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Heavy metal tolerance and localization in the moss *Physcomitrella patens*
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1 Abstract

Bryophytes are generally considered to be highly resistant to abiotic stress and specialist mosses like *Mielichhoferia elongata* are known to tolerate high levels of heavy metal contamination on natural- or antropogenous heavy metal sites. However, no such ecotype is known for *Physcomitrella patens* the model organism for bryophytes with its genome fully sequenced; therefore it was considered a heavy metal sensitive moss. In the present study *P. patens* was cultivated in vitro and exposed to graded heavy metal solutions of Cu-EDTA, CuCl₂, Zn-EDTA, ZnCl₂ and CdCl₂, ranging from 0.1 µM for CdCl₂ and up to 100 mM for zinc and copper EDTA.

Classical resistance experiments indicated a high resistance to copper and zinc, even higher than of the specialist moss *M. elongata*. Further tests confirmed this high tolerance to heavy metals and at low concentrations, a slightly positive effect in biomass accumulation could be observed in *P. patens*.

Energy dispersive X-ray spectroscopy (EDX) on a scanning electron microscope (SEM) confirmed an accumulation of zinc and copper in the leaf tissue. Cadmium could not be detected using this method; it seemed to be lethal at concentrations beneath the detection limit of the instrument used.

For the first time, using fluorescent dyes in a confocal laser scanning microscope (CLSM), it could be shown here that the uptake of zinc is in correlation to rising heavy metal concentrations. Zinc-containing vesicles as well as zinc accumulations in the vacuole and the cell wall could be observed.

To further determine the localisation of the heavy metals within the cell, an approach was used that combines transmission electron microscopy (TEM) and the electron energy loss spectroscopy (EELS) used. First micrographs showed electron dense precipitations in the vacuole and surrounding the chloroplasts, as well as the lack of starch grains in the cells grown above 1 mM zinc- or copper- EDTA. Small copper and zinc peaks could be detected in the chloroplasts.

The analyses of *P. patens* gametophytes by high performance liquid chromatography (HPLC) displayed the stress induced production of benzoic acid, a common stress response metabolite and precursor of salicylic acid corresponding linearly to rising heavy metal concentrations.

2 Zusammenfassung

Bryophyten werden in der Literatur als generell resistent gegen erhöhten abiotischen Stress beschrieben. Spezialisten unter den Moosen wie *Mielichhoferia elongata* sind bekannt dafür, erhöhte Schwermetallbelastung an natürlich oder menschlich verursachten Schwermetallstandorten zu tolerieren. *Physcomitrella patens* gilt als Modellorganismus, dessen Genom sequenziert ist. Bislang sind keine Schwermetallresistenten Ökotypen von *P. patens* bekannt und da es an natürlich belasteten Schwermetallstandorten nicht vorkommt, wird es als sensitives Moos angesehen. *P. patens* wurde unter in steriler Kultur auf abgestuften Konzentrationen von Cu-EDTA, CuCl₂, Zn-EDTA, ZnCl₂ und CdCl₂, ausgehend von 0.1 µM für CdCl₂ und bis zu 100 mM für Zink und Kupfer, ausgesetzt.

Klassische Resistenztests zeigten eine hohe Resistenz von *P. patens* gegenüber den verwendeten Schwermetallen Kupfer und Zink, welche sogar jene bekannter Schwermetallspezialisten einstellte oder sogar übertraf. Mit einer Wachstumskontrollversuchen konnte diese Resistenz bestätigt werden. In geringen Konzentrationen wurden sogar leicht positive Effekte der Schwermetalle im Biomassezuwachs festgestellt. Untersuchungen mit dem Rasterelektronenmikroskop und Energiedispersive Röntgenspektroskopie (REM-EDX) bestätigten eine Aufnahme von Kupfer und Zink in Blattzellen von *P. patens*. Für Cadmium wurde jedoch eine tödliche Konzentration für das Moos erreicht, die unter der Messgrenze des Gerätes lag. Mit der Verwendung von Fluoreszenzfarbstoffen im Konfokalmikroskop (CLSM) konnte zum ersten Mal nachgewiesen werden, dass die Zunahme der Fluoreszenz mit der Schwermetallkonzentration korreliert. Zink war außerdem in kleinen Vesikeln, in der Vakuole sowie in der Zellwand enthalten.

