinc (b) content to the mean growth of *P. patens*; correlations of tissue metal content to cell length (c for copper; d for zinc) and cell width show negative correlations to cell length for zinc (d) but no significant correlations of metal content to cell width neither for copper (Supplementary Fig. 3a) nor for zinc (Supplementary Fig. 3b). Correlation coefficients are shown only for significant correlations.

located within the cell walls. Here, the exchange of cations bound to cation-exchange sites is regulated by active transport of needed micronutrients but the avoidance of potentially harmful metals is impossible.

Nonetheless, metals can be retained to a certain amount at specific cation binding sites of the cell wall. To study cation binding to the wall, the sequential elution technique initially was proposed by Brown and Buck (1978a,b). This method is widely applied to mosses. However, such elution or filtration techniques comprise a risk of measuring artefacts (Foy et al., 1978). Sequential elution studies investigating the different cation exchange capabilities of different aquatic moss species suggested that 30–60 min is

sufficient to saturate the extracellular exchange sites (Vazquez et al., 1999). These authors used short term exposure in contrast to the present study where the plants are grown over a period of 5 weeks. Therefore, equilibrium to the cellular concentration is ensured. We are aware that the uptake measured here does not only implicate the intracellular metal content but states the non-water removable metal content of *P. patens* tissues.

4.1. Metal uptake and differences in zinc and copper

EDX-data showed rising levels of metal content of leaves and protonemata according to the supplied media concentration but only for low concentrations (Fig. 2). On $ZnCl_2$ we observed a limit of zinc uptake regardless of increasing media concentrations from 500 μM to 5 mM $ZnCl_2$. This effect was observed for both tissue types. In addition, no further increase after 10 weeks (Tab. 3; Supplementary Fig. 2) could be measured. We interpret this as the physiological limit where the cation exchange sites are saturated and the intracellular metal content reaches a toxic level at a total zinc uptake of 1.3 wt% for *P. patens*.

Furthermore, our data showed differences of copper and zinc metal content in leaves and protonema tissue. Both investigated tissues of *P. patens* showed a zinc content up to three times higher compared to copper. On one hand, this can be explained by the different toxicity levels of the metals as reviewed by Tyler (1990) and Brune et al. (1995). As a consequence, higher copper concentrations resulted in mortification of the moss tissues. As investigated in a preceding study, we also took the availability into account (Sassmann et al., 2015). Therefore, the effect is partially explained by a lower availability of copper compared to zinc at the same concentrations. The availability, estimated by the concentration of free metal cations, was 37% lower for $CuCl_2$ 100 μM than for $ZnCl_2$ 100 μM and 47% lower for $CuSO_4$ 100 μM than for $ZnSO_4$ 100 μM. In addition, the apoplastic metal binding sites need to be accounted for. Vázquez et al. (1999) stated a higher affinity of the extracellular binding sites to copper than to zinc for all three aquatic bryophyte species investigated. This is in accordance with the results for $CuCl_2$ and for $ZnCl_2$ 100 μM. Our results at higher metal concentrations are rather in accordance with other studies on bryophytes (Bengtson et al., 1982; Chen et al., 2010; Spagnuolo et al., 2011); here, the less toxic zinc was found to be accumulated more efficiently, even at sites where excess of both metals was available (Samaceka-Cymermann et al., 2002).

4.2. Anion dependent differences in uptake

Anion dependent toxicity can be explained by the differences in availability. Modelling of availabilities has shown much lower concentrations of free metal cations for EDTA chelates than for chlorides and sulfates (Sassmann et al., 2015). As a consequence, we found a dramatically decreased tissue metal content if the metal was offered as EDTA chelates when compared to the same concentrations applied as sulfates or chlorides (Fig. 2; Supplementary Fig. 1). This difference in uptake was observed for both metals and in both tissues of *P. patens*. Interestingly, these results differ from studies in higher plants where metal uptake was promoted by the addition of EDTA in order to form water soluble metal chelates which are available to the plant (Evangelou et al., 2007; Tanwar et al., 2013). But promotion is discussed critically by Nowack et al. (2006) and Custos et al. (2014) who reported a possible reduction in uptake after application of EDTA.

Nonetheless, we found a significantly increased metal content of leaves and protonemata at higher EDTA concentrations which cannot be explained by free metal cation concentrations only. We propose a possible exchange with cell wall binding sites indicating different affinities of the cell wall and EDTA metal chelates. Since copper and zinc have a high affinity to EDTA, replacement by other cations will only occur in a limited manner and our results indicate a high affinity of the cell wall as well. Further research is necessary to explain the metal content of *P. patens* grown on excess EDTA concentrations.

4.3. Metal content of protonemata exceeded the content of leaves

Comparing the metal content of leaves and protonemata, we found that metals in protonemata exceeded metals content in leaves when grown on the same metal concentrations (Table 4). As *P. patens* was transplanted on solid media over a period of five weeks, we simulated substrate metal pollution. Sidhu and Brown (1996) described an elevated metal content in basal parts of mosses if the substrate was the metal source. Contrary, if an atmospheric source of metal pollution was simulated, the tip cells contained higher levels of metal. In addition to the close contact with the substrate, a certain retention by protonema cells is possible as the protonemata seem to be more metal tolerant. Even augmentation or a shift to enhanced protonema formation can be induced by metal stress (Kapur and Chopra, 1989; Nomura and Hasezawa, 2011; Sassmann et al., 2015; Shaw, 1990).

4.4. Time dependency of metal uptake

Uptake kinetics of metals in the aquatic moss *Fontinalis antipyretica* varied in an element and concentration dependent manner but high bioconcentration factor values were already reached within a few days of exposure (Diaz et al., 2012). Although different metals were used in this study, we conclude that a 5 week retention of uptake is unlikely and this is in accordance with our results of constant metal values of *P. patens* after 5 and 10 weeks of exposure. Most samples grown on metal containing media did not show an unexpected time dependency in metal uptake (Table 3; Supplementary Fig. 2). We registered a significant increase of the metal content for plants grown on copper and zinc if they originally showed rather low sub limit metal content after 5 weeks. However, one exception occurred in plants grown on $CuSO_4$ 100 μM. They showed a decline in the metal content. But apart from plants grown on $CuSO_4$ 100 μM no plants from other tested copper and zinc concentrations showed decreased metal content. Even for $CuSO_4$ 100 μM, no clear sequestration or crystal formation of metals could be detected as described for ectohydric mosses by Shimwell and Laurie (1972). Therefore, the reduction of metal content in $CuSO_4$ 100 μM cannot be explained by excretion but could be a result of ongoing growth and propagation of the gametophyte on this concentration even when *P. patens* growth is generally diminished after week 6 (data not shown). However, concentrations with rather low initial uptake after 5 weeks showed a significant increase in metal content pointing to ongoing intracellular accumulation. Accordingly, Bleuel et al. (2005) described fast biosorption of Cd^{2+} but slower intracellular uptake (15 days investigated) in two *Fontinalis* species.

4.5. Metal content explained growth changes and differences in cell length

The metal content in leaves and protonemata was significantly correlated to the free metal ions for both metals (Fig. 3). This result endorsed the importance of free metal ions in general, as reviewed by Twiss et al. (2001). Nomura and Hasezawa (2011) described in *Scopelophila cataracte* that EDTA chelates inhibited gemma formation less than other copper species. Furthermore, the results confirmed the importance of free metal ion concentrations on growth and protonema development of *P. patens* as their availability resulted not only in inhibited growth (Sassmann

et al., 2015) but also in uptake into the tissue. In detail, cation availability seems to depend in similar ways on speciation in *P. patens* as in *Hordeum vulgare* (Bell et al., 1991). Finally, a strong difference between the added and the available copper and zinc of the added EDTA concentrations was found. Therefore, no correlation of plant metal content to the total metal content of media was found.

Interestingly, the effect of cell shortening was not limited to the tip growing protonema cells but also the physiologically different leaf cells were affected. Both formed shorter cells with rising metal content (Fig. 4). Possibly, metal cations bind to the pectin matrix of the newly formed cell wall and prevent the full elongation of the cell by stiffening. Krzeslowska et al. (2009) described the effects of lead on protonema cells and their cell wall in *Funaria hygrometrica*. Ultrastructural changes like an increased amount of plastoglobuli in the chloroplasts and changes of the endoplasmic reticulum, as well as a thickening of the cell wall and increased callose formation were reported (Krzeslowska and Woźny, 1996). This thickening may well correlate with reduced elongation.

Remarkably, the shortened leaf and protonema cells did not fully explain the reduced size of the plants. On the contrary, plants size was usually less affected by metal stress, and even a shift to augmented protonema growth could be observed in a preceding study (Sassmann et al., 2015). Thus, *P. patens* seems to compensate for reduced protonema cell size by increased cell division. Furthermore, we point out that no significant correlation of the cell width to tissue metal content was found. The different reactions regarding length and width indicate varying sensitivity of cell elongation in different dimensions which shall be addressed in a subsequent study.

5. Conclusions

The first hypothesis (H1) tested in this study was confirmed as *P. patens* showed differences in copper and zinc uptake and saturation in uptake of zinc regardless of the offered concentration if the concentration was between $ZnCl_2$ 500 μM and the sub-lethal concentration of $ZnCl_2$ 5 mM. Only for the highest tested $ZnSO_4$ concentration we found the same uptake for both metals, and no saturation could be found for metals added as EDTA chelates or the tested copper concentrations.

The second hypothesis (H2) was confirmed as well, as, we did not find sequestration or secretion of metals over a period of five and ten weeks.

The predictions of the third hypothesis (H3) were partly confirmed. Up to the uptake limit, protonemata constantly showed a higher metal content compared to the leaves in both copper and zinc tested plants. This suggests a higher uptake capacity and an elevated tolerance, although with the same limit of total metal content.

Hypothesis four (H4) was confirmed only for the cell length. The growth reduction of the leafy gametophyte and the shortening of both leaf and protonema cells were correlated significantly to the metal content of the respective tissue. Further, the available free metal ions of the media best explained the metal content of the two *P. patens* tissues. Surprisingly, the cell width was not significantly correlated with the metal content.

Our results indicate that the relation between metal exposition and metal uptake by mosses is not necessarily linear and not necessarily the same for all elements. Thus, the precision of bioindication using mosses could be increased if the uptake behavior of the used species for the element of interest were determined in more detail.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.envexpbot.2015.05.003.

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G. Zinc tolerance of Physcomitrella patens *evaluated by X-ray microanalysis*

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Microscopy Conference 2009 (MC 2009), Multinational Congress on Microscopy Graz, Austria

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Abstract and Poster

Contribution: 60%

Zinc tolerance of *Physcomitrella patens* evaluated by X-ray microanalysis

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Keywords: heavy metal, mosses, *Physcomitrella patens*, X-ray microanalysis, zinc

Physcomitrella patens is a non-vascular, multicellular land plant and belongs to the bryophytes. Due to the simple morphology of the moss and its facile *in vitro* cultivation, *P. patens* has become a model organism in plant and molecular biology [1]. Most mosses are specialists that inhabit ecological niches; some even live on heavy metal enriched substrates (e.g. *Pohlia drummondii*, *Mielichhoferia elongata*, [2]). We wondered if this potential is unique to certain moss species and tested *P. patens* that normally occurs on uncontaminated soil for its Zn-sensitivity. Zn^{2+} is an essential cation for eukaryotic cells with harmful effects if applied at high doses.

P. patens was grown on agar plates enriched with either zinc-EDTA (0.1 mM, 1 mM, 10 mM, 100 mM) or zinc-chloride (0.1 mM, 1 mM, 5 mM). Higher concentrations of Zn^{2+} showed to be lethal.

Semiquantitative analysis of heavy metal uptake was performed with X-ray microanalysis (EDX) on a scanning electron microscope (SEM). Moss samples were air dried, mounted and carbon-coated. During sample preparation, great attention was paid to avoid contamination with the Zn-containing growth medium. Figure 1a shows a typical EDX-spectrum with a distinct Zn-peak (arrow). At least seven measurements per plant were taken (Figure 1b) and the results are shown in Figure 1c. Student`s T-test was applied to indicate significant value differences. The uptake correlated with the amount of Zn offered in a gradient manner. $ZnCl_2$ is more harmful to *P. patens* than Zn-EDTA: on $ZnCl_2$-plates, the plants survived up to a concentration of 5 mM whereas the highest Zn-level for the EDTA-plates was shown in the probes containing 100 mM Zn-EDTA. Low amounts of $ZnCl_2$ accumulated stronger within the plants than low amounts of Zn-EDTA.

On the cellular level, we tested heavy metal resistance by exposure to gradient Zn-solutions. After 48 h, plasmolysis of the cells in 0.8 M mannitol reflected their viability and was determined in the light microscope. Cells of *P. patens* gametophytes showed a high tolerance to zinc up to concentrations of 100 mM Zn-EDTA (Figure 2).

In summary, the hypothesis that *P. patens* is sensitive to heavy metals could not be proven. By contrast, we observed an interestingly high tolerance to both, $ZnCl_2$ and Zn-EDTA. Further experiments are underway to specifically locate Zn storages within the cells and to show the impact of other heavy metals such as copper and cadmium.

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2. H. Stummerer, Österr. Bot. Z. **118** (1970) p189.
3. This research was supported by a grant of the „Verein zur Förderung für Pflanzenwissenschaften“ to S.S. and by the „Hochschuljubiläumsstiftung der Stadt Wien“, project H-1939/2008, to I.L.

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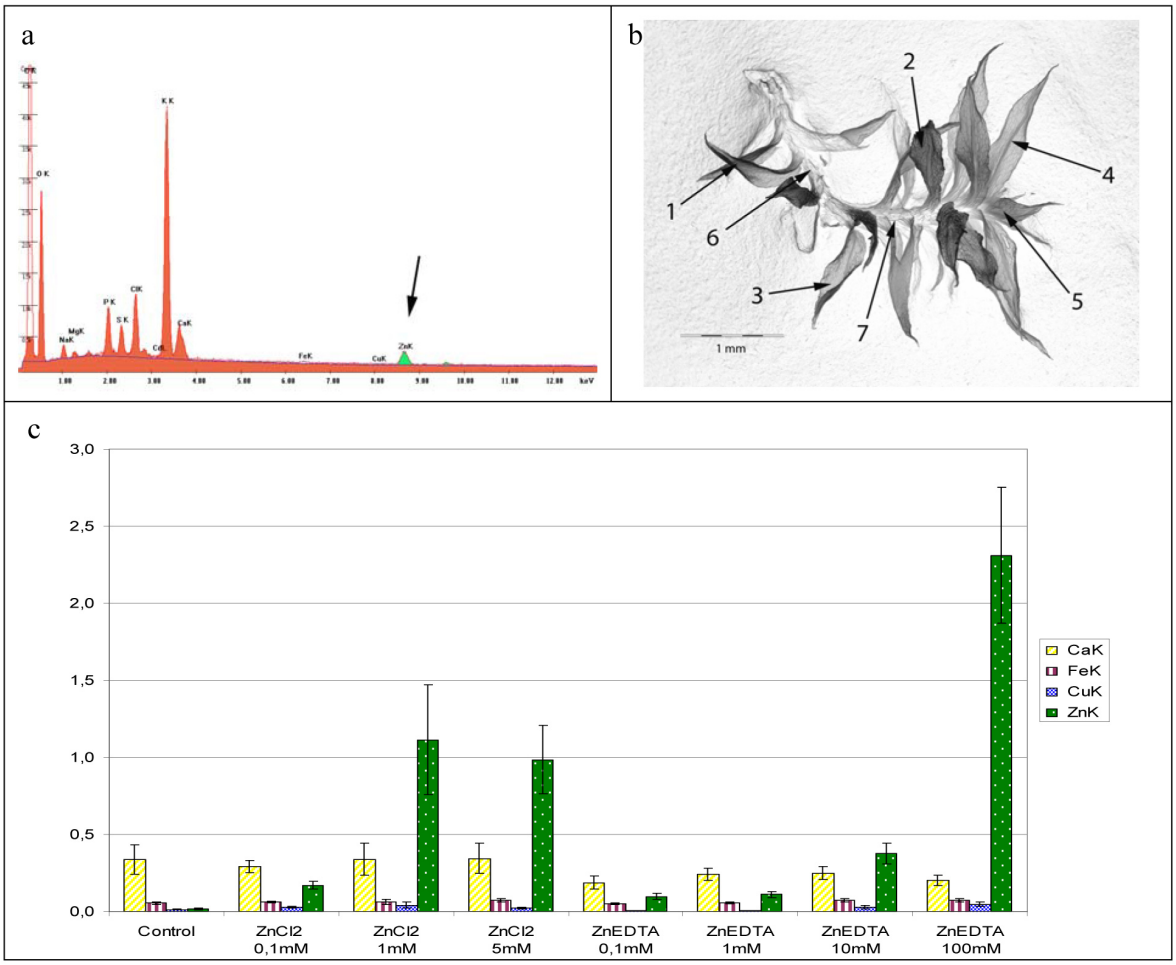

Figure 1. X-ray microanalysis of *P. patens* grown on gradient $ZnCl_2$ and Zn-EDTA plates. **a** Typical EDX-spectrum showing a distinct Zn peak (arrow). **b** SEM picture of gametophyte; numbers indicate EDX measurements per plant. **c** Gradient accumulation of Zn in the plants from both, $ZnCl_2$ and Zn-EDTA plates; leveling of $ZnCl_2$ occurs at 1 to 5 mM. Uptake of Zn-EDTA is less at low concentrations and EDX shows highest zinc amounts in plants from 100 mM Zn-EDTA plates.

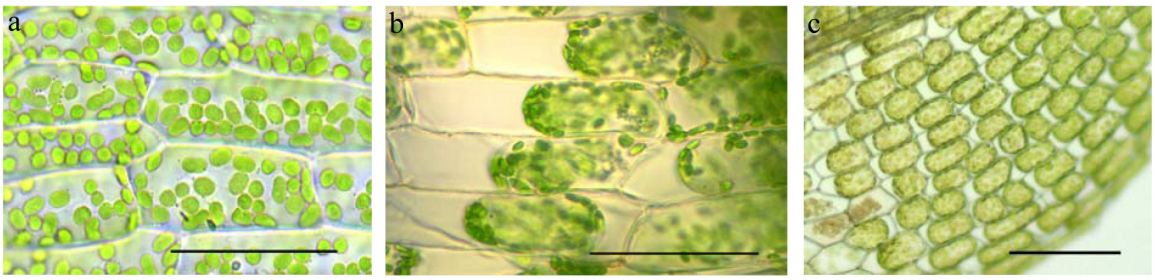

Figure 2. Resistance experiments of *P. patens* gametophyte: cells are exposed to gradient Zn-EDTA concentrations for 48 h; subsequent plasmolysis in 0.8 M mannitol reflects the viability of cells. **a** Control cells in water. **b** Control: plasmolysed cells. **c** 100 mM Zn-EDTA: the majority of cells is alive after 48 h and plasmolyses. Bar: **a, b**: 500 µm, **c**: 1000 µm.

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Zinc tolerance of *Physcomitrella patens* evaluated by X-ray microanalysis

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Introduction

Physcomitrella patens is a non-vascular, multicellular land plant and belongs to the bryophytes. Due to the simple morphology of the moss and its facile *in vitro* cultivation, *P. patens* has become a model organism in plant and molecular biology [1]. Most mosses are specialists that inhabit ecological niches; some even live on heavy metal enriched substrates (e.g. *Pohlia drummondii, Mielichhoferia elongata* [2, Poster: L4.P202 by S.W.]). We wondered if this potential is unique to certain moss species and tested *P. patens* that normally occurs on uncontaminated soil for its Zn-sensitivity. Zn^{2+} is an essential cation for eukaryotic cells with harmful effects if applied at high doses.

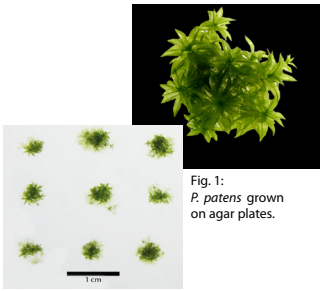
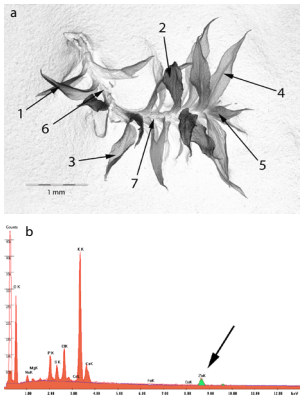


Fig. 1: *P. patens* grown on agar plates.

Material and Methods

P. patens was grown on agar plates of modified Beneke growth medium [3] enriched with either Zn-EDTA (0.1 mM, 1 mM, 10 mM, 100 mM) or Zn-chloride (0.1 mM, 1 mM, 5 mM). Higher concentrations of Zn^{2+} showed to be lethal. Semiquantitative analysis of heavy metal uptake was performed with X-ray microanalysis (EDX) on a scanning electron microscope (SEM). Moss samples were air dried, mounted and carbon-coated. During sample preparation, great attention was paid to avoid contamination with the Zn-containing growth medium. On the cellular level, we tested heavy metal resistance by exposure to gradient Zn-solutions.

EDX



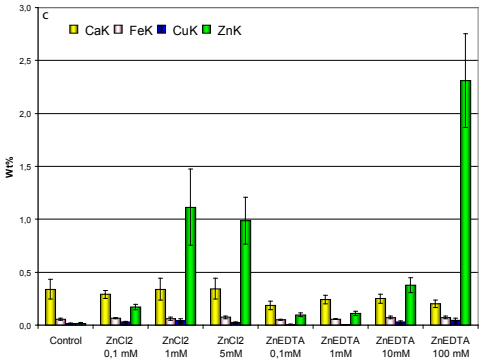


Fig. 2a-c: a *P. patens* with marked points of measurement. b EDX-Spectrum. c EDX-Results.

Figure 2b shows a typical EDX-spectrum with a distinct Zn-peak (arrow). This measurement was taken of a plant grown on $ZnCl_2$-5mM-plate. At least seven measurements per plant were taken (Figure 2a) and the results are shown in Figure 2c. Student`s T-test was applied to indicate significant value differences. (CI = 0,95) The uptake correlated with the amount of Zn offered in a gradient manner. $ZnCl_2$ is more harmful to *P. patens* than Zn-EDTA: on $ZnCl_2$-plates, the plants survived up to a concentration of 5 mM whereas the highest Zn-level for the EDTA-plates was shown in the probes containing 100 mM Zn-EDTA. Low amounts of $ZnCl_2$ accumulated stronger within the plants than low amounts of Zn-EDTA.

Resistance

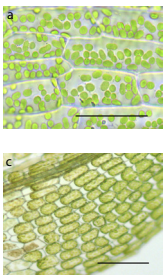


Fig. 3a-c: a Control cells in water. b Control: plasmolysed cells. c 100 mM Zn-EDTA: the majority of cells is alive after 48 h and plasmolyses. Bar: a, b: 50 µm, c: 100 µm.

Heavy metal resistance was tested by exposure to gradient Zn-solutions. After 48 h, plasmolysis of the cells in 0.8 M mannitol reflected their viability and was determined by deplasmolysis and observation in the light microscope. Cells of *P. patens* gametophytes showed a high tolerance to zinc up to concentrations of 100 mM Zn-EDTA (Figure 4 top).
This short time exposure of *P. patens* to heavy metal solutions showed an equal or even higher toxicity than long term exposure over 5 weeks on solid growth media (Figure 4 bottom).

	Concentration										
Liquid Media	1M	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	C
Zn-EDTA	-	-+	+	+	+	+	+	+	+	+	+
Cu-EDTA	-	-+	-+	+	+	+	+	+	+	+	+
$ZnCl_2$	-	-	-	-+	-+	+	+	+	+	+	+
$CuCl_2$	-	-	-	-	-	-	-+	+	+	+	+
$CdCl_2$	-	-	-	-	-	-	-+	-+	+	+	+
Solid Media	1M	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	C
Cu-EDTA	X	+	+	+	+						+
Zn-EDTA	X	+	+	+	+						+
$ZnCl_2$	X	-	-	+	+						+
$Zn-SO_4$	X	-	-	+	+						+
$CuCl_2$	X	-	-	-	+						+
$CdCl_2$	X	-	-	-	-	-	+	+			+

Fig. 4: Showing the survival at incresing heavy metal concentrations in liquid and solid media. Growing/living cells in green, nongrowing/dead cells in red.