Zur weiteren Lokalisation der Schwermetalle auf intrazellulärem Niveau wurde Transmissions-Elektronenmikroskopie und Elektronenenergieverlust-Spektroskopie (TEM-EELS) verwendet. Erste Ergebnisse zeigten elektrondichte Niederschläge in der Vakuole und nahe der Zellwand. Stärkekörner fehlten in den Chloroplasten jener Zellen, welche in höheren Konzentrationen als 1 mM Kupfer- und Zink-EDTA gewachsen waren. EELS-Messungen ergaben kleine Peaks für Kupfer und Zink in den Chloroplasten.

Mittels Hochleistungsflüssigkeitschromatographie (HPLC) Analyse konnte in *P. patens* ein erhöhter und mit zunehmender Schwermetallkonzentration korrelierender Gehalt an Benzoessäure, ein Stress-Metabolit und Vorstufe von Salicylsäure, gemessen werden.

3 Introduction

Heavy metal pollution is a well known fact in our environment. In the 1970's, lead pollution was in the focus of public interest, mainly because of the wide use of lead for industrial purposes and as an additive to fuel. But lead is not the only heavy metal pollution caused by men. Ongoing contamination of soils and wide landscapes by mining sites and industry are still a problem that we will have to deal with. Already slightly elevated level of heavy metals in the soil can render them unusable for agriculture. Besides the anthropogenic polluted sites, elevated heavy metal content can occur naturally. At such sites, an azonal vegetation of heavy metal tolerant specialist including mosses can be observed. Both, the soil conditions and the composition of tolerant plant species have been described extensively (Tyler, 1990). However, the mechanisms for survival are still unclear and only partially described (Pilon et al., 2009).

In this diploma thesis, the effects of heavy metals concerning resistance, uptake and cellular localizations in the putatively sensitive moss *Physcomitrella patens* are described and discussed.

3.1 Heavy metals

Heavy metals are naturally occurring elements with ubiquitary distribution and variable concentrations usually lower than 1 % in the bio- and lithosphere (with exceptions like iron and aluminum). In most cases, they are micronutrients and functional parts of enzymes but - depending on the element - they can have harmful and toxic effects (Tyler, 1990). In elevated concentrations, essential as well as non essential heavy metals become toxic.

3.1.1 Definition

Metals can be defined by weight and all metals with a density $> 5 \text{ g/cm}^3$ are heavy metals. However, some metals have lower density and although this definition is widely used, it is improper. For ecological investigations, there is another more relevant definition.

Nieboer and Richardson (1980), classified the elements by their affinity to complexes into three classes, as described below.

Class A (oxygen-seeking)

In this class are the elements with high affinity to oxygen-groups in macromolecules. To this group belong the alkaline earth metals, so called hard acceptors like Al^{3+} , Cr^{3+} , Co^{3+} , Fe^{3+} and also manganese (Mn^{2+}).

Class B (nitrogen/sulphur-seeking)

Elements with a high affinity to sulphur- or nitrogen groups in macromolecules are assigned to this group, so called soft acceptors like Cu^+ , Ag^+ , Au^+ and Hg^{2+} .

Class C (borderline-metals)

To class C all "borderline-metals" are integrated where no clear assignment to class A or B was possible. To this class belong Fe^{2+} , Cu^{2+} , Co^{2+} , Ni^{2+} , Zn^{2+} , Pb^{2+} and Cr^{2+} .