Conclusion

In summary, the hypothesis that *P. patens* is sensitive to heavy metals could not be proven. By contrast, we observed an interestingly high tolerance to both, $ZnCl_2$ and Zn-EDTA. This tolerance correlates with the Zn-content of leaf cells measured by EDX. Until now we can not fully explain why our data showed a higher Zn^{2+} uptake on $ZnCl_2$ media compared to the same concentration on Zn-EDTA media. Further experiments are underway to specifically locate Zn storages within the cells and to show the impact of other heavy metals such as copper and cadmium.

References

1. D. Lang et al., Trends Plant Sci. 13 (2008) p542.
2. H. Stummerer, Österr. Bot. Z. 118 (1970) p189.
3. Y.-Y. Gang et al., Acta Botanica Sinica 45(12) (2003) p 1475.
4. W. Url, Physiologia plantarum, Bd 10 (1955) p 1.

Acknowledgement

1.This research was supported by a grant of the „Society for the Advancement of Plant Sciences" to S.S.
2. This research was supported by the Hochschuljubiläumsstiftung der Stadt Wien, project H-1939/2008, to I.L.

D. A comparative study of heavy metal uptake by X-Ray micro-analysis and zinc localization in the moss Physcomitrella patens

Sassmann, S., Weidinger, W., Lichtscheidl, I., Lang, I.

RMS Botanical Microscopy 2011, Wageningen, The Netherlands

16/04/2011 - 21/04/2011

Abstract and Poster

Contribution: 70%

Abstract:

24.

A comparative study of heavy metal uptake by X-Ray microanalysis and zinc localization in the moss *Physcomitrella patens*.

STEFAN SASSMANN, MARIELUISE WEIDINGER, IRENE LICHTSCHEIDL & INGEBORG LANG
University of Vienna, Althanstraße 14, A-1090, Core Facility Cell Imaging and Ultrastructure Research

Mosses are discussed to be resistant to heavy metals and to accumulate heavy metals regardless of their original habitats. The reasons for this resistance are still poorly understood. By contrast to flowering plants, mosses take up nutrients by the entire surface and do not possess any exclusion mechanisms like the Casparian strip of roots. In preliminary experiments, we could confirm an exceptionally high zinc tolerance of moss gametophytes. Copper and zinc uptake was analysed by a semiquantitative method with X-ray microanalysis (EDX) in a scanning electron microscope (SEM). Moss samples were washed, air dried, mounted and carbon-coated. During sample preparation, great attention was paid to avoid contamination with the heavy metal containing growth medium. A gradient elevation of the heavy metal content in leaf tissue corresponding to the offered amount of zinc and copper in the growth media could be observed.
On the cellular level, zinc localization was performed on a confocal laser scanning microscope using a fluorescent heavy metal tracer dye (FluoZin™-3). Compared to controls, the leaf cells of plants grown on heavy metal spiked media indicated a strong relation of the fluorescence intensity to offered zinc concentrations. In low zinc concentrations, we found "doughnut-shaped" chloroplasts with conspicuous zinc staining; in some higher concentrations, small vesicles were labeled with FluoZin™-3.

Acknowledgements:
This research was supported by a grant of the „Verein zur Förderung der Pflanzenwissenschaften"to S.S,the Hochschuljubiläumsstiftung der Stadt Wien, project H-1939/2008, to I.L. and the "Forschungsstipendium 2010" of the University of Vienna to S.S.

A comparative study of heavy metal uptake by X-Ray microanalysis and zinc localization in the moss *Physcomitrella patens*

Stefan Sassmann, Marieluise Weidinger, Irene Lichtscheidl, Ingeborg Lang

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Introduction

The bryophyte *Physcomitrella patens* is a non-vascular, multicellular land plant. Due to the simple morphology of the moss and its facile *in vitro* cultivation (Fig. 1), *P. patens* has become a model organism in plant and molecular biology [1]. Most mosses are specialists that inhabit ecological niches and are discussed to be resistant to heavy metals and to accumulate heavy metals regardless of their original habitats. *P. patens* that normally occurs on uncontaminated soil showed exceptionally high zinc tolerance of moss gametophytes.

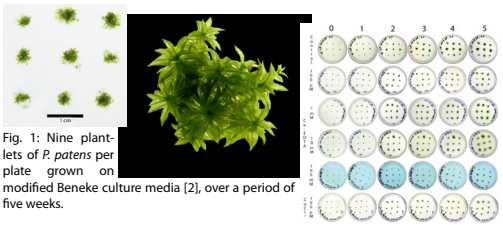

Fig. 1: Nine plantlets of *P. patens* per plate grown on modified Beneke culture media [2], over a period of five weeks.

Heavy metal uptake analysed by electron dispersive X-ray spectroscopy (EDX-SEM)

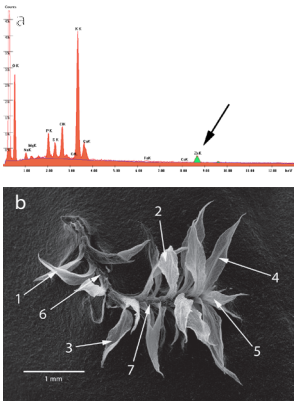

Fig. 2 (a-b): a) EDX-spectrum presenting the analysed elements, arrow points at zinc peak. b)SEM-Image of *P. patens*, numbers indicate the points of analysis.

Figure 2a shows a typical EDX-spectrum with a distinct zinc-peak (arrow). This element analysis was taken of a plant grown on agar spiked with $ZnCl_2$ 5 mM. At least seven measurements per plant were taken (Fig. 2b). The results for zinc are shown in Figure 3. The uptake correlated with the amount of zinc offered as Zn-EDTA in a gradient manner. Uptake of zinc when offered as $ZnCl_2$ reached a plateau between 1 and 5 mM. $ZnCl_2$ is more harmful to *P. patens* than Zn-EDTA: on $ZnCl_2$-plates, the plants survived up to a concentration of 5 mM whereas the highest zinc level for the EDTA-plates was shown in the probes containing Zn-EDTA 100 mM. Low amounts of $ZnCl_2$ accumulated stronger within the plants than low amounts of Zn-EDTA. The results for copper shown in Figure 4 were similar to zinc, $CuCl_2$ 100 µM showed stronger copper accumulation than plants grown on Cu-EDTA 100 µM. *P. patens* did not grow on $CuCl_2$ 1 mM. Copper is potentially more toxic than zinc for *P. patens*. Nevertheless in plants grown on Cu-EDTA 100 mM equivalent concentrations near 0.3 Wt% were observed.

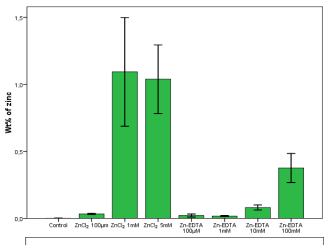

Fig. 3: EDX-Results of *P. patens* grown on differend zinc spiked media ($p<0.05$).

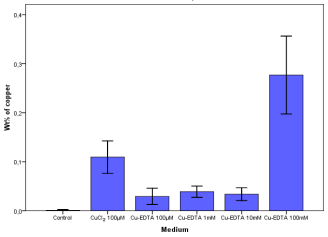

Fig. 4: EDX-Results of *P. patens* grown on differend copper spiked media ($p<0.05$).

Zinc localization by confocal laser scanning microscopy (CLSM)

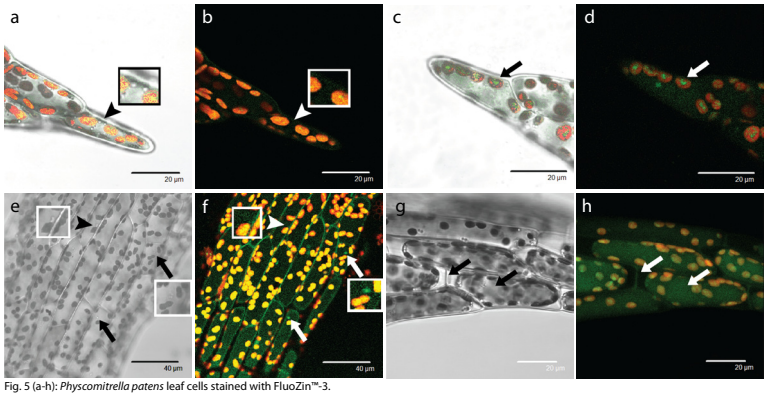

Fig. 5 (a-h): *Physcomitrella patens* leaf cells stained with FluoZin™-3.

In order to compare the changes of fluorescence intensity, the same adjustments of the CLSM where used for all samples. Compared to control (Fig. 5: a-b), the leaf cells of plants grown on heavy metal spiked media show a strong relation of the fluorescence intensity to offered zinc concentrations. In low zinc concentrations (100 µM - 1 mM), we found "doughnut-shaped" chloroplasts (Fig. 5: c-d) with conspicuous zinc staining. In some higher zinc concentrations (10 mM - 100 mM), small vesicles were labeled with FluoZin™-3. As shown in Figure 5: e-f these vesicles do not obligatory contain zinc. Localization of fluorescence between the cell wall and the protoplast was achieved by increasing the space between them by plasmolysis in 0.8 M D-Mannitol after staining with FluoZin™-3 (Fig. 5: g-h).

Conclusion

Results of EDX-spectroscopy show rising heavy metal concentrations in moss leaves in relation to the offered heavy metal concentrations but also indicate a plateau in uptake between $ZnCl_2$ 1 and 5 mM and show a dependency of uptake to the form the heavy metals are offered. As expected from the results of the EDX, we found a rising fluorescence and therefore rising zinc content in the cells in relation to the zinc concentration of the growth medium. In more detail, zinc could be localized to the cell wall, the protoplast and in small zinc enriched vesicles in cells grown on zinc spiked media whereas control cells stayed nearly unlabeled. Until now, we cannot fully explain why our data showed a higher zinc uptake on $ZnCl_2$ media compared to the same concentration on Zn-EDTA media. Further experiments are underway to specifically locate intracellular zinc storage sites by TEM-EELS and to further investigate the impact of other heavy metals such as copper and cadmium.

Acknowledgement

This research was supported by a grant of the „Society for the Advancement of Plant Sciences" , the "Forschungsstipendium 2010" of the University of Vienna to S.S, and by the Hochschuljubiläumsstiftung der Stadt Wien, project H-1939/2008, to I.L.

References

1. D. Lang et al., Trends Plant Sci. 13 (2008) p542.
2. Y.-Y. Gang et al., Acta Botanica Sinica 45(12) (2003) p 1475.

E. Cu and Zn trigger increased levels of H_2O_2 in cells of the moss Physcomitrella patens

Sassmann, S., Weidinger, W., Bock, B., Adlassnig, W., Lang, I.

Focus on Microscopy 2014, Sydney, Australia

13/04/2014 - 16/04/2014

Abstract and Poster

Contribution: 70%

Abstract:

Cu and Zn Trigger Increased Levels of H_2O_2 in Cells of the Moss *Physcomitrella patens*.

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KEYWORDS: Heavy metal, bryophytes, EDTA-coordinated metal, H_2DCFDA, SEM-EDX

ABSTRACT: In an earlier study [1], we described metal tolerance of moss gametophytes towards Cu-EDTA comparing *Mielichhoferia elongata,* known to grow on heavy metal contaminated mining sites, and *Physcomitrella patens* naturally not occurring on such sites. Here, we further investigate the tolerance and uptake of metals in *P. patens*. Despite of the high tolerance towards Cu-EDTA, we expect changes in tolerance, if the metal is offered with different anions. We tested zinc (as Na_2Zn-EDTA, $ZnCl_2$ and $ZnSO_4$) and copper (as Na_2Cu-EDTA, $CuCl_2$ and $CuSO_4$) and suggest increased levels of H_2O_2 induced by metal stress.

Gametophytes of *P. patens* were cultivated on sterile agar plates. After five weeks of cultivation the moss samples were stained with 2,7-dichlorofluorescein diacetate (H_2DCFDA) [2] and analyzed by X-ray microanalysis in a scanning electron microscope and by CLSM.

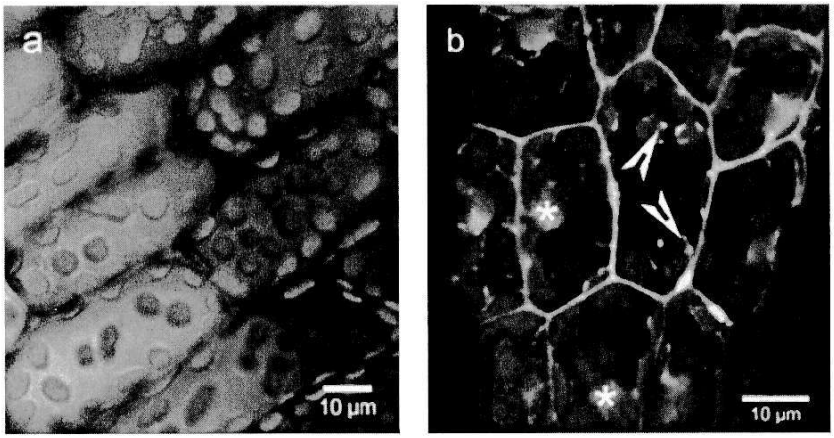

Figure1: *P. patens* leaf cells stained with H_2DCFDA for H_2O_2 detection. a, Control cells; b, Plants grown on 1 mM $ZnCl_2$ show increased fluorescence in close vicinity of the chloroplast, in the nuclear region (*), mitochondria (arrowhead) and the cell wall.

An elevation of the metal content of leaf tissue corresponding to the added amount of zinc and copper in the growth media was observed. Our results indicate strong differences in uptake and resistance depending on the metal as well as on the anion. Metals coordinated with EDTA prove to be significantly less available and therefore less toxic for *P. patens*. The relevance of coordinating agents for metal toxicity is discussed. In spite of the high metal tolerance, clear increase of H_2O_2 in metal treated cells indicates metal induced stress.

[1] Sassmann, S., S. Wernitznig, I. K. Lichtscheidl, I. Lang (2010): Comparing copper resistance in two bryophytes: *Mielichhoferia elongata* Hornsch. versus *Physcomitrella patens* Hedw. Protoplasma **246**:119–123.

[2] Darehshouri, A., U. Lutz-Meindl (2010): H_2O_2 localization in the green alga Micrasterias after salt and osmotic stress by TEM-coupled electron energy loss spectroscopy. Protoplasma **239**: 49-56.

ACKNOWLEDGEMENTS: This research was supported by the Vienna Anniversary Foundation for Higher Education (grant H-2486/2012 to S.S.), *Appear* (BIOREM FA 579003) and many thanks to Irene Lichtscheidl, University of Vienna.

Cu and Zn Trigger Increased Levels of H_2O_2 in Cells of the Moss *Physcomitrella patens*

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University of Vienna, Cell Imaging and Ultrastructure Research, Althanstrasse 14, A-1090 Vienna, Austria; Email: stefan.sassmann@univie.ac.at

Introduction

Bryophytes are non-vascular, multicellular land plants and inhabit extremely different habitats, ranging from temporary dry river banks (e.g. *Physcomitrella patens*, Fig. 1) to metal contaminated sites (e.g. *Mielichhoferia elongata*). Therefore, some bryophyte species are considered stress tolerant, and even the supposedly metal sensitive moss *P. patens* showed increased tolerance to Cu-EDTA in earlier studies[1]. Here, we further investigate the tolerance and uptake of metals in *P. patens*. We hypothesized that tolerance levels change if the metal is offered with different anions. *P. patens* was cultivated on sterile agar plates on modified Beneke growth medium. and we tested the effect of zinc (Fig. 2) and copper (both applied as EDTA-chelate, -chloride and -sulfate). To evaluate the plant available metal fraction, modelling of ion availability was performed using Visual MINTEQ 3.0 (Tab. 1).

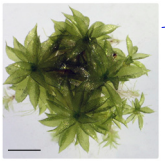


Fig. 1: *Physcomitrella patens* leafy gametophyte; (scale = 1mm)

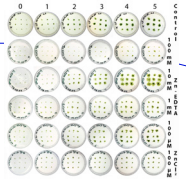
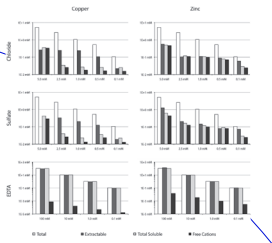


Fig. 2: Cultivation on solid medium in petri dishes from week 0 to 5 (Control, Zn-EDTA 100 mM, 10 mM, 1 mM, 0.1 mM and $ZnCl_2$ 100 µM as an example). Note the increase of biomass from the time of inoculation (week 0) to week 5



Tab. 1: Comparison of total added copper and zinc to growth media (Total), its measured water extractability after 24 hours (Extractable), the calculated total soluble metal ions (Total Soluble) as well as the calculated free Cu^{2+} and Zn^{2+} ions (Free Cations)

After five weeks of cultivation, the moss samples were air dried, mounted and carbon-coated. Semi-quantitative analysis of heavy metal uptake was performed by X-ray microanalysis (EDX) in a scanning electron microscope (SEM) (Fig. 3&4). To investigate the levels of H_2O_2, moss cells were stained with 2,7-dichlorofluorescein diacetate (H_2DCFDA) [2] and fluorescence intensity was measured on a Leica DM 6000CS confocal laser scanning microscope (Fig. 5-16).

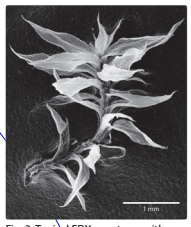


Fig. 3: Typical EDX-spectrum with a distinct Zn-peak (arrow). This measurement was taken of a sample grown on a 5 mM $ZnCl_2$ plate

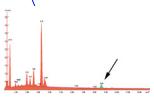


Fig. 4: Typical EDX-spectrum with a distinct Zn-peak (arrow). This measurement was taken of a sample grown on a 5mM $ZnCl_2$-plate

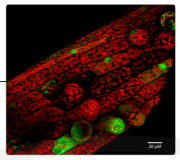


Fig. 16: *P. patens* grown on Zn-EDTA 100 mM with protonema emerging of the leaf cells.

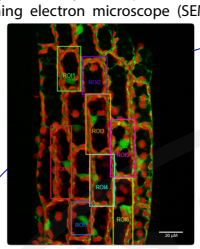
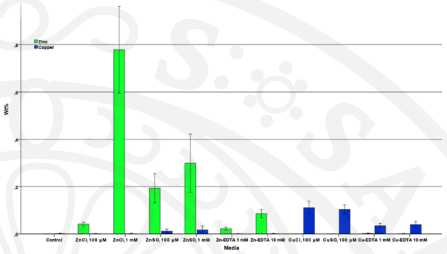


Fig. 15: Regions of interest (ROI) of H_2DCFDA fluorescence intensity measurements as indicator of H_2O_2 production in *P. patens* cells grown on $CuSO_4$ 0.1 mM



Tab. 2: In SEM-EDX analyses, metal uptake correlated well with the amount of added metals and the availability of free metal cations. Note the difference of uptake if metals are added as EDTA-chelates.

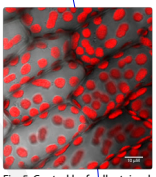


Fig. 5: Control leaf cells stained with H_2DCFDA; red autofluorescence of chloroplasts

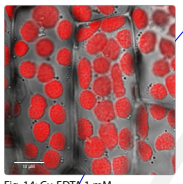


Fig. 14: Cu-EDTA 1 mM

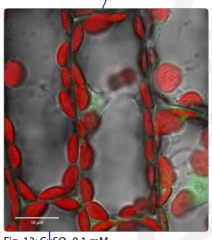
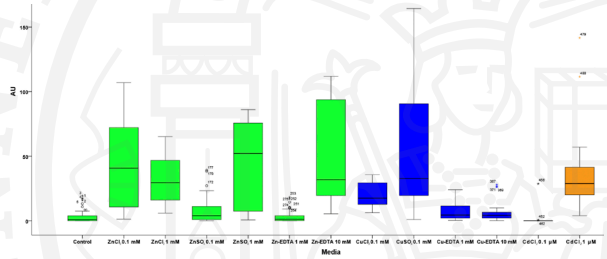


Fig. 13: $CuSO_4$ 0.1 mM



Tab. 3: Fluorescence intensity of H_2O_2 in *P. patens* leaf cells stained with H_2DCFDA, show treatment dependent differences of cellular H_2O_2 concentrations. Low fluorescence intensity was found in control, Zn-EDTA 1 mM, Cu-EDTA 10 mM and $CdCl_2$ 0.1 µM. Slightly but significantly enhanced levels were found in $ZnSO_4$ 0.1 mM and Cu-EDTA 1 mM. Even higher H_2O_2 levels were measured for $ZnCl_2$ 0.1 and 1 mM, $ZnSO_4$ 1 mM, Zn-EDTA 10 mM, $CuCl_2$ and $CuSO_4$ 0.1 mM. In addition to copper and zinc we tested the non-essential metal cadmium as additional control for metal induced increased H_2O_2 levels and found increased fluorescence in $CdCl_2$ 1 µM.

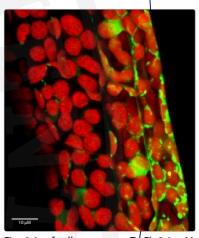


Fig. 6: Leaf cells grown on $ZnCl_2$ 0.1 mM; green fluorescent areas indicate positive H_2O_2 labeling with H_2DCFDA

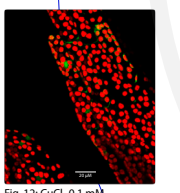


Fig. 12: $CuCl_2$ 0.1 mM

Conclusion

The most obvious general trend of our results is the simultaneous rise of metal toxicity, estimated by free cation concentration, metal uptake and stress level for the plants, estimated by ROS production. Both the results on metal uptake and H_2O_2 production indicate reduced uptake of EDTA-chelated metals compared to ionic salts. The zinc content of leaves was higher if the metal was offered as sulfate compared to chloride and EDTA. Interestingly, after staining with H_2DCFDA we found a plateau or even less fluorescence in plants grown on $ZnSO_4$ 0.1 mM when compared to $ZnCl_2$ 0.1 mM; the results on metal content, however, showed a plateau only at significantly higher zinc concentrations. No such difference could be found for $CuCl_2$ and $CuSO_4$ 0.1 mM. Furthermore, we could confirm the low fluorescence in Cu-EDTA and Zn-EDTA 1 mM treated plants which showed neither enhanced metal uptake nor symptoms of stress. Current investigations focus on the H_2O_2 production of plants grown on Zn-EDTA 10 mM compared to plants on Cu-EDTA 10 mM as well as on the differences of $ZnCl_2$ and $ZnSO_4$ treated plants.

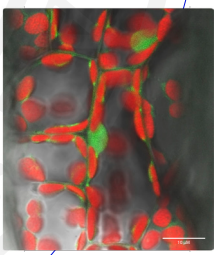


Fig. 7: $ZnCl_2$ 1 mM; H_2O_2 detection in nuclei and mitochondria

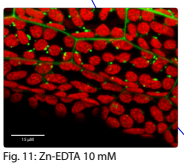


Fig. 11: Zn-EDTA 10 mM

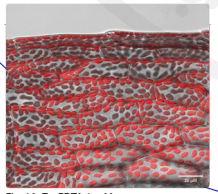


Fig. 10: Zn-EDTA 1 mM

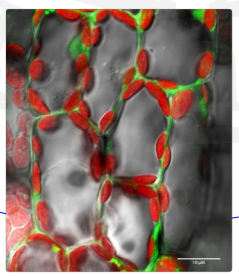


Fig. 9: $ZnSO_4$ 1 mM

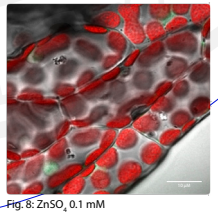


Fig. 8: $ZnSO_4$ 0.1 mM

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[1] Sassmann, S., S. Wernitznig, I. K. Lichtscheidl, I. Lang (2010): Comparing copper resistance in two bryophytes: *Mielichhoferia elongata* Hornsch. versus *Physcomitrella patens* Hedw. Protoplasma 246:119–123.

[2] Darehshouri, A., U. Lutz-Meindl (2010): H_2O_2 localization in the green alga Micrasterias after salt and osmotic stress by TEM-coupled electron energy loss spectroscopy. Protoplasma 239: 49-56.

Acknowledgements

This research was supported by the Vienna Anniversary Foundation for Higher Education (grant H-2486/2012 to S.S.) and the ŒAD (Appear-43/BIOREM). Many thanks are due to Irene Lichtscheidl, University of Vienna.

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F. *Metal Treatment on* Physcomitrella patens *Compared to two Bryophyte Species Naturally Occurring on Metal Contaminated Sites*

Sassmann, S., Weidinger, M., Bock, B., Antreich, S., Adlassnig, W., Lang, I.

18th International Microscopy Congres; IMC 2014, Prague, Czech Republic

07/09/2014 – 12/09/2015

Abstract and Poster

Contribution: 70%

Abstract:

Type of presentation: Poster

LS-1-P-3248 Metal Treatment on Physcomitrella patens Compared to two Bryophyte Species Naturally Occurring on Metal Contaminated Sites

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Bryophytes inhabit extremely different habitats, ranging from dry fallen river banks (e.g. *Physcomitrella patens*) to metal contaminated sites (e.g. *Pohlia drummondii* and *Mielichhoferia elongata*). Therefore, some bryophyte species are considered stress tolerant, and even the supposedly metal sensitive moss *P. patens* showed increased tolerance to Cu-EDTA in earlier studies.

For the present experiments, the bryophytes were cultivated on sterile agar plates and tested for zinc (as Zn-EDTA, $ZnCl_2$ and $ZnSO_4$) and copper (as Cu-EDTA, $CuCl_2$ and $CuSO_4$) over a period of five weeks (Fig. 1).

Despite of the high tolerance towards Cu-EDTA of *P. patens*, we measured changes in growth and metal uptake analyzed by X-ray microanalysis in a scanning electron microscope (Fig.2) if the metal is offered with different anions. Here, especially the uptake of EDTA chelated metals was significantly lower compared to metal offered as ionic salt. Modelling of ion availability explained most of the differences in toxicity.

Changes in the cellular content of reactive oxygen species (ROS) after staining with 2,7-dichlorofluorescein diacetate (H_2DCFDA) were analyzed in a confocal scanning microscope (Fig.3) and the three different bryophyte species compared. *P. patens* showed only low H_2DCFDA fluorescence in control cells, in contrast to metal treated cells were increased ROS could be detected for chloroplast associated mitochondria, the nuclear region and the cell wall region.

Further investigation of cellular localization of metal deposition was performed using FluoZin-3 and is ongoing in transmission electron microscopy studies.

Acknowledgement: This research was supported by the Vienna Anniversary Foundation for Higher Education (grant H-2486/2012 to S.S.) and the ŒAD (Appear/43/Biorem). Many thanks are due to Irene Lichtscheidl, University of Vienna.

Metal Treatment on *Physcomitrella patens* Compared to two Bryophyte Species Naturally Occurring on Metal Contaminated Sites

Stefan Sassmann, Marieluise Weidinger, Barbara Bock, Sebastian J. Antreich, Wolfram Adlassnig, Ingeborg Lang

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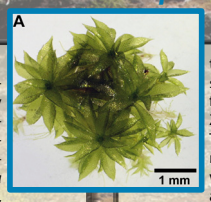
Introduction

Mosses inhabit extremely different habitats, ranging from dry fallen river banks (e.g. *Physcomitrella patens* **A**) to metal contaminated sites (e.g. *Mielichhoferia elongata* **I** and *Pohlia drummondii* **1**; Schwarzwand, Austria). Therefore, some bryophyte species are considered stress tolerant, and even the supposedly metal sensitive moss *P. patens* showed cosiderable tolerance to Cu-EDTA in earlier studies. For the present experiments, the mosses were cultivated on sterile agar plates and tested for zinc (as Zn-EDTA, $ZnCl_2$ and $ZnSO_4$) and copper (as Cu-EDTA, $CuCl_2$ and $CuSO_4$) over a period of five weeks. Metal uptake was measured by X-ray microanalysis in a scanning electron microscope (SEM-EDX). Intracellular reactive oxygen species (ROS) were stained with 2,7-dichlorofluorescein diacetate (H_2DCFDA) and analyzed in a confocal scanning microscope (Leica TCS SP5).

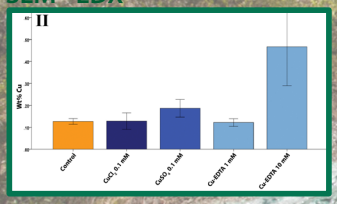
Physcomitrella patens



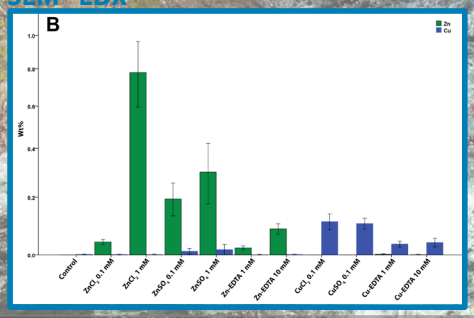
Mielichhoferia elongata

Pohlia drummondii

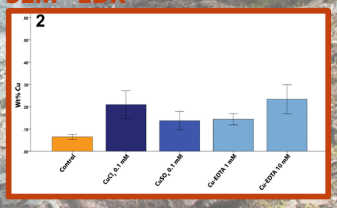
SEM - EDX



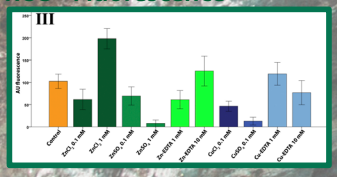
SEM - EDX



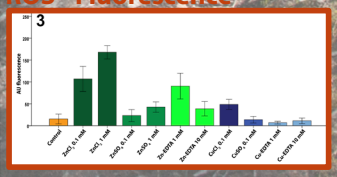
SEM - EDX



ROS - Fluorescence



ROS - Fluorescence

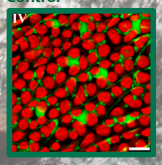


Results & Conclusions

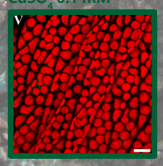
Despite the high tolerance towards Cu-EDTA in *P. patens*, we measured changes in growth and metal uptake analyzed by SEM-EDX if the metal was offered with different anions (**B**). Here, especially the uptake of EDTA chelated metals was significantly lower in *P. patens* compared to metal offered as ionic salt. Interestingly we did not find such difference for *P. drummondii* (**2**) and even found increased uptake for *M. elongata* for Cu-EDTA (**II**). Staining for ROS with H_2DCFDA in the three different bryophyte species (**III**; **C**; **3**) showed only low fluorescence in control cells of *P. patens* (**D**) and *P. drummondii* (**4**), but the control of the "copper moss" *M. elongata* (**IV**) showed increased ROS fluorescence compared to metal treated cells. While $ZnSO_4$ (**VI**) induces less ROS than $ZnCl_2$ (**6; E**) in all three species, no such difference could be found for copper in *P. patens* (**F**). While *P. drummondii* showed fluorescence near control level on $CuSO_4$ (**5; F**) and Cu-EDTA, *M. elongata* showed higher growth rates and less ROS fluorescence if grown on $CuSO_4$ (**V**) and $ZnSO_4$ (**VI**) spiked media.

Thus the sensitive moss *P. patens* suffers significantly under metal exposition whereas the facultative metallophyte *P. drummondii* shows little stress and the obligatory metallophyte *M. elongata* even benefits.

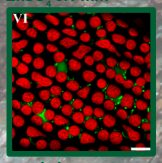
Control



$CuSO_4$ 0.1 mM

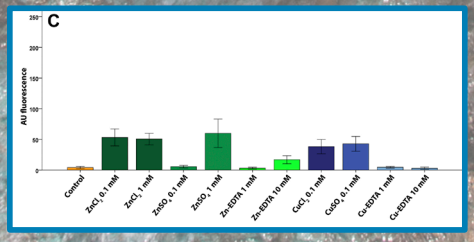


$ZnSO_4$ 0.1 mM

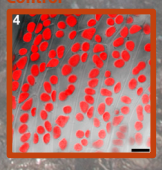


scale bar = 10 µm

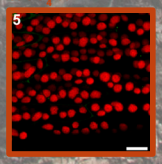
ROS - Fluorescence



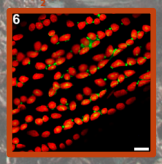
Control



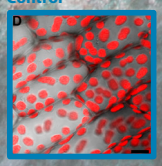
$CuSO_4$ 0.1 mM



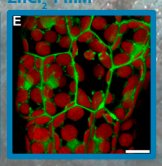
$ZnCl_2$ 0.1 mM



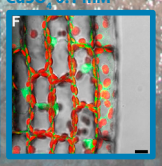
Control



$ZnCl_2$ 1 mM



$CuSO_4$ 0.1 mM



Acknowledgements

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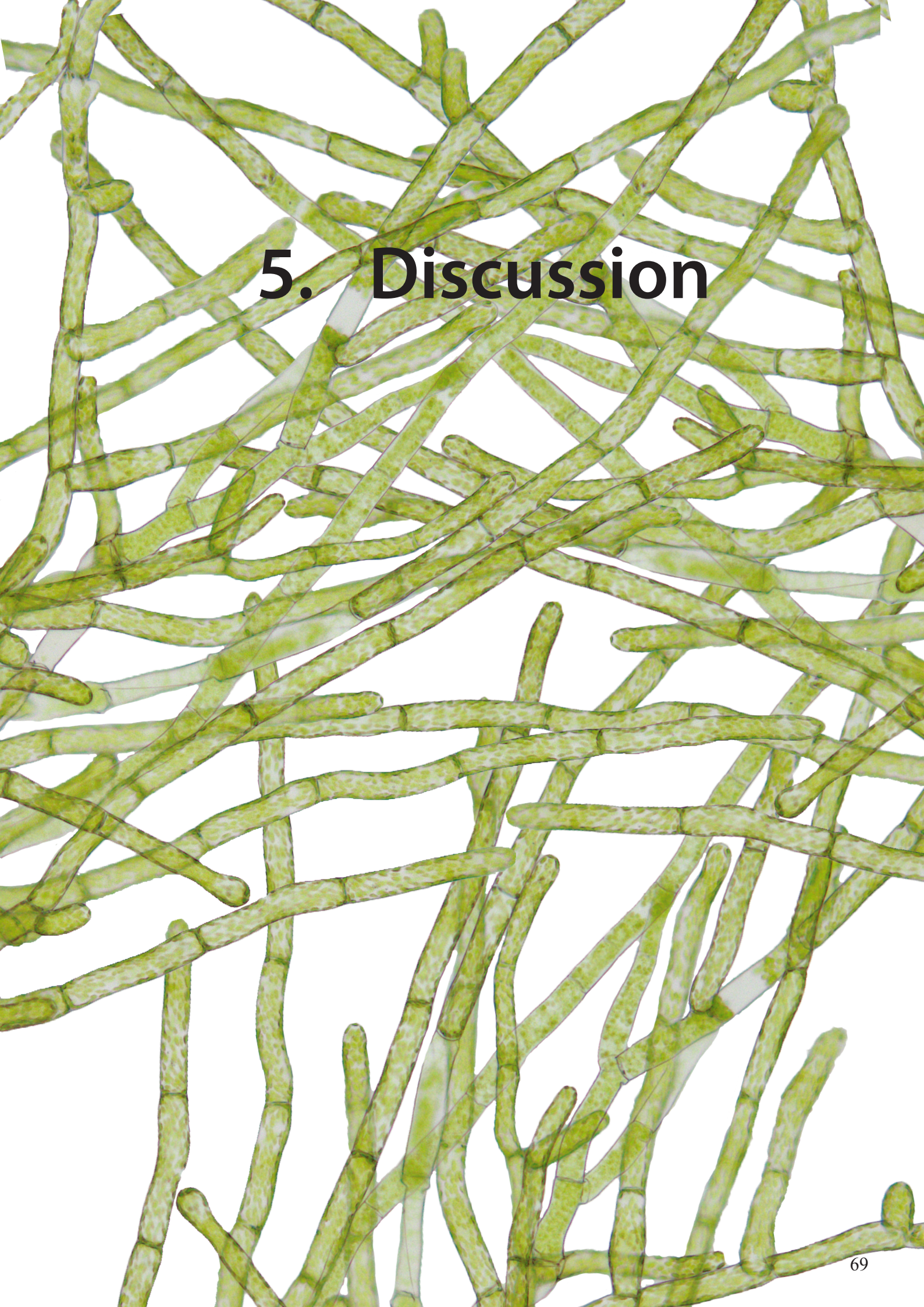

5. Discussion

5. Discussion

This thesis includes three manuscripts published as first author and four posters presented at scientific conferences and meetings. All those contributions cover different hypotheses and aspects of the same general topic: *Physcomitrella patens* and heavy metal stress.

In detail, we investigated the tolerance of *P. patens* towards different metals added with three different anions and compared the results to mosses originating from metal rich habitats (Sassmann et al., 2010). Further, we expanded the short term tolerance test to a long term growth experiment which continued over five weeks. Analyses of the water extractable metal portion and calculations of available free metal ion content of the growth media provided evidence for anion dependent differences. In Sassmann et al. (2015a) we correlated those results to the observed changes in growth and morphology of *P. patens*. Moreover, we analyzed the influence of metal availability on metal uptake and its impact on growth and cell size (Sassmann et al., 2015a; Sassmann et al., 2015b). Finally, taking into account the results of availability we investigated the intracellular localization of metals (Sassmann et al., 2009; Sassmann, 2011) and the resulting ROS formation (Sassmann, 2014a). We compared those data to observations on two mosses from metal rich habitats, *Pohlia drummondii* and *Mielichhoferia elongata* (Sassmann, 2014b). In the following our research is discussed in general. More specific topics are discussed in the respective sections of the publications included in this thesis.

5.1 Tolerance of Physcomitrella patens

The tolerance of plants towards metals is a complex system. For higher plants, Baker (1981) described different species dependent mechanisms with varying tolerance. Based on their occurrence on metal sites Frahm (2001) postulated a general high tolerance towards metal stress in bryophytes. However, bryophytes are highly diverse comprising approximately 26,000 species native to a great variety of habitats, and differences in tolerance therefore seem likely. Even intraspecies differences in tolerance of different ecotypes of *Funaria hygrometrica* have been reported; plants from a metal polluted habitat tolerated higher amounts of metal than plants from an unpolluted site (Shaw, 1988). However, it remained unclear if these differences were due to physiological adaptation or to the formation of metal tolerant ecotypes. Accordingly, in the present thesis, we used a homogenous strain of *P. patens* wild type which was always sub-cultured from control plates.

Tolerance tests of *P. patens* revealed neither special sensitivity nor high tolerance towards the tested metals per se but the tolerance was strongly dependent on the anion or complex conjugated to the offered metal. This anion dependent metal toxicity was already reported but not fully explained by Kaho (1933) for red cabbage (*Brassica oleracea* convar. *capitata* var. *rubra* L). He suggested that heavy metals are least harmful if applied as sulfates, i.e. as they usually occur in nature and chlorides and nitrates caused more damage to cells. Url (1956) found astonishing metal tolerance of

certain mosses, e.g. *Mielichhoferia elongata* and *Mielichhoferia nitida*, two mosses from copper rich areas towards copper if added as $CuSO_4$. Interestingly, no such differences in tolerance between chlorides and sulfates were found in the present thesis. We observed, however, an extraordinarily high tolerance towards EDTA-chelates, an effect that had been previously described in the liverwort *Marchantia polymorpha* by Coombes and Lepp (1974). When the metal was offered as Cu-EDTA, *P. patens* did even surpass the copper tolerance of the copper moss *M. elongata* (Sassmann et al., 2010). This remarkable tolerance of the 48 h exposure test was confirmed by long term growth experiments on solid media (Sassmann et al., 2009; Sassmann et al., 2010). However, questions remained on morphological characteristics, specific growth data after metal stress and the reasons for the reduced toxicity of EDTA-chelates.

5.2 The impact of metal availability on growth and morphology

Two principal reactions of the *P. patens* gametophyte were observed concerning growth and metal stress. First, the moss reduced its gametophore growth in favor of increased protonema formation. This consequently resulted in a general shift towards protonemata thereby changing the G : P ratio (Sassmann et al., 2015a). Secondly, a general growth reduction occurred; this had also been reported in other bryophytes as general reaction to metal stress (Chopra and Kumra, 1988; Glime, 2007; Tyler, 1990). However, *Scopelophila cataractae* showed promoted protonemata formation only after copper but not after zinc treatments (Konno et al., 2010; Nomura and Hasezawa, 2011). Therefore, metal as well as species specific differences have to be considered.

An important morphological alteration upon metals was the reduction of cell size. Rising metal concentrations led to a significant decrease in cell length with a significant correlation to zinc content of the tissue in both protonemata and gametophores (Sassmann et al., 2015b). However, no such significant correlations could be found concerning cell width. Copper treatments did not show any significant correlation in neither the length nor the width of cells in both tissues. A possible explanation could be that heavy metal adsorption by cell walls causes stiffening of the cell walls; such harder cell walls could hinder cell elongation by turgor pressure. In *F. hygrometrica* thicker cell walls were caused by deposition of callose (Krzesłowska and Woźny, 1996). In our *P. patens*, however, we could not observe thicker cell walls. The shorter cells did not necessarily result in an overall, proportionally reduced growth. A possible compensation of the reduced cell length by increased cell division rates is a possible explanation in *P. patens* but will need further investigation.

5.3 Metal uptake

Metal tolerance and growth changes were strongly anion dependent (Sassmann et al., 2015a; Sassmann et al., 2010); we expected a similar influence of free metal ion concentrations on metal uptake since they are considered as a major plant available fraction (Twiss et al., 2001). In sulfate and chloride solutions the amount of free metal ions is relatively high. In EDTA-chelates, however, metal ions are bound efficiently to the complex resulting in a very limited amount of free metal ions,

despite their extremely high water solubility. This might be the reason for the relatively low toxicity of metal EDTA chelates to *P. patens*; the addition of 1 mM Zn-EDTA to the medium resulted in only 0.0003 mM of free Zn^{2+} while an addition of 1 mM $ZnSO_4$ resulted in 0.1 mM of free Zn^{2+} (Sassmann et al., 2015a).

Metal uptake of the gametophore and the protonemata was determined using EDX on a SEM. Metal and anion dependent differences in metal uptake were found. As our calculation for free metal ion availability had predicted, metals from EDTA-chelates were taken up less than metals from chlorides or sulfates (Sassmann et al., 2015b). However, at higher concentrations (10 and 100 mM copper or zinc EDTA) a similar metal content was observed as in plants grown on media containing copper and zinc chlorides or sulfates (0.1 mM), while the available free metal ion concentration in the media was still comparatively low (Sassmann et al., 2015a).

Therefore, our results cannot only be explained by free metal ion concentrations but other players must be considered as well, for instance the apoplast or the cell wall. Exchange of the chelated metal to a highly affine cell wall is likely, but could not be calculated. Further, a possible capillary action or "wicking" needs to be considered; by transpiration and evaporation, nutrients but also heavy metals could be transported via the moss surface from the base to the upper parts. Although plantlets are grown in sterile culture dishes and transpiration was very limited, it cannot be fully excluded. However, simple surface metal enrichment without any binding to the cell wall is to be neglected as we thoroughly washed the plants prior to the measurements and any unbound metals were washed off. Further, we cannot exclude the possibility that *P. patens* is able to bind or store the whole EDTA-chelate apoplasticly, if only in a limited manner. How this uptake could be performed still needs to be evaluated. Further, research in this field is important especially since the use of EDTA is discussed for bioremediation although its role for plant availability and uptake remains unclear and both, promoted (Evangelou et al., 2007) as well as decreased metal uptake (Custos et al., 2014; Nowack et al., 2006) have been reported.

In addition to the anion dependent differences we also observed metal dependent differences for copper and zinc translocation. Zinc content was usually exceeding the copper content. *P. patens* is able to survive 5 mM $ZnCl_2$ in the growth media. Zinc content, however, usually reached a plateau both in gametophore and protonema cells, regardless of the added amount of zinc to growth media. Zinc uptake obviously was physiologically limited, remarkably already at added media concentrations of 500 µM $ZnCl_2$. This indicates active homeostasis and metal avoidance mechanisms or a limited number of binding sites in the cell wall. Such zinc homeostasis mechanisms were mostly investigated in *Arabidopsis thaliana* but some could be confirmed for *P. patens* (Sinclair and Kraemer, 2012). Boquete et al. (2011) reported decreased manganese uptake for *Pseudoscleropodium purum* under certain environmental conditions and questioned its suitability for biomonitoring of manganese. Therefore, knowledge of uptake decreasing factors is essential as they can influence the results of biomonitoring at highly polluted areas and lead to an underestimation of the actual deposition.

Apart from the increased protonemata formation under metal stress conditions, we also found highly significant correlations with the amount of metal uptake to the concentration of free metal ions. As protonemata formation seems to benefit from metal availability, we expected a general higher metal content in the protonemata in addition to a higher maximum of metal when compared to the gametophore. Our results confirmed a general higher metal content of the protonema as compared to the gametophore; maximum metal concentrations, however, reached the same maximum level. Sidhu and Brown (1996) described basal parts of mosses to contain more metal than the tip if grown on metal substrate. Since mosses possess no full vascular system, the proximity to the substrate may explain the higher metal content of the protonemata. However, it has to be considered that the protonemata may have other physiological tolerance mechanisms as than the gametophore. It has been reported that protonemata of *Funaria* can produce brachycytes, cells with a thickened cell wall formed under unfavorable environmental conditions (Decker et al., 2006; Schnepf and Reinhard, 1997). No such changes in gametophore cells were reported for *P. patens* but we observed conspicuously round protonema cells in some samples (data not shown).

5.4 Metal and ROS localization

Copper and zinc are both essential micronutrients and vital to plants. However, plants enforce different metal homeostasis mechanisms to keep metal concentrations in the cytoplasm within the physiological optimum (Cobbett, 2000; Hall, 2002). For zinc, those mechanisms were mostly discovered in the model plant *A. thaliana*. Zinc homeostasis can be maintained by a network of low molecular-weight ligands, membrane transport and zinc binding proteins with the vacuole as the main zinc storage site (Sinclair and Kraemer, 2012). In *Micrasterias denticulata*, metal compartmentalization of zinc, aluminum or copper after long term metal exposure was investigated by elemental energy loss spectroscopy on a transmission electron microscope (Volland et al., 2011). The authors localized all three metals in the cell wall but zinc occurred also in small vacuoles and mucilage vesicles. Copper was found in those compartments as well but was also incorporated into starch grains.

FluoZin-3, a zinc specific dye, was used to localize intracellular zinc in *P. patens* gametophore cells after 5 weeks long-term exposure to zinc. Confocal laser scanning micrographs indicated possibly zinc induced fluorescence in the cell wall, the central vacuole, small vesicles and in the chloroplasts (Sassmann, 2011). The cell wall as zinc retention and the vacuole as zinc storage sites were expected but the labeling of the chloroplasts was surprising as chloroplasts are not known to store zinc in mosses. Also, the role of the zinc containing small vesicles remains unclear. They may function as transport compartments for zinc or as zinc storage sites, similarly to the reported zinc containing small vacuoles found in *M. denticulata* (Volland et al., 2011). In contrast to *M. denticulata*, *P. patens* does not produce mucilage, rendering a secretion unlikely. However, transport into the apoplastic space is possible and further investigations on the ultrastructural level coupled with element analyses will hopefully shed more light on their function and zinc content in the near future.

Reactive oxygen species (ROS) such as H_2O_2 investigated here are important for plant signaling and occur regularly in physiological processes. In addition, H_2O_2 signaling (Rhee, 2006) and H_2O_2 bursts play an important role in plant defense towards pathogens, often in combination with the activation of antioxidant defense mechanisms (Low and Merida, 1996; Mehdy, 1994). However, high levels of H_2O_2 over a prolonged period cause damage to the cell and ultimately lead to cell death. Abiotic stress factors such as salinity and heavy metals are also known to induce augmented production of ROS in plant cells (Lutts and Lefevre, 2015; Viehweger, 2014), hence halophytes for instance are constitutionally well equipped to cope with oxidative stress (Bose et al., 2014; Ozgur et al., 2013). In the green alga *M. denticulata*, Darehshouri and Lutz-Meindl (2010) investigated H_2O_2 production after salt stress (KCl) using 2'7'-dichlorofluorescein (H_2DCFDA), a fluorescent ROS marker. The authors reported increased ROS induced fluorescence in the KCl treated cells and localized significantly more H_2O_2 at the plasma membrane, along the cell wall and chloroplasts, the cytoplasm and mitochondria, as compared to control. As metal stress has been reported to target the same physiological functions as salt stress (Lutts and Lefevre, 2015), we expected a similar localization of ROS labelling in *P. patens* after the application of the H_2O_2 specific dye H_2DCFDA.

ROS levels of *P. patens* corresponded well to the available amount of metal in the tested media. Localizing H_2O_2, we observed fluorescence along the cell wall, the nucleus and its surrounding cytoplasm as well as the chloroplasts. Intensive fluorescence was found in specific vesicles and most likely in chloroplast-associated mitochondria or peroxisomes (Sassmann, 2014a). Furthermore, we compared fluorescence signals from *P. patens* to those from *P. drummondii* and *M. elongata* from copper rich habitats. Although Cu- and Zn-EDTA grown plants (1 mM and 10 mM) of *P. patens* do not seem to be stressed, the other two moss species showed rising ROS levels either for both metals (M. elongata) or for Zn-EDTA only (*P. drummondii*). Interestingly, less fluorescence was observed when the metal was added as sulfate, indicating that both species from the metal habitats showed low ROS levels. ROS labeling in *M. elongata* showed even less fluorescence than in control cells. In *P. patens* it was different: only $ZnSO_4$ showed no increase of ROS, whereas $CuSO_4$ increased the fluorescence in a similar amount as $CuCl_2$(Sassmann, 2014b). A possible reason for these differences could lie in the sulfur content of *P. patens* which was dependent on copper or zinc treatments, respectively (EDX-data not shown). Higher sulfur content of cells might be explained by a high level of sulfur containing metallothioneines, proteins known to complex surplus metal in the cells (Cobbett and Goldsbrough, 2002). Adequate sulfur supply was guaranteed for all plants as the growth media always contained $MgSO_4$.

5.5 Conclusions

Our studies lead to a better understanding of the model organism *P. patens* under the influence of excessive metal concentrations. Although a great variety of studies uses molecular tools in order to study stress tolerance, significant gaps remain concerning reactions of the moss on the level of whole cells, tissues and organs. The studies presented here bridge this gap by combining experiments on metal tolerance, metal uptake and metal localization. We showed that neither the total nor

the soluble amount of metals allows for a good estimate of toxicity, but free metal ions were identified as a crucial factor. Free metal ion concentration offers the best explanation for the observed levels of tolerance, for the shift in growth patterns and for metal uptake. The metal uptake of zinc was generally exceeding the amount of copper but was limited regardless of further increase in media concentrations.

P. patens tolerated and survived similar metal concentrations as mosses occurring on metal enriched habitats. However, further investigations of ROS induced by metal stress revealed that it suffered significantly under metal exposition whereas the facultative metallophyte *P. drummondii* showed little stress signs and the obligatory metallophyte *M. elongata* even benefited. Thus, measurements of stress levels seem to reflect the habitat preferences of mosses more adequately than determination of survival rates. Metal and ROS localization in *P. patens*, *P. drummondii* and *M. elongata* are currently addressed in ongoing studies.

Finally, our results indicate that the relation between metal exposition and metal uptake by mosses is not necessarily linear and raise questions towards the general suitability of mosses for biomonitoring.

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6. Curriculum vitae

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Curriculum Vitae

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Born 1982 in Zwettl, Austria
Austrian citizenship

Education

since 2010	PhD in Biology (PhD thesis "The moss *Physcomitrella patens* under heavy metal stress: Morphological effects, intracellular localization and the detection of stress-induced metabolites.")
2006-2010	Master Ecology, University of Vienna, Austria (Graduation with distinction, Master thesis "*Heavy metal tolerance and localization* in the moss *Physcomitrella patens*")
2005-2006	Studies of Ecology, (Erasmus) Université Claude Bernard – Lyon 1, France
2002-2004	Studies of Biology, First Degree, University of Vienna, Austria
2001	Highschool graduation with distinction (Commercial highschool, Gmünd, Austria)

Career

since 2011	University Assistant at the Core Facility of Cell Imaging and Ultrastructure Research, University of Vienna, Austria
2010	Research Assistant at the Core Facility of Cell Imaging and Ultrastructure Research, University of Vienna, Austria

Awards and Grants

2015	1st Prize at the photo contest of the Faculty of Life Sciences (Vienna)
2011	Grant of the „Siegfried Ludwig-Fonds"
2010	"Research fellowship 2010" of the University of Vienna
2009	Scholarship for the diploma thesis of the „Society for the Advancement of Plant Sciences"
2008	Grant of the „Siegfried Ludwig-Fonds"
2008	Grant of the "Windhag-Stipendienstiftung für Niederösterreich"

Professional activities

2013-2014	Project leader of "Heavy metal stress in plant cells" a national research project of the "Vienna Anniversary Foundation for Higher Education, H-2486/2012"
2011-2014	Research Associate in the international bioremediation project "BIOREM"

Teaching

Lecturer

2010 - 2015 "Light and Video Microscopy in Theory and Practice"
2013 "The Plant Cell: Physiological and Ecological Aspects"
2010 "Ecology and Physiology of Cells"

Teaching Assistant

2009 - 2010 "Cells in the light microscope"
2009 - 2010 "Light and Video Microscopy in Theory and Practice "
2010 "Functional Cytology and Anatomy of Plants - Structure and function of glands of carnivorous plants"

Research interests

(1) Cell and molecular biology
(2) Plant metal interactions
(3) Microscopy

Review Activities Environmental and Experimental Botany
Acta Physiologiae Plantarum

Publications

Peer reviewed Journals

Sassmann, S, Weidinger, M, Adlassnig, W, Hofhansl, F, Bock, B & Lang, I 2015, 'Zinc and copper uptake in *Physcomitrella patens*: Limitations and effects on growth and morphology' Environmental and Experimental Botany, vol 118, no. 0, pp. 12-20., http://dx.doi.org/10.1016/j.envexpbot.2015.05.003

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Congress contributions

Turetschek, R, Holzbach, S, Epple, T, **Sassmann, S**, Desalegn, G, Kaul, H-P & Wienkoop, S 2015, 'Cultivar specific symbiotic teamwork in *Pisum sativum*' COST Workgroup Omics, Lisbon, Portugal, 25/02/15 - 26/02/15.

Sassmann, S, Weidinger, M, Bock, B, Antreich, S, Adlassnig, W & Lang, I 2014, 'Metal Treatment on *Physcomitrella patens* Compared to two Bryophyte Species Naturally Occurring on Metal Contaminated Sites', 18th International Microscopy Congress, Prague, Czech Republic, 7/09/14 - 12/09/14.

Steinacher R, Adlassnig W, **Sassmann S**, et al. 2014, 'Correlative microscopy of a metal accumulating biofilm', 18th International Microscopy Congress, Prague, Czech Republic, 7/09/14 - 12/09/14.

Koller-Peroutka M, Hefel B, Adlassnig W, Adamec L, **Sassmann S**, Lichtscheidl IK 2014, 'Element analysis by EDX in aquatic carnivorous plant', 18th International Microscopy Congress, Prague, Czech Republic, 7/09/14 - 12/09/14.

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Meyer I, Adlassnig W, Vrbecky L, **Sassmann S,** et al. 2014, '*Phragmites australis* as a pioneer on mine waste substrate', 20th Meeting of the Austrian Society of Plant Biology (ATSPB), Lunz am See, Austria, 19/06/14 - 21/06/14..

Vrbecky L, Adlassnig W, Meyer I, Steinacher R, **Sassmann S**, Lichtscheidl I 2014, '*Plantago* as a candidate for mine waste revegetation', 20th Meeting of the Austrian Society of Plant Biology (ATSPB), Lunz am See, Austria, 19/06/14 - 21/06/14

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Sassmann, S, Weidinger, M, Adlassnig, W, Lichtscheidl-Schultz, I & Lang, I 2013, 'X-Ray Microanalysis of Copper and Zinc Uptake in the Moss *Physcomitrella patens* HEDW', Oral presentation at the 5th Eurobiotech, Cracow, Poland, 8/10/13 - 11/10/13.

Adlassnig W, Grawunder A, **Sassmann S**, Horvath A, Koller-Peroutka M 2013, 'Amphibians at metal and metalloid contaminated habitats', In: Adlassnig W, Lichtscheidl I eds. 4th Biorem Meeting. Cracow, Poland, 11/10/13.

Adlassnig W, Pérez N, **Sassmann S**, Reipert S, Hofhansl F, Lichtscheidl IK 2013, 'Bioremediation by *Phormidium* biofilms. A case study from a historic mine site', 5th Eurobiotech, Cracow, Poland, 8/10/13 - 11/10/13.

Sassmann, S & Lang, I 2012, 'Protonema growth of *Physcomitrella patens* under zinc and copper stress', 19th Meeting of the Austrian Society of Plant Biology (ATSPB), Lienz, Austria, 7/06/12 - 10/06/12.

Sassmann, S, Weidinger, M, Lichtscheidl-Schultz, I & Lang, I 2011, 'A comparative study of heavy metal uptake by X-Ray microanalysis and zinc localization in the moss *Physcomitrella patens*', Botanical Microscopy Meeting, Wageningen, Netherlands, 16/04/11 - 21/04/11.

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Wernitznig S, **Sassmann S**, Lichtscheidl I, Lang I 2010, 'Uptake and cellular visualization of Zinc in the moss *Pohlia drummondii*', 18th Meeting of the Austrian Society of Plant Biology (ATSPB), Illmitz, Burgenland, Austria, 3/06/10 - 6/06/10.

Wernitznig S, Lang I, Weidinger M, **Sassmann S**, Lichtscheidl I 2009, 'The heavy metal distribution in two copper tolerant bryophytes *Pohlia drummondii* and *Mielichhoferia elongata*', Microscopy Conference 2009, Graz, Austria, 30/08/09 - 4/09/09.

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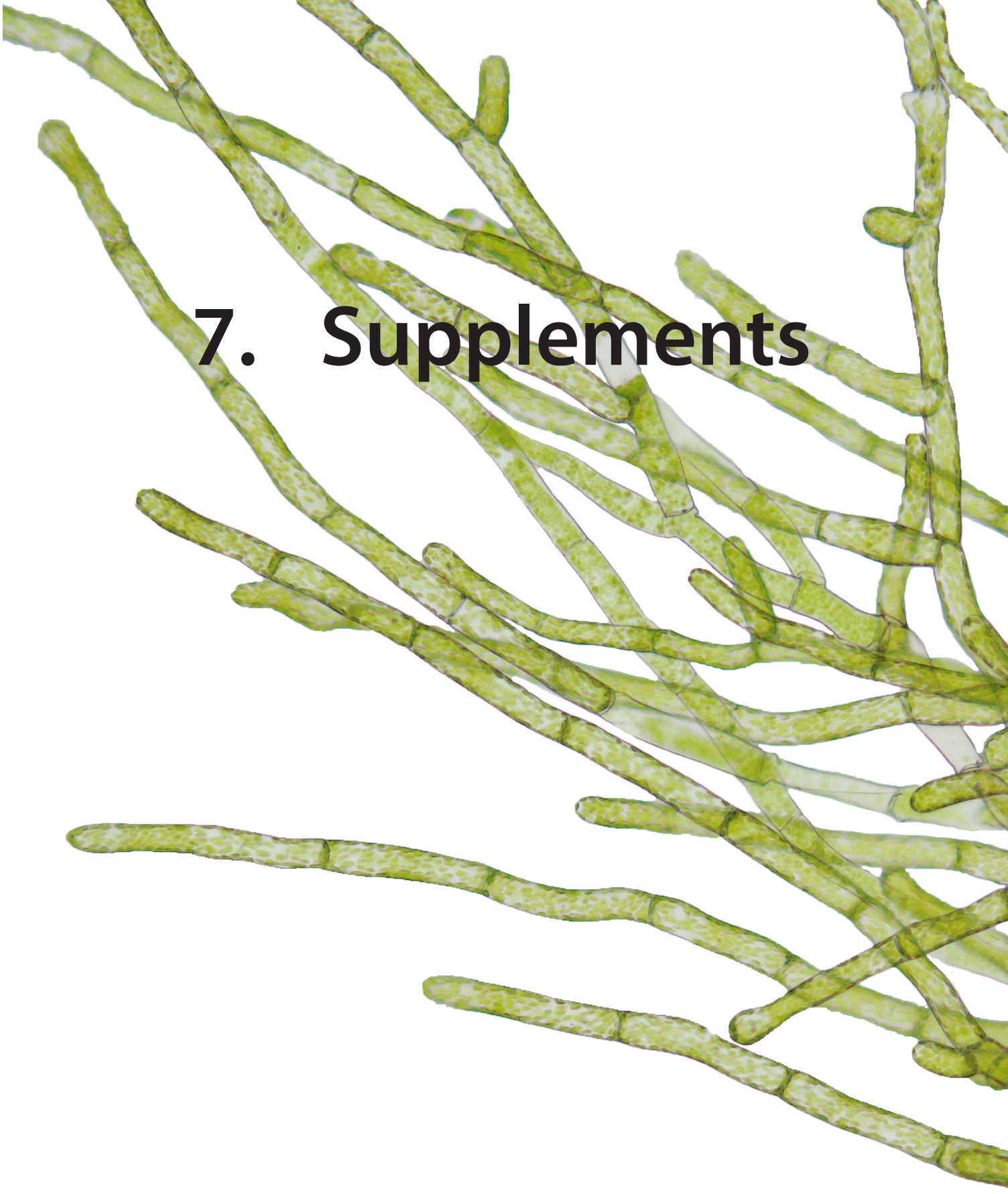

7. Supplements

7. Supplements

A. Lectures and Abstracts

19th Meeting of the Austrian Society of Plant Biology (ATSPB),

Lienz, Austria, 7/06/12 - 10/06/12

Lecture on:

"A Study on heavy metal uptake by X-Ray microanalysis in the Moss *Physcomitrella patens* Hedw."

Sassmann, S., Weidinger, M., Adlassnig, W., Lichtscheidl, I., Lang, I.

A Study on Heavy Metal Uptake by X-Ray Microanalysis in the Moss *Physcomitrella patens* Hedw.

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In the literature, mosses are discussed to be tolerant to heavy metals and to accumulate heavy metals regardless of their original habitats. The reasons for this resistance are still poorly understood. By contrast to vascular plants, mosses take up nutrients by the entire surface and do not possess exclusion mechanisms like the Casparian strip of roots. In preliminary experiments, we could confirm an exceptionally high zinc tolerance of moss gametophytes from heavy metal contaminated mining sites and also from *Physcomitrella patens*, not naturally occurring on heavy metal enriched habitats.
We offered zinc (as Zn-Ethylenediaminetetraacetic acid [EDTA], $ZnCl_2$ and $ZnSO_4$) and copper (as Cu-EDTA, $CuCl_2$ and $CuSO_4$). Gametophytes of *P. patens* were cultivated on sterile agar plates. After five weeks of cultivation the moss samples were washed, air dried, mounted and carbon-coated. During sample preparation, great attention was paid to avoid contamination with the heavy metal containing growth medium. Copper and zinc uptake of *P. patens* leaves was analysed by a semiquantitative method with X-ray microanalysis (EDX) in a scanning electron microscope (SEM).
We could observe differences in tolerance and uptake of copper and zinc. Furthermore, our results show a gradient elevation of the heavy metal content in leaf tissue corresponding to the added amount of zinc and copper in the growth media could be observed; however, our results indicate a difference in uptake depending on the anion of the heavy metal. For example P. patens did survive significantly higher amounts of zinc and copper if the metals were offered in combination with EDTA.

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5th Eurobiotech Meeting

Cracow, Poland, 8/10/13 - 11/10/13

Lecture on:
"X-Ray Microanalysis of Copper and Zinc Uptake in the Moss *Physcomitrella patens* HEDW"

Sassmann, S., Weidinger, M., Adlassnig, W., Lichtscheidl, I. & Lang, I.

Co-Author of the lecture on:
"Bioremediation by *Phormidium* biofilms. A case study from a historic mine site"

Adlassnig, W., Pérez, N., Sassmann, S., Reipert, S., Hofhansl, F., Lichtscheidl, I.

L7.5

X-Ray Microanalysis of Copper and Zinc Uptake in the Moss *Physcomitrella patens* Hedw

S. Sassmann, M. Weidinger, W. Adlassnig, I. Lichtscheidl, I. Lang

University of Vienna, Core Facility Cell Imaging and Ultrastructure Research, Althanstraße 14, A-1090 Vienna, Austria

Mosses are frequently regarded as tolerant to toxic metals and as accumulators of such metals regardless of their original habitats. The reasons for this tolerance are poorly understood. Unlike vascular plants, mosses absorb nutrients *via* the entire surface, and do not possess exclusion mechanisms like an exodermis or an endodermis. In preliminary experiments, we confirmed an exceptionally high zinc tolerance of moss gametophytes from heavy metal contaminated mining sites, and also for *Physcomitrella patens* Hedw. (Funariaceae) which avoids metal enriched habitats in nature.

Gametophytes of *P. patens* were cultivated on sterile agar plates. We offered zinc (as Na_2Zn EDTA, $ZnCl_2$ and $ZnSO_4$) and copper (as Na_2Cu EDTA, $CuCl_2$ and $CuSO_4$). After five weeks of cultivation the moss samples were washed, air-dried, mounted and carbon-coated. During sample preparation, great attention was paid to avoid contamination with the metal rich growth medium. Copper and zinc uptake of *P. patens* leaves were semi-quantitatively analysed by X-ray microanalysis in a scanning electron microscope.

We observed differences in tolerance and uptake of copper and zinc. A gradient elevation of the heavy metal content of leaf tissues corresponding to the added amount of zinc and copper in the growth media could be observed. Furthermore, our results indicate strong differences in uptake and resistance depending on the anion. E. g., metals coordinated with EDTA proved to be significantly less toxic for *P. patens*. The relevance of coordinating agents for metal toxicity is discussed.

Acknowledgements

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L7.6

Bioremediation by *Phormidium* Biofilms a Case Study from a Historic Mine Site

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Mining and ore processing frequently results in the formation of metal rich drainage water, representing a serious threat for people and environment. The remediation of such waters is difficult and expensive, and alternatives to conventional water treatment are intensively investigated.

At a historic mining site in the Austrian Alps, a Cu contaminated creek is remediating *via* constant binding of metals to microbial mats. This study deals with the localisation of the absorbed copper, as well as the isotopic signature and species composition of the microbial community.

The biofilm is almost exclusively dominated by the Cyanobacterium *Phormidum* sp. and contains 3.9 ± 1.8% Cu. The constant absorption from the water reduces the Cu content of the creek from 0.6 ± 0.1 mg · l^{-1} to 0.2 ± 0.2 mg · kg^{-1} after only 300 m, thereby exceeding abiotic Cu precipitation by far. Ancient dead layers of biofilm covered by humus but still rich in Cu indicate that the immobilisation is permanent. In spite of the variable Cu content of the biofilm, it exhibits a remarkably constant $\delta^{65}Cu$ of 0.5 ± 0.1‰ which is significantly higher than in the contaminated water (−0.9 ± 0.2‰) making uptake of the Cu into the living cell improbable. This is confirmed by the ubiquitous occurrence of electron dense mineral particles in the gelatinous sheath of *Phormidium*, partly identified as the secondary Cu mineral sampleite.

Fixed mats of *Phormidium* have been frequently used for metal absorption under laboratory conditions. The spontaneous self-remediation of a creek by *Phormidium*, however, is a novel feature and suggests new strategies for the application of Cyanobacteria in the field of bioremediation.

Acknowledgements

We are grateful to Prof. Dr. A. Beran (University of Vienna) for the identification of sampleite. Thanks are due to Forstmeister R. Schilcher, who made scientific research in the Schwarzwand possible. This study was supported by the EU project Umbrella (EU 226870), the Appear project Biorem and the OEAD project Promote.

20th Meeting of the Austrian Society of Plant Biology (ATSPB)

Lunz am See, Austria, 19/06/14 - 21/06/14

Lecture on:
"Response of Three Different Bryophyte Species to Cu and Zn Treatment"

Sassmann, S., Bock, B., Weidinger, M., Adlassnig, W., Antreich, S., Lichtscheidl, I. & Lang, I.

Response of three different bryophyte species to Cu and Zn treatment

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Bryophytes are non-vascular, multicellular land plants and inhabit extremely different habitats, ranging from temporary dry river banks (e.g. *Physcomitrella patens*) to metal contaminated sites (e.g. *Pohlia drummondii* and *Mielichhoferia elongata*). Therefore, some bryophyte species are considered stress tolerant, and even the supposedly metal sensitive moss *P. patens* showed increased tolerance to Cu-EDTA in earlier studies [1].

In this study, the bryophytes were cultivated on sterile agar plates and tested for zinc (as Zn-EDTA, $ZnCl_2$ and $ZnSO_4$) and copper (as Cu-EDTA, $CuCl_2$ and $CuSO_4$) over a period of five weeks.

Despite of the high tolerance towards Cu-EDTA of *P. patens*, we measured changes in growth and metal uptake by X-ray microanalysis (EDX) in a scanning electron microscope if the metal is offered with different anions. Here, especially the uptake of EDTA chelated metals was significantly lower compared to metal offered as ionic salt. Modelling of ion availability using Visual MINTEQ [2] explained most of the differences in toxicity. In addition to EDX, the fluorescent dyes FluoZin-3 for zinc and Phen Green SK for copper were used to ensure intercellular metal uptake.

Reactive oxygen species (ROS) are a general indicator for stress and changes in the cellular content of ROS after staining with 2,7-dichlorofluorescein diacetate (H_2DCFDA) [3] were analyzed and the three different bryophyte species compared. *P. patens* showed only low H_2DCFDA fluorescence in control cells, in contrast to metal treated cells were increased ROS could be detected for chloroplast associated mitochondria, the nuclear region and the cell wall region.

Further investigation of cellular localization of metal deposition was performed using FluoZin-3^{tm} and is ongoing in transmission electron microscopy studies.

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This research was supported by the Vienna Anniversary Foundation for Higher Education (grant H-2486/2012 to S.S.) and the ŒAD (*Appear*-43/BIOREM)

20th Meeting of the Austrian Society of Plant Biology (ATSPB)

Lunz am See, Austria, 19/06/14 - 21/06/14

Co-Author of the lecture on:
"Cytosceletal elements and plasmolysis: stabilisation under exceptional circumstances"

Lang, I., Sassmann, S., Komis, G.

Cytoskeletal elements and plasmolysis: stabilisation under exceptional circumstances

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Many will know the process of plasmolysis from their student days: hyperosmotic solutions extract water from the plant cell, turgor pressure is lost and finally, the living protoplast becomes detached from the cell wall. This process is reversible (deplasmolysis) and serves as a proof for the vitality of plant cells. The phenomenon of plasmolysis is known for a long time [1, 2] and still, the use of GFP-transformed plants opened up new possibilities to analyse this process and trace e.g. cytoskeletal elements in living cells. Closely considered, some questions are still not fully answered, even to date.

In plasmolysed cells, a membranous network (Hechtian reticulum) remains attached to the inner side of the cell wall and provides the anchor and contact of the plasma membrane to the wall. Hechtian strands [3] span the space between the Hechtian reticulum and the plasmolysed protoplast and partly preserve the membrane material that is lost in plasmolysis. This is impressively shown after staining with the membrane dye DiOC6 [4] or in the scanning electron microscope. However, structural hints for the anchoring of the plasma membrane at the cell wall are rare [4] and only a few studies exist on the role of the cytoskeleton during plasmolysis [5,6]. In a turgid cell, cortical cytoskeletal elements are located in close vicinity to the plasma membrane and cell wall; how are microtubules and actin microfilaments behaving in plasmolysing cells and how do they accommodate in the smaller shape of a plasmolysed protoplast? In Arabidopsis expressing cytoskeletal GFP-tags, we analysed microtubules and actin microfilaments during plasmolysis and deplasmolysis and documented their reorganisation in time lapse studies. We want to follow the dynamics of cytoskeletal elements in plasmolysis and deplasmolysis and analyse their role in the stabilisation of the plasmolysed state.

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B. Posters and Abstracts

18th Meeting of the Austrian Society of Plant Biology (ATSPB)

Illmitz, Austria, 03/06/10 - 05/06/10

Poster title: "Zinc uptake and localization in the moss *Physcomitrella patens*"

Sassmann, S., Wernitznig, S., Lang, I., Lichtscheidl, I.

Abstract:

Zinc uptake and localization in the moss *Physcomitrella patens*

STEFAN SASSMANN, STEFAN WERNITZNIG, INGEBORG LANG, IRENE LICHTSCHEIDL

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Mosses are discussed to be resistant to heavy metals and to accumulate heavy metals regardless of their original habitats. The reasons for this resistance are still poorly understood. By contrast to flowering plants, mosses take up nutrients by the entire surface and do not possess any exclusion mechanisms like the Casparian strip of roots. In preliminary experiments, we could confirm an exceptionally high zinc tolerance of moss gametophytes. Zinc uptake was analysed by a semiquantitative method with X-ray microanalysis (EDX) on a scanning electron microscope (SEM). Moss samples were air dried, mounted and carbon-coated. During sample preparation, great attention was paid to avoid contamination with the zinc-containing growth medium. A gradient elevation of the zinc content in leaf tissue corresponding to the offered amount of zinc in the growth media could be observed.

On the cellular level zinc localization was performed on a confocal laser scanning microscope using a fluorescent heavy metal tracer dye (FluoZin™-3). Compared to controls, the leaf cells of plants grown on heavy metal spiked media indicated a strong relation of the fluorescence intensity to offered zinc concentrations. In low zinc concentrations, we found "doughnut-shaped" chloroplasts with conspicuous zinc staining; in some higher concentrations, small vesicles were labeled with FluoZin™-3.

This research was supported by a grant of the „Gesellschaft zur Förderung für Pflanzenwissenschaften" to SS.

Zinc uptake and localization in the moss *Physcomitrella patens*

Stefan Sassmann[1], Stefan Wernitznig[1], Marieluise Weidinger[1], Irene Lichtscheidl[1], Ingeborg Lang[1]

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Introduction

Physcomitrella patens is a non-vascular, multicellular land plant and belongs to the bryophytes. Due to the simple morphology of the moss and its facile *in vitro* cultivation, *P. patens* has become a model organism in plant and molecular biology [1]. Most mosses are specialists that inhabit ecological niches and are discussed to be resistant to heavy metals and to accumulate heavy metals regardless of their original habitats. *P. patens* that normally occurs on uncontaminated soil showed exceptionally high zinc tolerance of moss gametophytes.

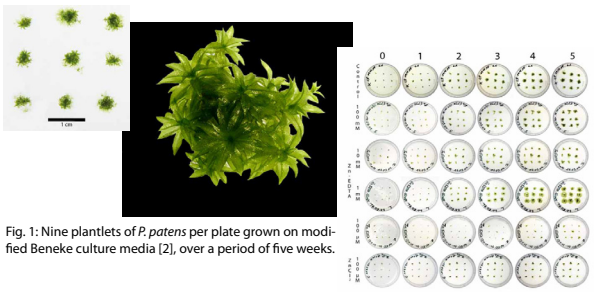


Fig. 1: Nine plantlets of *P. patens* per plate grown on modified Beneke culture media [2], over a period of five weeks.

Electron dispersive X-ray spectroscopy (EDX-SEM)

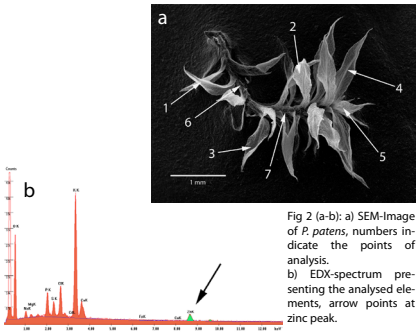


Fig 2 (a-b): a) SEM-Image of *P. patens*, numbers indicate the points of analysis.
b) EDX-spectrum presenting the analysed elements, arrow points at zinc peak.

Figure 2 shows a typical EDX-spectrum with a distinct zinc-peak (arrow). This element analysis was taken of a plant grown on agar spiked with $ZnCl_2$ 5 mM. At least seven measurements per plant were take and the results are shown in Figure 3. The uptake correlated with the amount of zinc offered in a gradient manner. $ZnCl_2$ is more harmful to *P. patens* than Zn-EDTA: on $ZnCl_2$-plates, the plants survived up to a concentration of 5 mM whereas the highest Zn-level for the EDTA-plates was shown in the probes containing Zn-EDTA 100 mM. Low amounts of $ZnCl_2$ accumulated stronger within the plants than low amounts of Zn-EDTA.

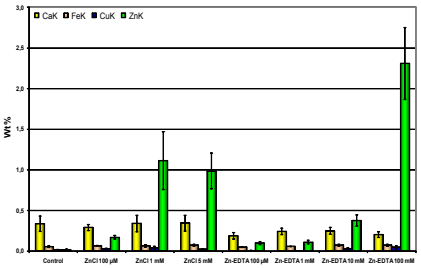
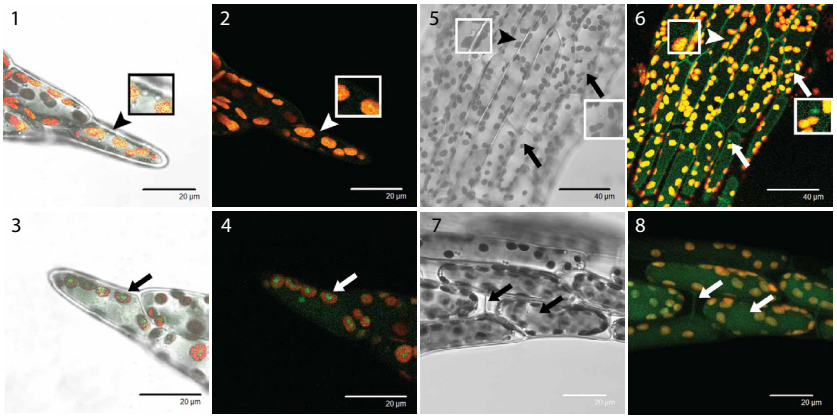


Fig. 3 EDX-Results of *P. patens* grown on differend zinc spiked media ($p<0.05$).

Confocal laser scanning microscopy (CLSM)



In order to compare the changes of fluorescence intensity, the same adjustments of the CLSM where used for all samples. Compared to control (1-2), the leaf cells of plants grown on heavy metal spiked media indicated a strong relation of the fluorescence intensity to offered zinc concentrations. In low zinc concentrations (100 µM - 1 mM), we found "doughnut-shaped" chloroplasts (3-4) with conspicuous zinc staining. In some higher zinc concentrations (10 mM - 100 mM), small vesicles were labeled with FluoZin™-3. These vesicles as shown in 5-6 do not obligatory contain zinc. The differentiation of fluorescence between the cell wall and the protoplast was achieved by increasing the space between them by plasmolysis in 0.8 M D-Mannitol after staining with FluoZin™-3 (7-8).

Conclusion

As expected from the results of EDX-data, *P. patens* showed a rising fluorescence and therefore rising zinc content in the cells in relation to the zinc concentration of the growth medium. In more detail, zinc could be localized to the cell wall, the vacuole and to small zinc enriched vesicles in cells grown on zinc spiked media whereas control cells stayed nearly unlabeled. Until now, we cannot fully explain why our data showed a higher zinc uptake on $ZnCl_2$ media compared to the same concentration on Zn-EDTA media. Further experiments are underway to specifically locate zinc storages within the cells by TEM-EELS and to show the impact of other heavy metals such as copper and cadmium.

Acknowledgement

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This research was supported by the Hochschuljubiläumsstiftung der Stadt Wien, project H-1939/2008, to I.L.
This research was supported by the "Forschungsstipendium 2010" of the University of Vienna to S.S.

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18th Meeting of the Austrian Society of Plant Biology (ATSPB)

Illmitz, Austria, 03/06/10 - 05/06/10

Poster title: "Uptake and Cellular Visualisation of Zinc in the moss *Pohlia drummondii*"

Wernitznig, S., Sassmann, S., Lichtscheidl, I., Lang, I.

Abstract:

Uptake and cellular visualization of zinc in the moss *Pohlia drummondii*

STEFAN WERNITZNIG, STEFAN SASSMANN, IRENE LICHTSCHEIDL, INGEBORG LANG

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The moss *Pohlia drummondii* grows naturally on heavy metal rich soils. Previous investigations on heavy metal tolerance, showed a high uptake and also tolerance, especially for copper. However in comparison to the model organism *Physcomitrella patens*, neither in their uptake behaviour nor in their heavy metal tolerances no differences could be found. Due to the fact that *P. patens* does not occur naturally on heavy metal soils, a comparison study with *P. drummondii* was carried out to find similar or different characteristics towards zinc. Since there are no high zinc concentrations in the soil of their habitat known, it would be interesting to known how *P. drummondii* reacts towards this particular heavy metal.

In vitro grown *P. drummondii* gametophytes were cultivated on solid media with 1 mM and 10 mM Zn-EDTA. Analysis of elemental compositions of the gametophytes was carried out using X-ray microanalysis combined with SEM (Scanning Electron Microscopy). The results showed a higher content of zinc in the metal treated mosses. Further localization of the metal at the cellular level was carried out after FluoZn™-3 labeling at the CLSM. At both concentrations fluorescence could be detected in the cell walls of the gametophyte stems and leafs. Any accumulation of zinc ions in the plasma, vacuole or other cell compartments could not be confirmed.

Comparison to the results of *P. patens* suggests that most of the zinc is bound to the cell walls of *P. drummondii* and only a small amount of it reaches the plasma. These results illustrate the essential role of the cell wall in the tolerance mechanism of *P. drummondii*.

This work was financially supported by grant H-1939/2008 of the "Hochschuljubiläumsstiftung der Stadt Wien" to IL.

Uptake and Cellular Visualisation of Zinc in the Moss *Pohlia drummondii*

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Introduction

The moss *Pohlia drummondii* grows naturally on heavy metal rich soils. Previous investigations on heavy metal tolerance showed a high uptake, especially for copper. Recent studies about zinc tolerance and uptake were carried out with the model organism *Physcomitrella patens*. Since there are no high zinc concentrations in the soil of their habitat is interesting to know how *P. drummondii* as a heavy metal moss reacts towards this particular heavy metal.

Material & Methods

In vitro grown *P. drummondii* gametophytes were cultivated on solid Benecke media with 1 mM and 10 mM Zn-EDTA. At first analyses of elemental compositions of the gametophytes were carried out using X-ray microanalysis (EDX) combined with SEM. Further localization of the metal at the cellular level was carried out after FluoZn™-3 labeling (1h) at the CLSM. For better differentiation between cell wall and plasma membrane, leaves were plasmolyzed with 0,8 M D-Mannitol after FluoZn™-3 staining.

Results

EDX: The results showed a higher content of zinc in the metal treated mosses. A clear uptake could be found but it was not possible correlate a higher accumulation of zinc with a higher concentration in the media (Fig.1).

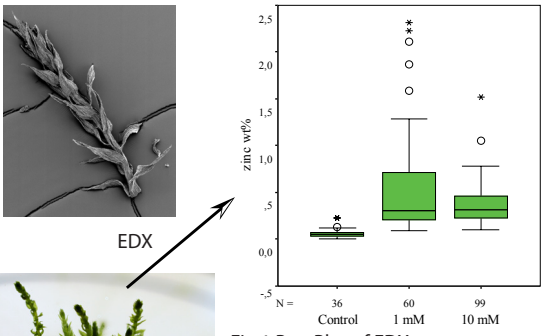
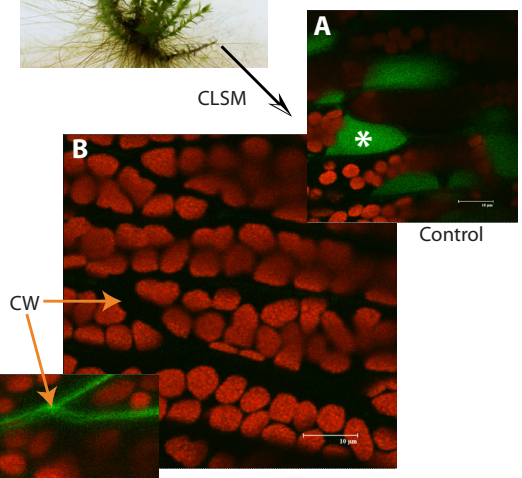
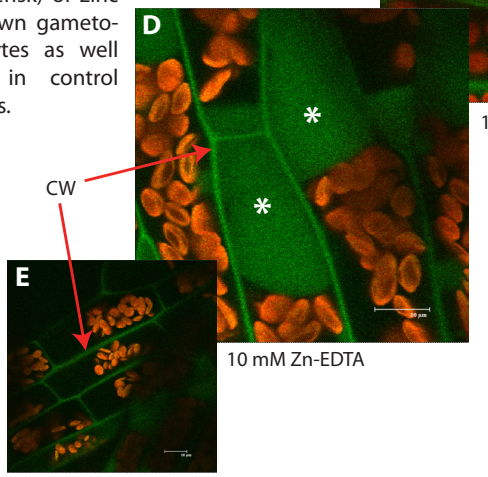


Fig.1 Box-Plot of EDX measurements

CLSM: At both zinc concentrations (C,D) fluorescence could be detected in the cell walls (CW) of the gametophyte stems (E) and leaves. Equally to the EDX results, the CLSM results did not show increased zinc levels in the cell wall when grown on higher concentrated media.

Any accumulation of zinc ions in the plasma, vacuole or other cell compartments could not be confirmed. In plasmolyzed cells, fluorescence could be detected in the plasmolytic space (A,D; asterisk) of zinc grown gametophytes as well as in control cells.





1 mM Zn-EDTA

10 mM Zn-EDTA

Stem 10 mM Zn-EDTA

Conclusion

It was possible to show that *Pohlia drummondii* is able to take up zinc from the media. Though the EDX data clearly confirmed an uptake, only the use of the CLSM and the specific labeling of zinc with FluoZn™-3 made it possible to show its cellular location. The clear detection in the cell walls of leaves and stem cells indicates them as the main barrier and important defense mechanism against toxic levels of ions. Due to this barrier, high concentrations of the metal ions in the plasma can be avoided. *P. drummondii* naturally grows on heavy metal contaminated soil; it seems that a mechanism to bind harmful heavy metal ions outside of the cell, e.g. in the cell wall, is the best way to tolerate high heavy metal concentrations. So it can be postulated that differences of the cell wall chemistry between *P. drummondii* and *Physcomitrella patens* (Poster Sassmann) are one reason for their different tolerance strategies.

Acknowledgement

This research was supported by the Hochschuljubiläumsstiftung der Stadt Wien, project H-1939/2008, to I.L.

19th Meeting of the Austrian Society of Plant Biology (ATSPB),

Lienz, Austria, 7/06/12 - 10/06/12

Poster title: "Protonema of *Physcomitrella patens* under copper stress"

Bock, B., Sassmann, S., Lang, I.

Abstract:

Protonema growth of *Physcomitrella patens* under zinc and copper stress

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The moss *Physcomitrella patens* belongs to the family Funariaceae.
After sequencing the whole genome of *P. patens* in 2008, this moss got a well established model organism, especially for studies in cell physiology and genetics. Additionally *P. patens* is easy to cultivate, has only a short lifecycle and the simple morphology makes direct intracellular observations possible.
Protonema is defined as multicellular filaments. It is a very primal, haploid structure, which is developed after spore germination.
The Protonemata of mosses show similar polar tip growth of root hairs of higher plants. Due to its direct exposure to the environment this cells show a faster response to stress factors. The Protonema of *P. patens* therefore is best suitable for my investigation concerning alterations of cell morphology and physiology.
Zinc and Copper in low concentrations are essential micronutrients. However, like all micronutrients there are deficient, optimal and toxic concentration levels for plant cells.
My thesis concentrates on the investigation of the effects on P. patens concerning zinc and copper in elevated concentrations reaching to the toxic level.
Physcomitrella patens not naturally occurring on heavy metal enriched soils. Therefore it is not considered as heavy metal moss, like the copper moss *Mielichhoferia elongata*. However, previous studies have shown that *Physcomitrella patens* showed a clear tolerance to heavy metals like zinc and copper. This study was mainly focussed on gametophyte growth and leaf cells.
My master thesis will concentrate on the tolerance of the protonema of *Physcomitrella patens* to zinc and copper stress. Different effects on the physiology and the tolerance of *P. patens* towards zinc and copper will be studied in short term (24 and 48 h) and long term experiments.

Protonemata of *Physcomitrella patens* under copper stress

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Introduction

The protonema is the first developmental stage of mosses, which is developed after spore germination or initially from fragments of the gametophyte. It shows polar tip growth very similar to root hairs of higher plants and pollen tubes. Due to its direct exposure to the environment, cells show a fast response to stress factors. Therefore, the protonema of the model plant *Physcomitrella patens* is best suitable for my investigation concerning alterations of cell morphology and physiology after copper treatments.

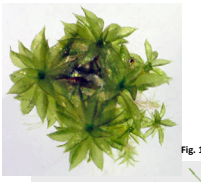

Fig. 1: Habitus of *Physcomitrella patens*

Fig. 2: Protonema growing from a single leave

Material & Methods

The moss *Physcomitrella patens* (Hedw.) was used in this study. Plants were grown under sterile conditions on solid Benecke medium modified according to Gang et al. (2003). The mosses grew in a growth chamber at 21.5° C under a light-dark regime of 14 h/10 h. Plants were exposed to different copper compounds (Cu-EDTA, $CuSO_4$, $CuCl_2$) in concentrations ranging from 0.1 to 100 mM for Cu-EDTA and from 0.05 to 0.1 mM for $CuSO_4$ and $CuCl_2$ were added to the medium.

An Olympus BX41 light microscope was used for documentation. CellD software was used to measure the cells (length and width). Due to the cylindrical shape of protonema cells the plasmometric method after Höfler (1918) was used to determine the osmotic value. The KS-test indicated normal distribution of all data, thus t-test and Pearson-test were used to test for differences and correlations.

Cell measurements

Fig. 3: Control protonema

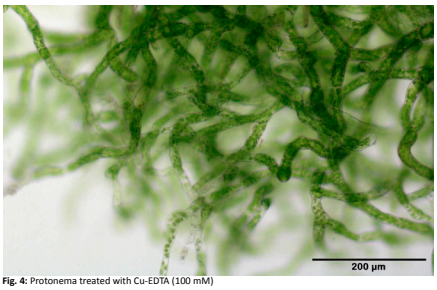

Fig. 4: Protonema treated with Cu-EDTA (100 mM)

Comparison of control and copper treated cells showed significant differences in the growth structure of the whole protonema as well as in protonema cells (see Fig. 3-6).

All copper treatments resulted in significantly reduced cell length ($p < 0.01$). Treatments with higher concentrations of Cu-EDTA (10 mM, 100 mM) as well as all treatments with $CuSO_4$ and $CuCl_2$ showed a significant increase in width ($p_{Cu\text{-}EDTA} < 0.05$, $p_{CuSO4} < 0.01$, $p_{CuCl2} < 0.01$).

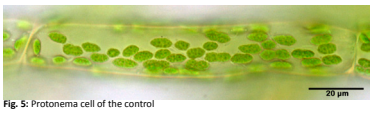

Fig. 5: Protonema cell of the control

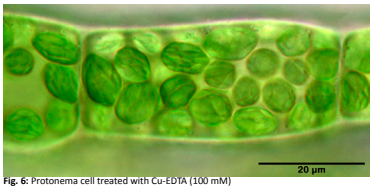

Fig. 6: Protonema cell treated with Cu-EDTA (100 mM)

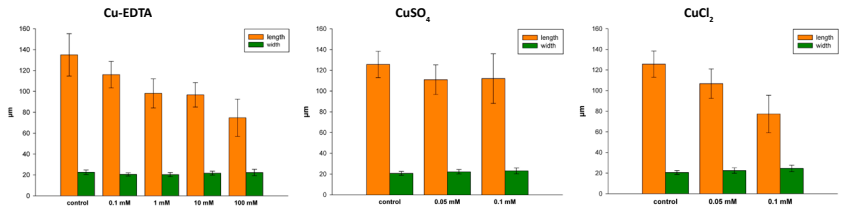

Fig. 7: Length and width of copper treated protonema cells in comparison to control cells

Osmotic value

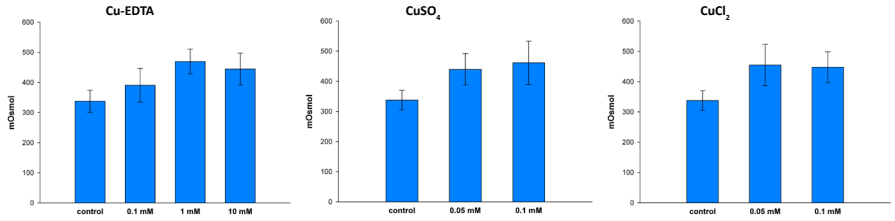

Fig. 8: Mean osmotic values of copper treated protonema cells in comparison to control cells

The protonema cells were plasmolysed using 90% KCl and 10% $CaCl_2$ of 800 mOsmol for a maximum of 20 minutes (Fig. 9). The mean osmotic values of the control cells was 337.7 mOsmol. Osmotic value increased significantly in all copper treated protonema cells ($p < 0.01$). A maximum value of 469.7 mOsmol was achieved in cells treated with 1 mM Cu-EDTA.

Fig. 9: Convex plasmolysis of protonema cells in 800 mOsmol KCl/$CaCl_2$

Conclusions

According to Fig. 7 and 8 copper has a major impact on the cell dimensions and the osmotic value of the cells. There are different approaches to explain these differences:

1. Due to the high stress caused by the heavy metal treatment, cells stay smaller thus the vacuole content is less diluted. This would lead to an increase in the osmotic value of the cell.
2. Due to binding of copper ions cell walls stiffen, thus cells must develop a higher turgor pressure to perform growth
3. Stress induced metabolites may additionally increase their osmotic value.

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Acknowledgements

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19th Meeting of the Austrian Society of Plant Biology (ATSPB),

Lienz, Austria, 7/06/12 - 10/06/12

Poster title: "Die Wirkung erhöhter UV-B Strahlung auf das Moos Physcomitrella patens"

Ruplitsch, B., Sassmann, S., Lichtscheidl, I., Lang, I.

Abstract:

The effects of enhanced UV-B radiation on the moss *Physcomitrella patens*

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Although small in size, mosses show a great ability to adapt to various stress factors enabling them to inhabit ecological niches. However, the fully sequenced genome of the model moss *Physcomitrella patens* L. (Funariaceae) has not yet provided satisfactory results to answer these ecological questions and cell biological experiments are still missing to backup genetic and molecular studies. Here, we analysed the effects of enhanced UV-B radiation which would occur due to a depletion of the ozone layer. Even in the northern hemisphere, the reduced ozone layer is becoming an increasing concern.
Moss plantlets of *Physcomitrella patens* L. (Funariaceae) were grown in sterile tissue culture under constant light and temperature conditions. Apart from these controls, plates were irradiated with additional UV-B (290-315nm; 48µMs^{-1}m^{-2}). Differences at the cellular level were analysed microscopically. Furthermore, the probes were tested for enhanced secondary metabolites in High Performance Liquid Chromatography (HPLC).
After four weeks of UV-B radiation, we found a strong morphological effect. Older moss plants reacted in an increased production of biomass. By contrast, the radiation of young samples resulted in reduced growth and the plantlets produced less protonemata and biomass. HPLC analyses showed increased levels of benzoic acid, a common stress indication.
In a set of ongoing experiments, we will look further into the subcellular architecture to detect possible effects of UV-B on actin microfilaments or the endoplasmic reticulum.

Acknowledgements: For the construction of the shelf for UV radiation, we greatly acknowledge the help and expertise of Robert Kartusch. Franz Hadacek kindly provided access to the HPLC equipment. This work was supported by a grant of the "Verein zur Förderung der Pflanzenwissenschaften" to B.R.

Die Wirkung erhöhter UV-B Strahlung auf das Moos *Physcomitrella patens*

Barbara Ruplitsch, Stefan Sassmann, Irene Lichtscheidl, Ingeborg Lang

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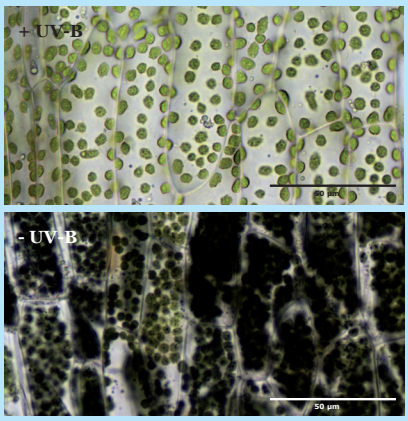
EINLEITUNG

Durch die Abnahme der Ozonschicht in der Stratosphäre steigt der Anteil der UV-B Strahlung (290-315 nm), der die Erdoberfläche erreicht - auch in der nördlichen Hemispäre. In der vorliegenden Versuchsreihe haben wir diese Tatsache simuliert, Moospflänzchen von *Physcomitrella patens* unter erhöhter UV-B Strahlung gezogen und verschiedene Auswirkungen auf die Zellen analysiert.

MATERIAL & METHODEN

Die Gametophyten von *Physcomitrella patens* L. (Funariaceae) wurden als Sterilkultur in Petrischalen auf Kontrollmedium gezogen (14 h Tag/10 h Nacht, 25 °C). Die erhöhte UV-B Strahlung (290-315 nm, Lichtintensität 48 µM pro sek und m²) erfolgte für vier Wochen, wobei die Biomassezu-/abnahme wöchentlich dokumentiert wurde. Anzahl der Zellkerne nach DAPI-Färbung, physiologische Veränderungen und die Produktion von sekundären Inhaltsstoffen wie z.B. Benzoesäure (HPLC-Analyse) wurden gemessen.

Langsamer Energiestoffwechsel

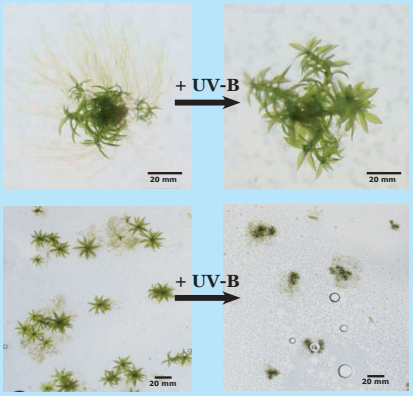


Obwohl die Zellen optisch nicht beeinträchtigt erscheinen, zeigt eine Färbung mit dem Redox-Farbstoff NBT, dass Stoffwechselvorgänge der Atmungskette verlangsamt werden. In unbestrahlten Zellen bewirkt NBT (Nitroblau-Tetrazolimchlorid, SERVA; 0.004%) eine blauschwarze Färbung von unslöslichem Di-Formazan. Nach Bestrahlung der Zellen mit 310 nm für 45 sek bleibt diese Färbung aus.

DANKE

Herzlichen Dank an Robert Kartusch für seine Expertise beim Aufbau der UV-Bestrahlungseinrichtung. Franz Hadacek ermöglichte die Benutzung der HPLC. Die Arbeit wurde durch den "Verein zur Förderung der Pflanzenwissenschaften" unterstützt.

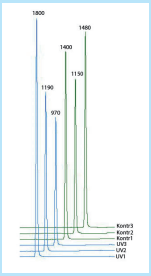
Altersunterschiede



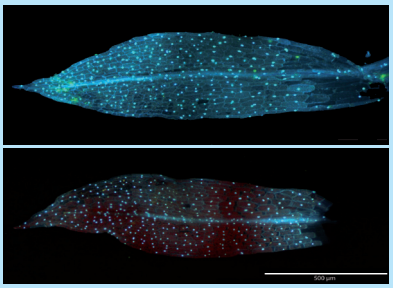
Das Alter der Zellen zum Zeitpunkt der erhöhten UV-B Belastung spielt eine entscheidende Rolle: Transfer von vier Wochen alten Gametophyten führt zu Biomassezuwachs, während die Überimpfung von ganz jungen Pflänzchen zu einer Schädigung durch UV-B führt.

Erhöhte sekundäre Inhaltsstoffe

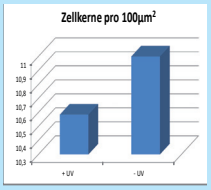
Mittels HPLC (High pressure liquid chromatography) wurde ein Anstieg des sekundären Inhaltstoffes Benzoesäure gemessen, was als Schutzreaktion vor zu hoher UV-B Strahlung gewertet werden kann. Allerdings sind auch die Werte in den unbehandelten Kontrollpflanzen erhöht.



Gleiche Anzahl der Zellkerne



Nach DAPI-Färbung wurden Blattflächen von 100 µm² der bestrahlten und unbestrahlten Gametophyten verglichen. Da sich die Anzahl der Zellkerne in adulten Pflanzen vor und nach der Bestrahlung nicht signifikant unterscheidet, werden bei Biomassezuwachs nach UV-B Behandlung nicht mehr, sondern größere Zellen gebildet.
n=153 für Kontrolle; n=154 für UV-B.



DISKUSSION

Auf den ersten Blick kann *P. patens* durchaus mit erhöhter UV-B Strahlung umgehen und bestätigt dadurch die generell hohe Toleranz von Moosen gegenüber Umwelteinflüssen. Allerdings gibt es Einschränkungen, die erst bei genauerer Analyse deutlich werden:
P. patens schützt sich durch erhöhte Produktion sekundärer Inhaltsstoffe und zeigt zudem verlangsamte Stoffwechselvorgänge der Atmungskette. Mit diesen Fakten kommen nur adulte Gametophyten zurecht; junge Pflänzchen halten dem erhöhten UV-Stress nicht stand.

ABSTRACT

Although small in size, mosses show a great ability to adapt to various stress factors enabling them to inhabit ecological niches. However, the fully sequenced genome of the model moss *Physcomitrella patens* L. (Funariaceae) has not yet provided satisfactory results to answer these ecological questions and cell biological experiments are still missing to backup genetic and molecular studies. Here, we analysed the effects of enhanced UV-B radiation which would occur due to a depletion of the ozone layer. Even in the northern hemisphere, the reduced ozone layer is becoming an increasing concern.
Moss plantlets of *Physcomitrella patens* L. (Funariaceae) were grown in sterile tissue culture under constant light and temperature conditions. Apart from these controls, plates were irradiated with additional UV-B (290-315 nm; 48 $\mu Ms^{-1}m^{-2}$). Differences at the cellular level were analysed microscopically. Furthermore, the probes were tested for enhanced secondary metabolites in High Performance Liquid Chromatography (HPLC).
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In a set of ongoing experiments, we will look further into the subcellular architecture to detect possible effects of UV-B on actin microfilaments or the endoplasmic reticulum.

Focus on Microscopy 2014 (FOM2014)

Sydney, Austrialia, 13/04/14 - 16/04/14

Poster title: "Metal Tolerance Strategies of Mosssses Differ at the Cellular Level"

Antreich S., Sassmann, S., Wernitznig, S., Weidinger, M., Lang, I.

Abstract:

METAL TOLERANCE STRATEGIES OF MOSSES DIFFER AT THE CELLULAR LEVEL

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KEY WORDS: Bryophytes, heavy metal tolerance, living cells, starch, CLSM, fluorescence labelling, polarized light, TEM, x-ray microanalysis

ABSTRACT

Many mosses inhabit ecological niches and have adapted to stress conditions like drought, irradiation, flooding or metal contamination. Our growth data on zinc spiked substrate show a very similar behaviour of *Physcomitrella patens* and *Pohlia drummondii*. However, specific dyes as well as diverse microscopy methods indicate different tolerance strategies at the cellular level. Fluorescence labelling with the zinc-specific dye FluoZin3 shows the retention of the metal in the cell wall of *P. drummondii* which might enable the occurrence of this species at former mining sites [1]. In *P. patens*, normally living at non-contaminated sites, the zinc-specific dye enters the cell and is apparently scavenged in the cytoplasm. Recent x-ray microanalyses confirm this phenomenon: mosses from metal habitats show less uptake than *P. patens*. In a new set of experiments, we are now analysing the influence of metal contamination on the photosynthetic pathway. In control cells, autochthonic starch can be detected at the TEM-level (Fig. 1) as well as by the use of polarized light microscopy or specific dyes like Lugol`s iodine and safranin. On copper and zinc spiked media, less autochthonic starch grains are observed in the chloroplasts. Interestingly, the chlorophyll a/b ratio remains rather constant in control and metal treated cells over a period of six weeks. Fluorescence data detecting H_2O_2 with 2,7-dichlorofluoresceindiacetate (H_2DCFDA) show a clear localisation of this reactive oxygen species in close vicinity to the chloroplasts, as well as in the nuclear region and the cell wall of metal stressed cells. Further experiments are underway to correlate these fluorescence data with structures in EM micrographs.

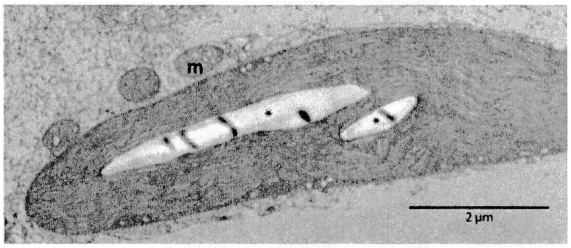

Figure 1: TEM micrograph of a *P. patens* chloroplast with two autochthonous starch grains (*) and associated mitochondria (m).

[1] I. Lang, and W. Wernitznig •Sequestration at the cell wall and plasma membrane facilitates zinc tolerance in the moss *Pohlia drummondii*.••*Environ. Exp. Bot.* **74**, 186••193 (2011).

ACKNOWLEDGEMENTS: Many thanks to Ursula Lütz-Meindl, the University of Salzburg, Austria, for fruitful discussions and technical support in high pressure freezing and freeze substitution. This work was supported by the Vienna Anniversary Foundation for Higher Education (grant H-1939/2008 to IL and H-2486/2012 to SS).

Metal Tolerance Strategies of Mosses Differ at the Cellular Level

Sebastian Antreich, Stefan Sassmann, Stefan Wernitznig, Marieluise Weidinger, Ingeborg Lang

University of Vienna, Cell Imaging and Ultrastructure Research, Althanstrasse 14, A-1090 Vienna, Austria
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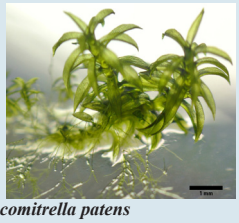

Physcomitrella patens

Introduction

The moss *Physcomitrella patens* (Funariaceae) lives on argillaceous soil whereas *Pohlia drummondii* (Mniaceae) occurs naturally on metal spiked substrate. Growth data of both species show a similar metal tolerance under sterile culture conditions but labelling with the zinc-specific dye FluoZin3 revealed differences and the localisation of zinc to specific compartments [1]. The photosynthetic system is also affected and we observed structural modifications of the chloroplasts as well as changes in the *Chla/Chlb* ratio.

Pohlia drummondii

Strategy I: Compartmentation

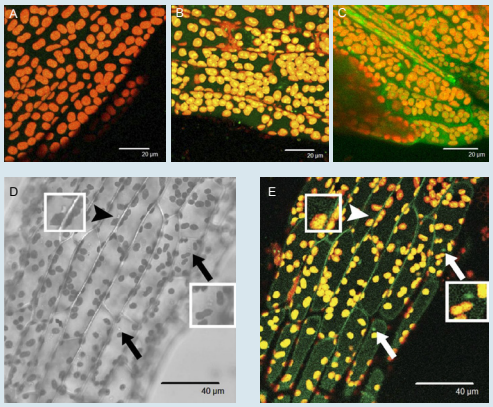

Figure 1: FluoZin3 labelling in *P. patens*: A no labelling in plantlets from control plates; B, C the dye is taken up into the cytoplasm in samples grown on 0.1 mM ZnEDTA and 10 mM ZnEDTA, respectively.

D, E some vesicular structures are labelled (arrows), some are not (arrow head).

Strategy II: Exclusion

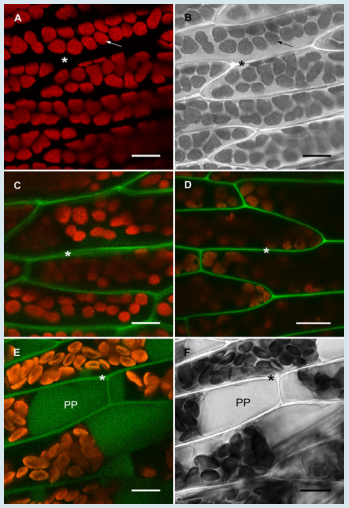

Figure 2: Zinc detection in *P. drummondii* leaf cells after application of the fluorescent dye FluoZin3.

A Control cells without zinc; autofluorescence of chloroplasts with grana thylakoids (arrow). B Corresponding bright field image.

C Sample from 1 mM zinc-spiked agar plate and D from 10 mM zinc-spiked plates; the cell walls are brightly labeled with the zinc-specific dye.

E, F Cells from a gametophyte grown on a 10 mM ZnEDTA plate, staining with FluoZin3 followed by plasmolysis in 0.8 M D-mannitol. E The cell wall and the periplasm (PP) is labeled but not the cytoplasm nor the vacuole. F Corresponding bright field image. Asterisks: cell wall; bar: 10 µm

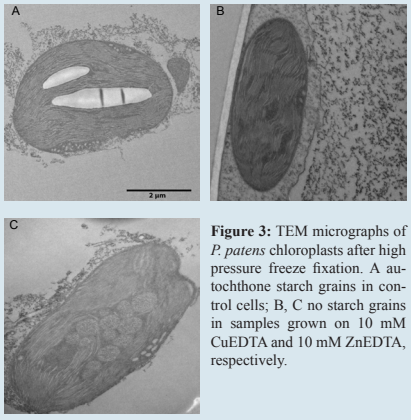

Figure 3: TEM micrographs of *P. patens* chloroplasts after high pressure freeze fixation. A autochthone starch grains in control cells; B, C no starch grains in samples grown on 10 mM CuEDTA and 10 mM ZnEDTA, respectively.

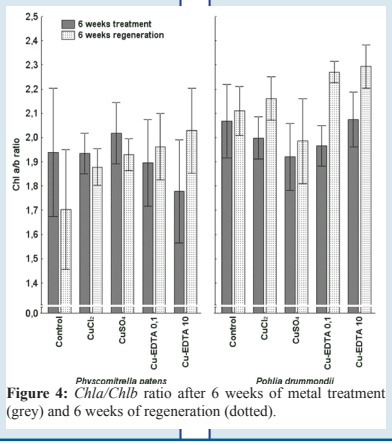

Figure 4: *Chla/Chlb* ratio after 6 weeks of metal treatment (grey) and 6 weeks of regeneration (dotted).

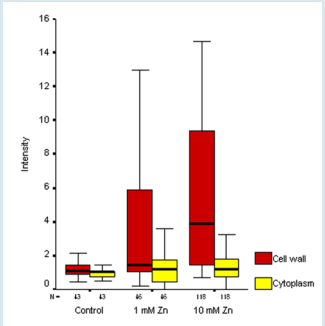

Figure 5: Box plot diagram of fluorescence measurements comparing the fluorescence intensity of the cytoplasm and the cell wall from control, 1 mM ZnEDTA and 10 mM ZnEDTA probes.

Conclusion

We propose two tolerance strategies:

I *Physcomitrella patens* is not used to metal contamination but utilizes compartmentation to dispose the excessive metal.

II *Pohlia drummondii* from metal contaminated substrate uses exclusion mechanisms that prevent the entering of the metal into the cells.

In both species, the *Chla/Chlb* ratio is affected by copper and depends on the ligand (-Cl^-, -SO_4^{2-} or -$EDTA^{4-}$, respectively); a regeneration after 6 weeks is more effective in *P. drummondii* than in *P. patens*.
TEM micrographs of *P. patens* indicate structural changes of the chloroplasts after metal treatment.

Acknowledgements

Many thanks to Ursula Lütz-Meindl, the University of Salzburg, Austria, for fruitful discussions and technical support in high pressure freezing and freeze substitution and to Wolfram Adlassnig for the help in poster design. This work was supported by the Vienna Anniversary Foundation for Higher Education (grant H-1939/2008 to IL and H-2486/2012 to SS).

Reference

[1] I. Lang, and W. Wernitznig „Sequestration at the cell wall and plasma membrane facilitates zinc tolerance in the moss *Pohlia drummondii"*. Environ. Exp. Bot. 74, 186– 193 (2011).

universität wien

20th Meeting of the Austrian Society of Plant Biology (ATSPB)

Lunz am See, Austria, 19/06/14 - 21/06/14

Poster title: "Uptake and localization of copper in three different moss species"

Antreich S., Sassmann, S., Weidinger, M., Lichtscheidl, I., Lang, I.

Abstract:

Differences in the uptake and localization of copper in three different moss species

S. J. Antreich[(1)*], S. Sassmann[(1)], M. Weidinger[(1)], I. K. Lichtscheidl[(1)] and I. Lang[(1)]

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Higher plants growing on heavy metal contaminated sites can be classified into excluders or accumulators, depending on whether the root serves as a barrier against heavy metal uptake or not. For mosses, this classification is not applicable due to the absence of a root system and the potential uptake of nutrients and water *via* the whole moss surface. In this experiment, the influence of different copper media on the allocation of the metal in three different mosses were compared.

For this purpose, the mosses were grown on agar medium supplemented with 0.1 mM $CuCl_2$, 0.1 mM $CuSO_4$, 0.1 mM or 10 mM Cu-ethylenediaminetetraacetate (CuEDTA), respectively. After six weeks the mosses were harvested, dried and the top and bottom parts of the stem were analyzed by means of energy dispersive X-ray microanalysis (EDX). In the $CuCl_2$ and $CuSO_4$ treatments, *Pohlia drummondii* and *Mielichhoferia elongata* both showed a strong decrease of copper from the base toward the top parts of the stem, while in contrast *Aphanorhegma patens* presented constant high amounts in both parts. In all three species, treatment with 10 mM CuEDTA resulted in more copper in the top parts of the stem than in the bottom parts. Especially in *P. drummondii*, the amount in the top was significantly higher than in the bottom parts. In this species, the same trend could also be observed in the 0.1 mM CuEDTA treatment, whereas the other two moss species showed no difference.

The copper adapted species *P. drummondii* and *M. elongata* strongly excluded the copper in the $CuCl_2$ and $CuSO_4$ treatment from the top parts. This was probably caused by the strong absorbance capacity of the outer cell walls. In contrast, the metal sensitive *A. patens* seemed to not have such a strong exclusion ability, which was supported by the fact that many moss plantlets died before the sixth week. The strong accumulation of copper in the EDTA treatments likely resulted from the shielding of the Cu^{2+} by the $EDTA^{4-}$ and therefore its easier penetration into the moss. As the shielded Cu^{2+} could not bind to cell walls, it was transferred into the top parts of the stem together with the water flow.

In conclusion, it was shown that the heavy metal adapted mosses *P. drummondii* and *M. elongata* were able to exclude copper from the sensitive growing bud in contrast to the non-adapted control moss *A. patens*.

Acknowledgement: This study was funded by the ÖAD project APPEAR 43/BIOREM

Uptake and localization of copper in three different moss species

Sebastian J. Antreich, Stefan Sassmann, Marieluise Weidinger, Irene K. Lichtscheidl and Ingeborg Lang

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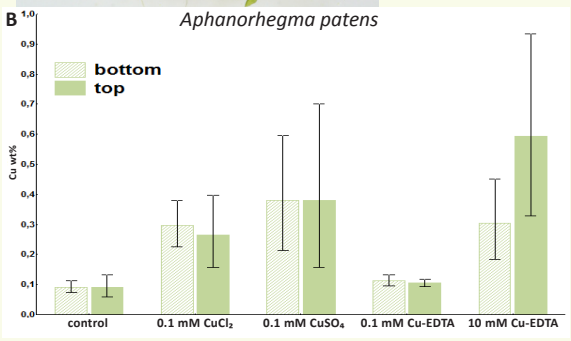
Introduction

Flowering plants of metal contaminated habitats can be classified into excluders or accumulators, depending on whether the root serves as a barrier against heavy metal uptake or not. For mosses, this classification is not applicable due to the absence of a proper root system; nutrients and water are taken up *via* the whole moss surface. Specialised mosses are able to grow on heavy metal sites. In this experiment, the allocation of different copper compounds in the moss *Aphanorhegma patens* (syn. *Physcomitrella patens*), a model for many physiologic and genetic questions, was compared with two metal adapted species *Mielichhoferia elongata* and *Pohlia drummondii*.

(**A**) habitus of the moss *A. patens* (scale bar: 1 mm)

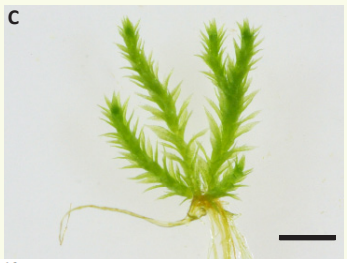
(**B**) EDX results of each treatment in weight percent (wt%) copper, comparing the bottom with the top parts of the moss (error bars: 95% confidence intervall)

Aphanorhegma patens



Method

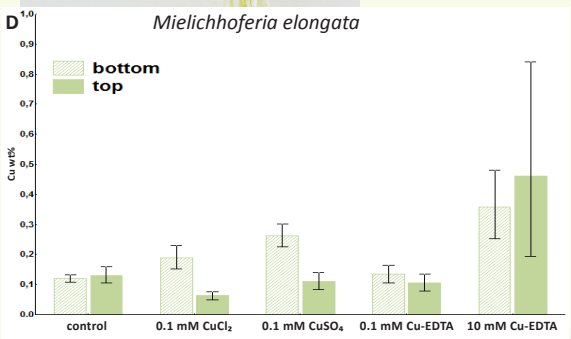
The three moss species were grown on agar supplemented with 0.1 mM $CuCl_2$, 0.1 mM $CuSO_4$, 0.1 mM or 10 mM Cu-ethylenediaminetetraacetate (Cu-EDTA). After six weeks the mosses were harvested, dried and the top and bottom parts of the stem were analyzed by means of energy dispersive X-ray microanalysis (EDX), n = 8-14. Differences were statistically analysed with Student t-test for significance.



Conclusion

In contrast to the non-adapted control moss *A. patens* the heavy metal adapted mosses *P. drummondii* and *M. elongata* were able to exclude copper from the sensitive growing bud.

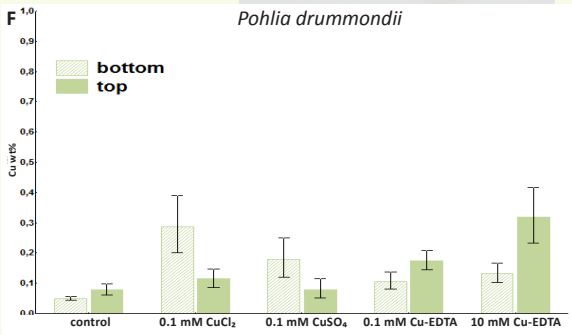
Mielichhoferia elongata



(**C**) habitus of the moss *M. elongata* and (**E**) *P. drummondii* (scale bar: 1 mm)

(**D**) & (**F**) EDX results of each treatment in weight percent (wt%) copper, comparing the bottom with the top parts of the moss (error bars: 95% confidence intervall)

Pohlia drummondii



Discussion

The copper adapted species *P. drummondii* and *M. elongata* strongly excluded the copper from the top parts in the $CuCl_2$ and $CuSO_4$ treatments. This indicates a strong absorbance capacity of the outer cell walls which has been already observed for zinc [1]. In contrast, *A. patens* was lacking such effective exclusion ability and showed also high amounts of copper in the top parts. The same concentration of EDTA-complexed copper had no strong effect on the uptake of the metal. The accumulation of copper in the top parts in the 10 mM Cu-EDTA treatments possibly resulted from the chelation of copper by the EDTA-complex causing easier translocation into the moss. This effect was already shown for flowering plants, where lead chelated with EDTA increased the translocation into the shoot [2]. As the EDTA-complex lowered the binding to cell walls, it was transferred into the top parts of the stem together with the water flow.

Acknowledgement

Many thanks are due to W. Adlassnig. This study was funded by the ÖAD project APPEAR 43/BIOREM.

References

[1] Lang I & Wernitznig S (2011), Sequestration at the cell wall and plasmamembrane facilitates zinc tolerance in the moss *Pohlia drummondii*. Environmental and Experimental Botany, 74, 186– 193

[2] Jarvis MD & Leung DWM (2002), Chelated lead transport in *Pinus radiata*: an ultrastructural study. Environmental and Experimental Botany, 48, 21–32

20th Meeting of the Austrian Society of Plant Biology (ATSPB)

Lunz am See, Austria, 19/06/14 - 21/06/14

Poster title: "*Phragmites australis* as a Pioneer on Mine Waste Substrate"

Meyer, I., Adlassnig, W., Vrbecky, L., Sassmann, S., Steinacher, R., Puschenreiter, M., Lichtscheidl, I.

Abstract:

Phragmites australis as a pioneer on mine waste substrate

I. Meyer[(1)*], W. Adlassnig[(1)], L. Vrbecky[(1)], S. Sassmann[(1)], R. Steinacher[(1)], M. Puschenreiter[(2)] and I. K. Lichtscheidl[(1)]

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Industrial ore processing leads to the formation of huge mine tailings consisting of milled ore minerals and bedrock. These mine waste material is an extremely hostile substrate for plant growth as it still contains elevated concentrations of toxic metals, remnants of chemicals used for metal extraction but virtually no nutrients or humus. Furthermore, the substrate is highly mobile and either water saturated or completely dry due to a low water holding capacity. Such mine tailings form a major threat for people and environment as toxic material can be easily distributed by wind or water, either in large catastrophic events like in 2000 in Baia Mare (Ro) or in 2010 in Kolontár (Hu) or by chronical release of heavy metals. The formation of a closed vegetation is a crucial step in the remediation but only few plant species are suitable.

This study investigates the potential of *Phragmites australis* (Poaceae) to colonise metal rich mine tailings and its influence on the mine waste substrate. The extensive mine tailing landscape at Baia Mare (Ro) served as test sites and were compared to a non-contaminated site in Breitenbrunn (At).

In Baia Mare, *P. australis* is able to tolerate extremely acidic pH, high concentrations of total, exchangeable and water-soluble heavy metals, especially lead, high salinity and a substrate that is dominated by the silt fraction. It requires, however, a high ground water level, and is therefore restricted to the banks of the central ponds as well as to the escarpment at the periphery of the mine tailings. As a first coloniser, it improves the soil quality by stimulating the formation of humus and moderating the pH. Thereby, *P. australis* creates suitable conditions for the colonisation of the mine tailing by other plant species, such as *Typha latifolia*, *Salix alba*, *S. purpurea*, *S. viminalis* *Populus alba*, *Equisetum ramosissium*, *Bolboschoenus* sp., *Juncus effusus* and also by a remarkably diverse community of animals.

By aerating the soil *via* its rhizomes rich in aerenchyma, *P. australis* alters the oxidations state of metals in the soil, which becomes apparent by the formation of a layer of red ferric iron around the rhizomes instead of the otherwise predominant grey ferrous iron. Similar oxidation processes are involved in the mobilisation of metals during the weathering of mine waste material. The rhizomes of *P. australis* are extremely resistant against the influence of metal ions but the effects of aeration on the metal household of the whole mine tailing need to be thoroughly discussed. Altogether, however, *P. australis* shows an extraordinary potential in initialising the formation of a closed vegetation cover even on the most toxic mine tailings. By appropriately designing the topography of the mine tailings, the area accessible for *P. australis* could be significantly expanded at low costs.

Phragmites australis as a Pioneer on Mine Waste Substrate

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Introduction

Industrial ore processing leads to the formation of huge mine tailings consisting of milled ore minerals and bedrock. This mine waste material is extremely toxic for plants, and virtually no nutrients or humus are available. The substrate is highly mobile and either water saturated or completely dry due to a low water holding capacity. Therefore, such mine tailings form a major threat for people and environment as toxic material can be easily distributed by wind or water, either in large catastrophic events like in 2000 in Baia Mare (Ro) or in 2010 in Kolontár (Hu), or by constant release of heavy metals. The formation of a close vegetation cover is a crucial step in remediation but only a few plant species are suitable.

This study investigates the potential of *Phragmites australis* (Poaceae) to colonise metal rich mine tailings and its influence on the mine waste substrate. The following hypotheses are tested:

- *P. australis* is a first coloniser on mine tailings where conditions are too hostile for other plant species.
- By aerating the soil *via* its rhizomes rich in aerenchyma, *P. australis* alters the oxidation state of metals in the soil. The oxidation of Fe^{2+} to Fe^{3+} leads to an acidification of the soil and increases the availability of toxic metals.
- The subsoil parts tolerate high metal concentrations by (a) minimizing aeration of the soil by reducing aerenchyma formation; (b) formation of a protective barrier or (c) high cytoplasmic tolerance.
- *P. australis* is suitable for remediation of mine tailings.

Materials and Methods

Three metal tolerant populations of *P. australis* were studied at lead rich mine tailings in Baia Mare (Romania) and were compared to an uncontaminated site at the Neusiedlersee (Austria). Flooded and dry growing sites were distinguished. Soil (pH, humus, grain size distribution) and water parameters (pH, oxygen saturation, conductivity) were analysed, together with plant and algal diversity. On site performance of the plants was estimated by measuring chlorophyll fluorescence.

In order to clarify the residence strategy of *P. australis,* the formation of aerenchyma and surface tissues was studied in paraffin sections. Cellular metal tolerance was estimated by incubating sections of the rhizomes in graded solutions of Fe-, Mn-, Cu-, Zn-, Co-, Pb- and Ni–chloride. Cytoplasmic tolerance was tested after two days by plasmolysis.

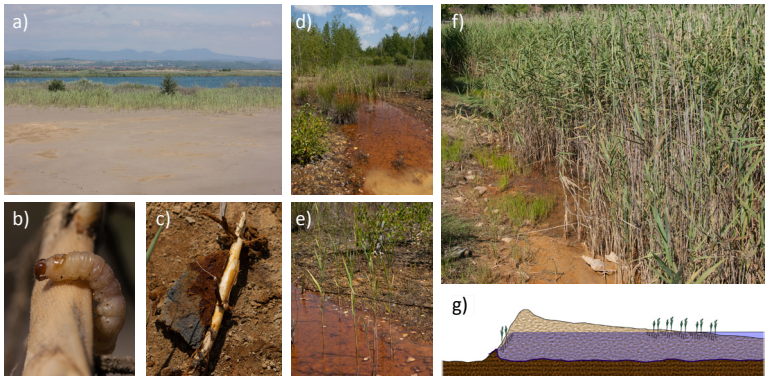
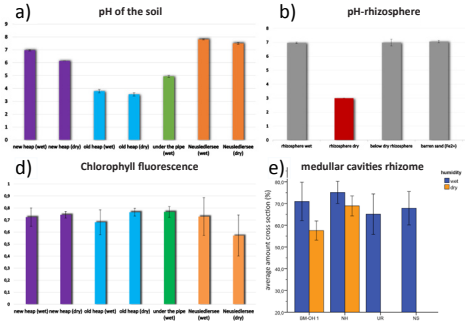


Fig. 1: a) New heap (Baia Mare); b) *Chilo phragmitellus* living in rhizomes of *P.australis*; c) rhizosperic red Fe^{3+} ; d) Old heap (Baia Mare); e) *P. australis* at the old heap; f) "under the pipe" (Baia Mare); g) schematic view of ground water level at the new heap (Baia Mare) and the distribution of *P. australis*.



	Zn	Mn	Cu	Fe	Ni	Co	Pb
new heap	10^{-2}	10^{-4}	10^{-3}	10^{-7}	10^{-3}	10^{-2}	10^{-4}
Neusiedlersee	10^{-2}	10^{-5}	10^{-5}	10^{-7}	10^{-3}	10^{-2}	10^{-4}

Fig. 2: a) pH of the soil; b) pH meassured in the rhizosphere and barren sand; c) pH of the water; d) Stresslevel estimated with Fv/Fm; e) average amount of medullar cavities of the rhizomes, in percent of the cross section; f) metal resistance of epidermal cells of the rhizome from *P.australis* from a selected mine waste site in Baia Mare and the control site Neusiedlersee.

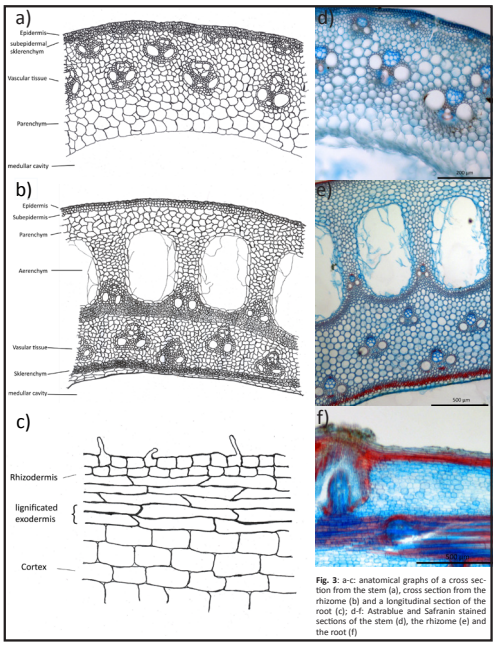


Fig. 3: a-c: anatomical graphs of a cross section from the stem (a), cross section from the rhizome (b) and a longitudinal section of the root (c); d-f: Astrablue and Safranin stained sections of the stem (d), the rhizome (e) and the root (f)

Results

The metal contaminated sites at Baia Mare (Fig. 1) were dominated by fine sand and silt and therefore, the substrate was highly mobile and either completely dry or water saturated. *P. australis* was omnipresent at all parts of the mine tailing where the ground water level was only a few dezimeters below the surface (Fig. 1 f), including shallow ponds. Both, the top soil and the water were acidic and contained increased concentrations of lead and other metals (Zn: 718 ± 513 ppm; Pb: 2352 ± 1067 ppm; Mn: 1768 ± 1566 ppm) as well as high amounts of salts (conductivities up to 2,770 µS ; salts blooming at the soil surface). In deeper soil layers, the pH was moderate and the transition from red Fe^{3+} to gray Fe^{2+} was visible (Fig . 1 c). Similary, the pH was more moderate at wetter habitats (Fig. 2). With populations of *P.australis*, especially at wet substrate, more humus was found than on the barren mine tailing. At dry habitats the rhizomes were surrounded by a reddish and extremely acidic layer of substrate which was not observed under flooded conditions. At wet habitats chlorophyl fluorescence (F_v/F_m) was not different to the uncontaminated site at the Neusiedlersee, wheras it was significantly lower at the dryer and more acidic sites (Fig. 2 d).

Only in the centre of extended populations, *P. australis* was accompanied by other vascular plants, such as *Typha latifolia, Salix purpurea*, *S. viminalis*, *Bolboschoenus maritimus*, *Juncus capitatus*, *Populus* sp., *Betula pendula* and *Juncus effuses*, and algae like *Klebsormidium* sp., *Navicula* sp., *Chlamydomonas* sp., *Mougeautia* sp., *Zygnemopsis* sp. and *Tribonema* sp. Furthermore, the populations of *P. australis* hosted surprisingly diverse animal communities including *Orthretum cancellatum*, *Chilo phragmitellus* (Fig. 1 b), *Rana ridibunda* and *Bufo viridis*.

The anatomy of the rhizomes and stems did not differ in the samples from the mine tailings of Baia Mare and the Neusiedlersee. The medullar cavities occupied the same portion of stems and rhizomes, indicating comparable oxygen transfer to the substrate. However, the medullar cavities were more pronounced under wet conditions (Fig. 2 e). Protective barriers like the exodermis were equally developed (Fig. 3), indicating no enhanced shielding of the living tissues under metal stress. Formation of an increased number of shorter roots under metal influence indicate stress for the root. On the cellular level however all populations of *P.australis* exhibit an extraordinary degree of tolerance against metals (Fig. 2 f), significantly exceeding the resistance of other species from the mine tailings such as *Rumex acetosella* and *Plantago lanceolata*. The populations from mine tailings, however were only slightly more resistant than the population from the Neusiedlersee.

Oxidation and reduction of iron

On mine tailings, Fe is present as grey Fe^{2+} and red Fe^{3+}; according to redox conditions, both species are easily convertable according to

$$4\,FeS_2 + 15\,O_2 + 2\,H_2O \leftrightarrow 4\,Fe^{3+} + 8\,SO_4^{2-} + 4\,H^+$$

$$Fe^{3+} + 2\,H_2O \rightarrow FeO(OH)\downarrow + 3H^+$$

Thus, the formation of Fe^{3+} is accompanied by an acidification of the substrate, leading to an increased solubility of other metals. Red FeO(OH) (Limonite) is dominant at the surface of mine tailings and around rhizomes of *P. australis*.

Discussion

P.australis is able to colonize mine waste substrate that is far too toxic for other plant species. By stimulating humus formation and moderating the pH, more favourable conditions could be created that enable the establishment of more diverse communities. High salinity, low pH, increased levels of lead and other metals, lacking humus and unnaturally fine grain size are tolerated but a high ground water level is a prerequisite. At habitats with a comparatively low water level, aereation of the soil via the rhizome leads to addition acidification and poorer performance of the plants, though aerenchyma formation is reduced under dry conditions. On flooded habitats, no acidification of the rhizosphere was observed.

No evidence for increased barrier formation could be found and root growth is visibly affected by metals. The extraordinary cytoplasmic tolerance however, still enables survival and growth of the plants. This tolerance is also found in plants from uncontaminated habitats and not restricted to specific genotypes.

Phragmites australis is therefore a suitable candiate for revegetation of wet, heavy metal contaminated mine tailings. As a cosmopolite, it can be used on all continents and under all climatic conditions. Under rather dry conditions, aereation of the soil may lead to undesired side effects which are not observed under flooding. A proper design of the mine tailing will facilitate the establishment of *P.australis* and prevent undesireable acidification.

Acknowledgements

We thank Dr. Ingeborg Lang, Amadea Horvath and Teresa Luftensteiner for helping us, whenever we needed their help.

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20th Meeting of the Austrian Society of Plant Biology (ATSPB)

Lunz am See, Austria, 19/06/14 - 21/06/14

Poster title: " A Cyanobacteria-Plant Consortium Remediating Mine Drainage Water"

Steinacher, R., Adlassnig, W., Sassmann, S., Reipert, S., Hus, K., Puschenreiter, M., Lichtscheidl, I.

Abstract:

A cyanobacteria-plant consortium remediating mine drainage water

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Mine galleries frequently produce drainage water and may continue to do so for a long time after closure of the mine. In the case of mining for ore minerals, the drainage water usually contains elevated concentrations of toxic metals, which may affect people and the environment a long way downstream. At the historical copper mine Schwarzwand (Hüttschlag/Salzburg), copper rich drainage water is still feeding a creek 200 years after the depletion of the ore deposit. The copper affected area supports a unique vegetation dominated by lower plants and retaining most of the metal load of the creek.

This study analyses the biodiversity of the copper rich creek, as well as the metal content of water, sediments and organisms in order to understand the fate of metals in the drainage water and the role of plants and microorganisms in the metal household of the Schwarzwand. Special attention was paid to the localisation of elements in plants and microorganisms by EDX and EELS.

The bed of the copper creek is covered by bluish layer, which was so far misinterpreted as precipitations of $Ca/CuCO_3$ but consists of thick mats of the blue-green alga *Phormdium* growing in clearly distinct layers. Exposed to running water, the mechanical stability of the biofilm is increased by *Saxifraga stellaris* (Saxifragaceae) and *Pohlia spp.* (Bryaceae), stabilising the biofilm with a network of roots and dead stems. Though both *Saxifraga* and *Pohlia* accumulate increased amounts of Cu, their contribution to water remediation is negligible compared to the role of *Phormidium*: the biofilm contains $3.9 \pm 1.8\%$ of Cu, partly as globular, micro-sized particles between the filaments, and partly as nano-sized particles covering the surface of the mucilaginous sheath surrounding the filaments. Though the deeper layers of the biofilm are dead, little evidence for decomposition is found, and the immobilisation of Cu seems to be permanent. Accordingly, the Cu load of the creek decreases from 0.6 ± 0.1 mg $\cdot l^{-1}$ to 0.2 ± 0.2 mg $\cdot l^{-1}$ over a course of 330 m.

Thick layers of the soft biofilm alone would not be stable in the swiftly running waters of the creek, and the mosses and vascular plants do not immobilise relevant amounts of copper. Thus, the biological remediation of the creek becomes only possible by the interaction of metal resistant plants and microorganisms. The process of copper mineralisation is far from clear but depends obviously on a specific chemical milieu created by *Phormidium* and possibly also by *Pohlia*, therefore stressing the need for a combined mineralogical-biological approach in order to understand this remarkable case of self-cleaning.

Acknowledgment: This study was funded by the ŒAD (Appear 43/Biorem).

A Cyanobacteria-Plant Consortium Remediating Mine Drainage Water

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Introduction

At the former copper mine Schwarzwand (Hüttschlag/Salzburg, Fig. 1), main drainage water is still feeding a creek, 200 years after the depletion of the ore deposit. At the entrance of the galley, the creek contains 0.6 ± 0.1 mg · l^{-1} copper. Over a course of only 330 m, however, the copper content decreases to 0.2 ± 0.2 mg · l^{-1}.

The bed of the copper creek is covered by a bluish, gelatinous layer overgrown with a few plants, like *Saxifraga stellaris* (Fig. 2A) and *Pohlia* (Fig. 2B). This study investigates the structure of this layer and aims to localize the copper sink.

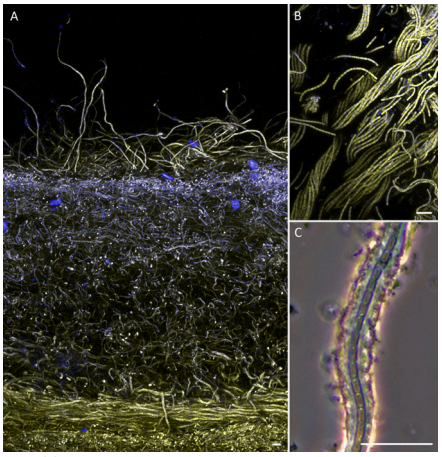

Fig. 3: (A) CLSM: cross section of the biofilm; blue: DAPI and mineral particles, yellow: autofluorescence of *Phormidium*; **(B) CLSM**: pigtails of *Phormidium*; **(C) phase-contrast**: single filament of *Phormdium* surrounded by a gelatinous sheath; (scale bar: 10 µm).

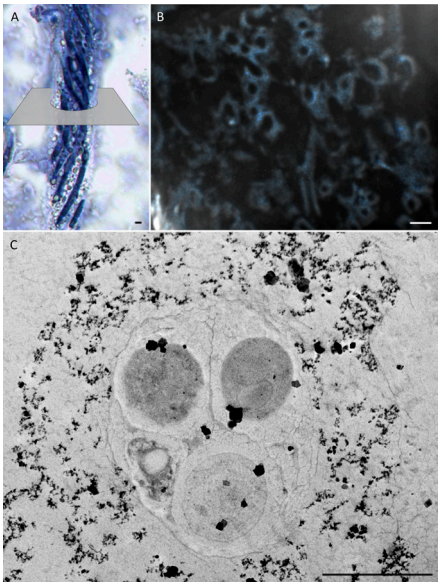

Fig. 6: (A) bright-field: pigtail; sectional plane of **(C)** is indicated; **(B) EDX:** semithin-sections, copper (blue) is localized exclusively in the sheaths, but not within the cells; **(C) TEM:** ultrathin-section of a pigtail containing four *Phormidium* filaments; the outer layers of the sheaths contain nano-sized electron-dense particles, propably copper; (scale bar: 2 µm).

Acknowledgement

Thanks are due to Marieluise Weidinger, Norbert Cyran, Ingeborg Lang, Anton Beran, Kevin Hallbeck and Robert Schilcher.

This study was funded by the ŒAD (Appear 43/Biorem).

Fig. 1: Schwarzwand (Hüttschlag/Salzburg); with creek of the former copper mine; the blue biofilm and inorganic copper precipitations are clearly visible.

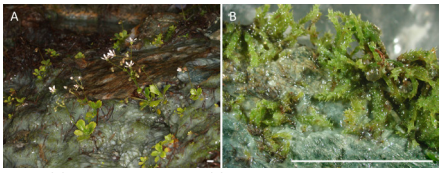

Fig. 2: (A) *Saxifraga stellaris* and **(B)** *Pohlia* spp. are growing within the biofilm (scale bar: 1 cm).

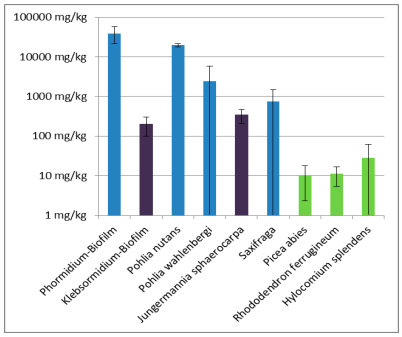

Fig. 7: Copper uptake of selected organisms from the Schwarzwand; blue bars: blue creeks; violette bars: creeks without *Phormidium*; green bars: surrounding area. By far the highest copper amount is accumulated in *Phormdium*-biofilms, followed by *Pohlia nutans* incrusted with *Phormdium*.

Discussion

The Schwarzwand hosts a high diversity of organisms [1] but only a few species are able to grow in the copper-rich creek: *Phormidium* sp. accomponied by *Saxifraga stellaris*, *Pohlia* spp. and a few other bryophtyes. The plants stabilize the biofilm by their network of roots and stems, thus enabeling the formation of extra ordinary thick microbial mats.

Phormidium has been described to absorb various heavy metals [2]. Here we show that this absorbtion is not caused by passive adsorbtion but by precipitation of mineral particles. On the one hand, micro-sized aggregates of sampleite are formed, on the other hand, nano-sized particles of unknown speciation, possibly reduced copper as described by [3]. Obvisouly the process of copper mineralisation depends on a specific chemical milieu created by *Phormidium*. The predominance of insoluable copper minerals explains why *Phormidium* and other organisms are able to survive in a matrix containing up to 4% copper.

The high biomass formed by *Phormidium* together with the high concentration of precipitated copper sufficiently explains the extremly fast decrease of copper in the creek, which leaves the habitat virtually clean. These specific capabilities of *Phormidium* are not only fascinating for science, but might also be used for the remediation of metal-rich waste water.

Material & Methods

The gelatinous layer was identified as a cyanobacterial biofilm. Samples were taken directly from 8 points all over the Schwarzwand, where water parameters were analyzed as well (not shown).

For structural analysis of the biofilm, light- and electron microscope techniques were used. For light microscopy, samples were fixed in buffered formaldehyde. Paraffin-sections stained with toluidin-blue (Fig. 6A) were studied in the bright field. Whole mounts stained with DAPI were studied in the Confocale Laser Scanning Microscope (CLSM; Fig. 3A/B). The following electron microscopy techniques were used: Scanning Electron Microscope (SEM), Environmental Scanning Electron Microscope (ESEM), Energy Dispersive X-ray Spectroscopy (EDX) and Transmission Electron Microscope (TEM). For SEM, sections of the biofilm were coated with carbon; simultaneously, copper was localized by EDX. In the ESEM, fresh material was used.Samples for the TEM were plunge frozen in liquid propane, cryosubstituted in acetone and OsO_4, infiltrated with low-viscosity resin and sectioned.

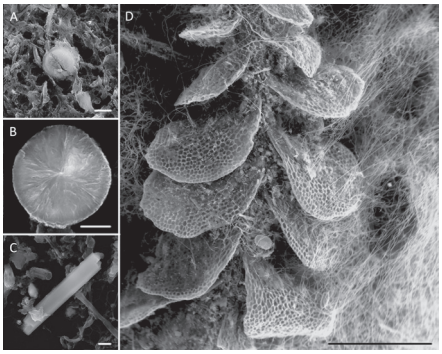

Fig. 4: SEM: (A) & (B) copper-rich mineral-particles, probably sampleite, and **(C)** gypsom from the biofilm (scale bare: 5 µm); **(D) ESEM:** front view of a liverworth within the biofilm (scale bar: 500 µm).

Results

The biofilm was overwhelmingly dominated by *Phormidium* sp. frequently accomponied by mosses and liverworths (Fig. 2B & 4D). On some locations a thickness of more than 20 cm was achieved. The copper content of the biofilm exceeded other copper-tolerant organisms in one to two orders of magnitude (Fig. 7).

The biofilm exhibited several, clearly distinct layers, partly caracterized by pigtailed-like structures of the filaments (Fig. 3B & 6A). Deeper layers were dead and consisted of heavily compressed filaments (Fig. 5).

Each filament was surrounded by a thick gelatinous sheath. EDX showed that the bacterial cells contained much less copper, than these sheaths. At higher magnification it became apparent that the copper was not distributed evenly within the sheaths but concentrated as nano-sized particles (Fig. 6B/C).

Furthermore, the biofilm contained a varity of mineral particles. Besides quartz and feldspar from the bed load of the creek, limonite and gypsum were found which probably originated from the weathering of sulfidic-ores.

On the other hand, globular aggregates of a copper-rich mineral were restricted to the biofilm and possibly formed under the influence of *Phormidium*. The EDX-data fitted well to the rare secondary copper-mineral sampleite ($NaCaCu_5(PO_4)_4Cl \cdot 5\,H_2O$).

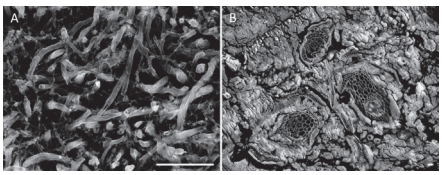

Fig. 5: SEM: (A) upper layer of the biofilm formed by a loose network of filaments; **(B)** deeper layer of the biofilm containing compressed remnants of cyanobacteria as well as intacted moss-plants (leaves and stems); (scale bar: 20 µm).

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20th Meeting of the Austrian Society of Plant Biology (ATSPB)

Lunz am See, Austria, 19/06/14 - 21/06/14

Poster title: "*Plantago spp.* as a Candidate for Mine Waste Revegetation"

Vrbecky, L., Adlassnig, W., Meyer, I., Steinacher, R., Sassmann, S., Lichtscheidl, I.

Abstract:

Plantago as a candidate for the mine waste revegetation

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Without proper vegetation, tailing dams, resulting from ore mining are an environmental risk, leading to wind-blow dispersal of heavy metal contaminated dust. Enhanced concentrations of toxic metals in the substrate result in hostile conditions for plant growth. Metal toxicity is frequently accompanied by extreme pH, low humus content and various symptoms of soil degradation. Natural metal rich habitats are usually colonized by a highly specialized endemic vegetation; anthropogenic metal sites, however, frequently present themselves as barren, desert-like landscapes. The establishment of a closed vegetation is a key step in the remediation of such sites. *Plantago spp.* is one of the few species that are found both on natural and anthropogenic metal sites, and is therefore a promising candidate for revegetation.

This study investigates the occurrence of *Plantago* on the natural Ni site Ochsenriegel (AT), the Cu spoil heaps of the Slovak Ore Mountains and polymetallic mine tailings in Baia Mare (RO). Data on soil condition, metal tolerance and the surrounding vegetation, combined with greenhouse experiments using spiked soil shall clarify the remediation potential of *P. lanceolata*, *P. media* and *P. major*.

All three native species of *Plantago* are found on heavy metal rich substrate. *P. media* occurs on the natural Ni site Ochsenriegel. *P. lanceolata* colonizes the Ochsenriegel as well as Cu and Pb rich mine waste. *P. major* was found exclusively on Cu and Pb rich mine waste. *P. major* and *P. lanceolata* from Baia Mare exhibit a strongly increased resistance against Pb, the dominant metal at the mine tailings. *Plantago* from Ni and Cu rich substrate, however, showed the same cytoplasmic tolerance against these metals as plants from the control site. In all habitats, roots were colonized by mycorrhiza which may facilitate coping with the substrate. Besides enhanced levels of toxic metals, soil conditions were not as extreme as expected: extremely acidic pH was found only at one site in Baia Mare. Humus ranged from low at Baia Mare to very high at Ochsenriegel, but was always present. However, all plants from metal rich sites exhibit reduced chlorophyll fluorescence, indicating an increased level of stress.

Of the three investigated species, *P. major* and *P. lanceolata* colonize the periphery of mine waste habitats and seem to have a potential for revegetation. As perennial plants, they contribute to the stabilization of the substrate. Due to their readiness to form mycorrhiza, *Plantago* increases the abundance of mycorrhizal fungi and therefore may facilitate the establishment of other plant species. However, *Plantago* seems to be unable to cope with extreme pH and a complete lack of humus. The manipulation of soil composition [1] may be required to enable growth of *Plantago* in the central parts of mine tailings.

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Plantago spp. as a Candidate for Mine Waste Revegetation

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Introduction

Enhanced concentrations of toxic metals in the substrate result in hostile conditions for plant growth. Metal toxicity is frequently accompanied by extremely high or low pH, low humus content and various symptoms of soil degradation. Natural metal rich habitats are usually colonized by a highly specialized endemic vegetation; anthropogenic metal sites, however, frequently present themselves as barren, desert-like landscapes. The establishment of a closed vegetation is a key step in the remediation of such sites (e.g. preventing erosion). *Plantago* spp. is one of the few taxa that are found both on natural and anthropogenic metal sites, and is therefore a promising candidate for revegetation.

This study investigates the occurrence of *Plantago* spp. on Ochsensattel (AT), where Ni occurs naturally in elevated concentrations, the Cu spoil heaps of the Slovak Ore Mountains (Banská Štiavnica, SK) and the polymetalic mine tailings in Baia Mare (RO). The following hypotheses are tested:

1. *Plantago* spp. is no typical metallophyte but still far more tolerant to the hostile mine waste environment than the vast majority of vascular plants.
2. An enhanced cytoplasmic tolerance to toxic metals partly explains the ability to colonise mine waste habitats.
3. On the most extreme mine waste habitats, *Plantago* spp. reaches the limits of its tolerance. However, minimal soil improvement would enable establishment of *Plantago* spp.

Material and Methods

Plantago lanceolata, *P. major* and *P. media* were tested for their heavy metal tolerance.

Three techniques were used to evaluate the performance of *Plantago* under heavy metal stress:

1. In the field, chlorophyll fluorescence (F_v/F_m) was measured using a fluorimeter (Hansatech Handy Pea; n = 40 per site).
2. Cytoplasmatic metal tolerance of leaf epidermis cells was tested by incubating leaf sections in graded solutions (10^{-1}- 10^{-6}) of Ni-, Co-, Cu-, Pb-, Mn-, Zn- and Fe(III)-chlorides. After two days, vitality of the cells was checked by plamolysis in 0,8 M mannitol (Fig. 2).
3. The most promising species, *P. lanceolata* was grown on nutrient poor potting soil + sand (4:1) spiked with 500 ppm Ni, Cu and Pb as chlorides after six weeks of ageing. Seeds collected from Pb rich habitats in Baia Mare and from the uncontaminated Wienerwald were compared. Here first observations after 3 month of growth are shown.

The situation on the growth site was evaluated by documentation of the accompanying vegetation and by soil analyses. Soil pH was measured in NH_4NO_3 extracts. Humus content was determined by wet oxidation using $Cr_2O_7^{2-}$, grain size distribution by sieving and sedimentation. Total metal content of the soils was quantified by digestion in boiling *aqua regia*; bioavailable metals were estimated by extraction in 1 molar NH_4NO_3.

Results

Fig. 1: a) old heap (Baia Mare); b) under a pipe (Baia Mare); c) spoil heaps (Richtárová)

Sampling Sites and Plant Communities

In Baia Mare (BM) high levels of Pb (2352±1067 ppm, 7.2% extractable) and Cu (349±174, 10% extractable) were present, accompanied by enhanced levels of Mn, Zn and As in the soil. *P. lanceolata* was found at the escarpment of an old heap (>20 years; OH) and under a pipe (R), formerly leaking with a metal rich mineral suspension (Fig. 1 a, b). Nearby, on a road side, *P. major* occurred as well. Both species were accompanied by *Rumex acetosella*, under the pipe also by *Arabidopsis halleri*, *Achillea millefolium* and *Agrostis capillaris*.

In the Slovak Ore Mountains, *P. major* was collected on a Zn-Pb rich meadow (Terézia) where the biocoenosis showed little evidence for metal stress. The accompanying species were *Artemisia vulgaris*, *Silene* sp., *Dactylis glomerata*, *Noccaea caerulescens*, *Equisetum sylvaticum*, *Trifolium pratense* and *Cardamine impatiens*. Furthermore, *P. major* was found at the bottom of the rocky copper spoil heap Richtárová (Fig.1 c) accompanied by *Agrostis capillaris*, *Rumex acetosella*, *Equisetum arvense*, *Rumex crispus*, *Echium vulgare*, *Galium album*, *Taraxacum* sp. and *Silene* sp. At Richtárová, *P. lanceolata* colonized flat areas within the spoil heaps where a stronger influence of copper was visible (938 - 8224 ppm Cu, 84 - 284 ppm Zn and 50 - 131 ppm Pb). It was accompanied by seedlings of *Carpinus betulus*, *Betula pubescens*, *Picea abies*, respectively, as well as by *Rumex acetosella*, *Galium album*, *Campanula* sp., *Silene vulgare* ssp. *vulgaris*, *Abietinella abietina* and *Aegopodium podagraria*.

At the Ochsensattel, Ni rich (238±3 ppm Ni, 64±6 ppm Zn) substrate is found over serpentine rock. Here, *P. lanceolata* and *P. media* were found in a woodland dominated by *P. silvestris* with *Noccea goesingensis*, *Rumex acetosella*, *Potentilla alba*, *Myosotis stenophylla* and other typical serpentine species in the understorey.

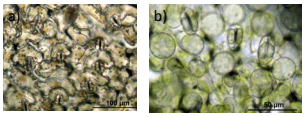

Fig. 2: a) Incubation of plant epidermis cells of *P. media* in 10^{-4} M $CuCl_2$ solution (dead); b) Incubation of plant cells in 10^{-4} M $MnCl_2$ solution (plasmolysed)

			Zn	Mn	Cu	Fe	Ni	Co	Pb
Ochsensattel	*P. media*	x	10^{-3}	10^{-3}	10^{-2}	10^{-3}	10^{-3}	10^{-3}
	P. lanceolata	x	10^{-4}	10^{-4}	10^{-3}	10^{-3}	10^{-3}	10^{-4}
Baia mare	*P. lanceolata*	10^{-4}	10^{-2}	10^{-3}	10^{-3}	10^{-3}	10^{-4}	10^{-3}
	P. major	10^{-4}	10^{-2}	10^{-4}	10^{-3}	10^{-3}	10^{-4}	10^{-3}
Banská Štiavnica	*P. major*	10^{-4}	10^{-3}	10^{-3}	10^{-4}	10^{-4}	10^{-3}	10^{-3}
Richtárová	*P. lanceolata*	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}
	P. major	10^{-4}	10^{-2}	10^{-3}	10^{-3}	10^{-3}	10^{-4}	10^{-4}
Control	*P. major*	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}
	P. lanceolata	10^{-4}	10^{-4}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-4}
	P. media	10^{-4}	10^{-4}	10^{-5}	10^{-5}	10^{-4}	10^{-4}	10^{-5}

Tab. 1: Metal tolerance of three *Plantago* species of different sites. The extraordinary tolerances of some populations towards Pb and Mn are highlighted.

Reactions of the Plants

In the natural habitat, plants from metal rich sites showed a significantly lower F_v/F_m ratio than plants from control sites (Fig. 3 a), indicating an increased level of stress. The plants from Baia Mare showed a drastic increase of their cytoplasmic Pb tolerance, indicating adaption to the dominant metal in the substrate. Similarly, most plants from mine waste showed a higher tolerance to Mn. Plants from the Ochsensattel, however, were not more tolerant to Ni than control plants and even less tolerant to other metals (Tab. 1). The roots of all tested plants were colonised by mycorrhizal fungi (data not shown).

In the pot experiment, the poorest plant growth occurred on Ni spiked soil, the cotyledons became necrotic at an early stage and the growth of the surviving plants was heavily retarded. Cu was nearly as toxic as Ni. Pb, however, was tolerated much better than the other heavy metals but still affected plant growth when compared to the control. No differences between the seeds of the sampling sites Baia Mare and Wienerwald were found (Fig. 4).

Soil

Mine waste substrates are usually characterised by extremely high or low pH, lack of humus and unusual patterns of grain size distribution. On the tested growing sites of *Plantago*, however, rather moderate soil conditions were observed: with one exception (*P. lanceolata* at OH), pH was always between 5.5 and 7.5. Sand, silt, clay and humus occurred in well balanced mixtures (Fig. 3 b, c). Thus, the substrate resembled natural uncontaminated soils rather than typical mine waste where either the coarse or the extremely fine fraction are dominant.

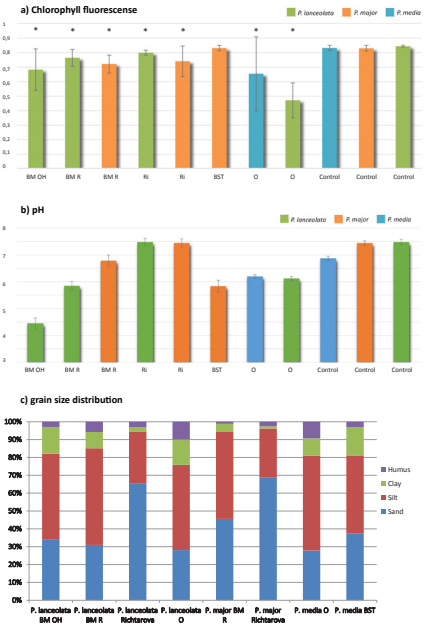

Fig. 3: a) Chlorophyll fluorescence of *Plantago* at different sites: Baia Mare old heap (BM OH), Baia Mare under the pipe (BM R), Richtárová (Ri), Banská Štiavnica/Terézia (BST), Ochsensattel (O) and the control site in the Wienerwald (* indicates a significant decrease compared to the control); b) pH of the soil; c) distribution of humus, clay, silt and sand

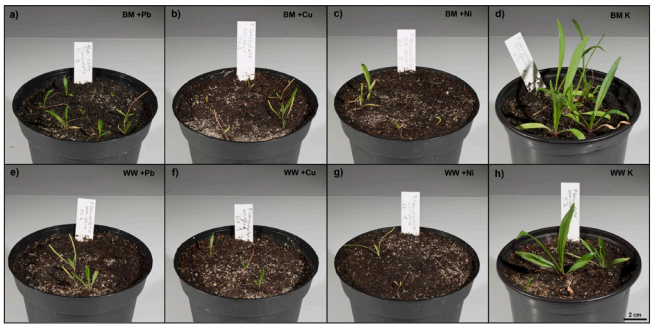

Fig. 4: a - d: *P.lanceolata* from Baia Mare (BM) growing on soil spiked with Pb, Cu, Ni and a control (K); e-h: *P.lanceolata* from the control site Wienerwald (WW) growing under the same conditions

Discussion

Plantago spp. is part of the typical vegetation in the periphery of metal rich sites. *P. lanceolata* occurs on Cu, Pb, Zn and Ni rich soils and penetrates comparatively far into the barren center of the heaps. *P. major* is restricted to road sides within metal rich areas, probably due to its trampling resistance. *P. media* was not found at mine waste habitats but over serpentine were Ni is only poorly available. Chlorophyll fluorescence (F_v/F_m) indicates that all three species suffer from serious stress on metal rich soil. Compared to other species from the same habitat, the cytoplasmatic metal tolerance of *Plantago* is rather low (compare poster by Meyer *et al.* in the same session) which may be partly compensated by a high degree of mycorrhization. The cytoplasmic tolerance to Pb, however, can obviously be increased by a Pb rich environment.

Of the three investigated species, only *P. lanceolata* seems to have a potential for the revegetation of spoil heaps. As a perennial plant, *P. lanceolata* contributes to the stabilisation of the substrate. Due to its readiness to host mycorrhiza, *Plantago* spp. increases the abundance of mycorrhizal fungi and therefore may facilitate the establishment of other plant species. However, *Plantago* spp. seems to be unable to cope with extreme pH, a complete lack of humus and unusual patterns of grain size distribution. Manipulation of the soil composition is therefore required to enable growth of *Plantago* spp. in the central parts of mine tailings. Furthermore, the transfer of metals from the soil to the aboveground organs of *Plantago* spp. needs to be clarified in order to evaluate both, its potential for phytoextraction and the risk of metals entering the food chain.

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Lisbon, Portugal, 26/02/15 - 27/02/15

Poster title: "Cultivar specic symbiotic teamwork in *Pisum sativum*"

Turetscek, R., Holzbach, S., Epple, T., Sassmann, S., Desalegn, G., Kaul H.-P., Wienkoop, S.

Abstract:

Cultivar specific symbiotic teamwork in *Pisum sativum*

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(3) University of Natural Resources and Life Sciences, Department of Crop Sciences

Legume species account for a big part of agricultural production because of their nutritional value to man and life stock. Moreover, due to their symbiotic interactions (*Rhizobia* & arbuscular mycorrhizal fungi - AMF) which enhance nutritional uptake, they substantially contribute to sustainable agriculture. Each legume is capable of forming symbiosis with particular *Rhizobia* and commonly several species of AMF. The interaction with *Rhizobia* is to a great extent controlled by the plant and each species shows different nodule morphology. With regard to breeding strategies, agronomy is interested in the effect of below ground parts on above ground traits (e.g. biomass, pathogen resistance levels, and yield). We tested the effect of single and co-inoculation with *Rhizobia* and AMF on the plants´ morphology as well as the leaf proteome and metabolome in two cultivars of *P. sativum*. The nodulation profile (weight and number of nodules) is remarkably distinct among cultivars and the proteome shows predominantly cultivar rather than symbiotic effects. However, we found that single *Rhizobia* inoculation shows the utmost effect on the proteome in a cultivar specific manner. As the intensity of the host-symbiont interaction over a plants´ lifespan usually varies between cultivars, we further aim to elucidate the nodules´ morphology as well as its proteome in a time series. These insights about cultivar specific symbiotic interaction provide knowledge for advanced sustainable breeding strategies.

Cultivar specific symbiotic teamwork in *Pisum sativum*

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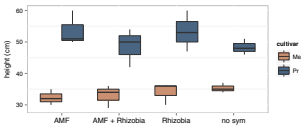
Background

Lower needs for chemical fertilizer make *Pisum sativum sp. sativum* well suited for sustainable agriculture and the protein rich seeds are a valueable food source for man and life stock. During the past decades yield losses happened mainly due to pathogenic infestations, with Ascochyta blight as the most severe disease. In this fungal disease complex *Didymella pinodes* is the most aggressive agent. Despite the great number of varieties absolut resistance was not yet found which indicates the involvement of numerous genomic regions for resistance [1]. Besides improving the nutritional status, the symbiotic interactions of *P.* sativum (*Rhizobia* & arbuscular mycorrhizal fungi - AMF) hold the potential to prime the plants immune system for pathogen encounters. In our study, AMF inoculated plants were phosphorus deprived and *Rhizobia* inoculated plants did not receive nitrogen fertilizer. Plants without any symbionts were fully fertilized. Here, we analyzed morphological and proteomic features under different symbiotic conditions of three cultivars (Model, Protecta and Messire - kindly provided by the Institute for Sustainable Agriculture, Spain) with different resistance levels to *D. pinodes*.

Morphology & Physiology

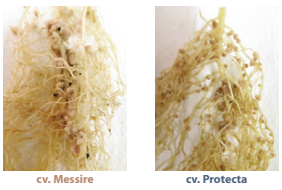
Both cultivars exhibit a leafy-type morphology (showing stipes, leaflets and tendrils) with different above and below ground habitus.

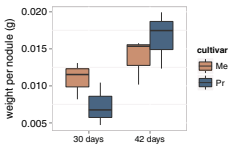
Shoot morphology



Habitus: dense in cv. Messire vs. tall in cv. Protecta
Cv. Messire shows a dense habitus with short internodes and sometimes branched shoots. Cv. Protecta hardly branches and has longer internodes with a significantly taller habitus than Messire. Among the symbionts *Rhizobia* showed the most effects in both cultivars. Co-inoculated plants with AMF and *Rhizobia* showed slightly shorter shoot length. Single AMF inoculated plants exhibited little cultivar distinct performance, beeing slightly shorter in Messire and on average in Protecta.

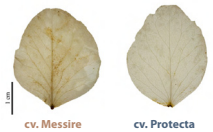
Nodulation pattern

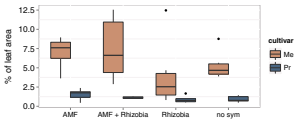




Dispersed nodules in cv. Protecta
In accordance to Ludidi (2007), the shorter shoot length of cv. Messire correlates with less but larger nodules, mainly situated on the primary root. Cv. Protecta shows more but lighter nodules, dispersed along secondary roots. By time nodules of cv. Protecta gain more weight than in cv. Messire, which number of nodules still increases. This results in similar total fresh weight of nodules but slightly lighter nodules in Messire.

H_2O_2 - levels

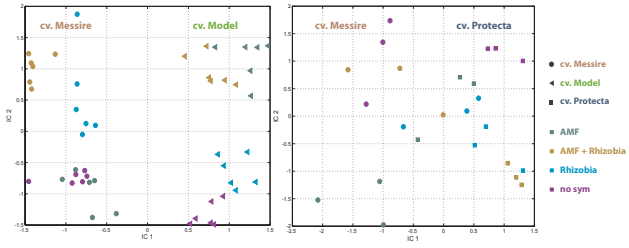




Higher ROS-level in cv. Messire
Levels of H_2O_2 were determined by the relative amount (colouring) of DAB-staining over the leaf area. In cv. Protecta levels were substantially lower compared to cv. Messire. However, AMF showed the highest concentrations among the treatments. In cv. Messire *Rhizobia* showed the lowest levels.

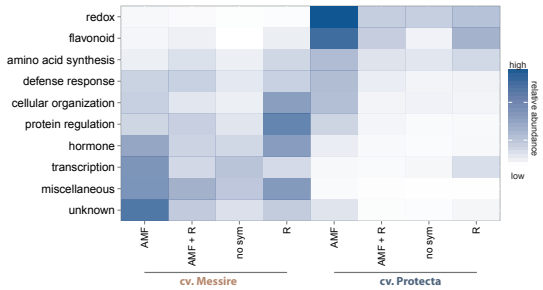
Label free proteomics

The soluble proteome of whole shoots or leaves was extracted with TRIzol and measured on a LTQ-Orbitrap XL/Elite instrument. Quantification over MS1 intensity was performed with MaxQuant LFQ software.



Impact of morphotype on the proteome
Preliminary experiments with cv. Model (low resistant, semi-leafless) and cv. Messire (leafy) showed remarkable distinct shoot proteomes (119 proteins). This great difference possibly derives from the distinct morphology of the two cultivars. IC2 separates the treatments of each cultivar: *Rhizobia* as factor responsible for separation in cv. Messire and AMF explaining separation in cv. Model. To exclude main morphological effects on the proteome cv. Protecta was chosen as similar phenotype to cv. Messire. Despite the greater number (276) of significant different proteins (t-test) the proteomes of cv. Messire and Protecta cluster more with notable higher variance on IC1. The treatments of cv. Protecta show effect on IC1 (AMF) as well as on IC2 (no sym) which indicates differing symbiotic compatibility.

Functional grouping



Redox and flavonoid pronounced in cv. Protecta
Proteins with significant difference (t-test, $p<0.05$) in abundance (2 fold) between the cultivars are categorized according to their biological function. Each tile on the map represents the relative abundance in its category. The higher abundant categories in cv. Protecta are redox involved proteins as well as proteins associated with flavonoid synthesis. In these and some more categories (amino acid synthesis, defense response, cellular organization) cv. Protecta shows a pronounced effect in the AMF treatment. This AMF derived effect is as well present in cv. Messire. However, AMF affects other biological processes in cv. Messire. Noteably, the treatment with *Rhizobia* showed more effect in cultivar Messire. Co-inoculation with both symbionts or no symbionts at all seems to hamper synthesis of cultivar specific proteins.

Conclusion

Comparing the susceptible cv. Messire with the low resistant cv. Protecta in non-infected condition we observe, in accordance to other studies [2], that above ground morphology affects nodulation patterns. This possibly results from variation in maturity, with protecta being a later cultivar. We see a noticeably effect of the symbionts on the proteome in a cultivar dependence: cv. Protecta affected by AMF and cv. Messire by either AMF or *Rhizobia*. We found that cultivar distinct proteins being higher abundant in cv. Protecta are biologically categorized in flavonoid synthesis and redox processes. Flavonoids are suggested to have ROS-quenching functions [3] which we believe to account for the lower levels of H_2O_2 in the cv. Protecta. The cultivar and treatment effects in infected plants need to be evaluated.

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