This classification is more relevant for biological investigations than the simple definition by density because in literature aluminum (Al) as a light metal, Arsen (Ar) and tin (Sn) as half-metals and Se as non-metallic element are all considered as "heavy metals" (Prasad, 2004).

The elements used in this work (copper, zinc, iron and cadmium) meet both definitions and are therefore consolidated as heavy metals.

3.1.2 Macro- and micronutrients

The heavy metals used in this work (copper, zinc, iron), with the exception of cadmium, can also be described as micronutrients.

Besides macronutrients (C, H, O, N, P, S, K, Ca, Mg) plants need also small amounts of such micronutrients (B, Cl, Cu, Mn, Fe, Zn, Mo), and some also need Si, Co and Se (Hansch and Mendel, 2009). In contrast to macronutrients, these micronutrients are only needed in very small amounts, whereas availability in high concentrations results in harmful effects. Non-essential elements like cadmium cause highly toxic effects even in very low concentrations (Wang et al., 2009).

3.1.3 Copper

Copper (Cu; density: $8.94 \text{ g}\cdot\text{cm}^{-3}$) is a very ductile metal and due to its high electrical and thermal conductivity, it is a very important industrial metal. In biological context it is an essential micronutrient for the enzymes plastocyanin, superoxide-dismutase or cytochrome-oxidase (Sakurai, 2006). Copper is known to be toxic for plants in higher concentrations of $3.6 \text{ }\mu\text{M}$ (LD50) (Baker, 1992). Extensive studies of naturally and anthropogenic habitats with copper contamination have a tradition within our group, Cell Imaging and Ultrastructure Research. The *Schwarzwand*, (Salzburg, Austria) has been well studied (Nadubinská et al., 2005; Hus, 2008) and is still part of ongoing investigations.

On natural habitats, copper occurs as native copper, CuS_2 , Cu_2O , CuFeS_2 (Chalcopyrite), CuCO_3 and $\text{Cu}(\text{OH})_2$ (Malachite).

3.1.4 Zinc

Zinc (Zn; density: $7.14 \text{ g}\cdot\text{cm}^{-3}$) is a silvery metal widely used in the production of anti-corrosive zinc-plating of steel. In biology, it is a very important micronutrient for zinc containing enzymes like the zinc-finger, carbonic anhydrases and the superoxide-dismutase (Coleman, 1992). It is considered to be less toxic for plants than copper (Shakya et al., 2008). Some plants are hyper-accumulators for zinc like *Arapidopsis*

halleri and *Thlaspi caerulescens*. Those species occur naturally on heavy metal sites and have been shown to survive very high levels of zinc (Banasova et al., 2006). In contrast to iron or copper, zinc is highly mobile in the soil.

Naturally, zinc is occurring as Zn(Fe)S (Sphalerite or *Zinkblende*), and ZnCO_3 (Smithsonite).

3.1.5 Cadmium

Cadmium (Cd; density: $8.65 \text{ g}\cdot\text{cm}^{-3}$) is a silvery grey metal which, despite its ecological harmful potential, is still widely used for industrial purposes. It is neither a macronutrient nor a micronutrient; in fact it is a non-essential element for life.

However, its affinity to bind onto organic molecules combined with its high mobility in the soil and also within the eukaryotic cells makes cadmium highly toxic. In some compounds, it is considered to be carcinogen (Andujar et al., 2010).

Cadmium is naturally occurring as CdS (Greenockite) and mostly as contamination in phosphor and zinc ores. It is produced mainly as a byproduct in the mining of zinc ores.

3.1.6 Iron

Iron (Fe; density: $7.874 \text{ g}\cdot\text{cm}^{-3}$), the sixth most abundant element in the earth crust, is a silvery-grey metal but oxidizes to a rusty brown in contact with air. It is the most important metal in everyday use. Iron is the core element in hemoglobin of our red blood cells, and furthermore a micronutrient for plants (Hansch and Mendel, 2009). Iron is relatively immobile in the soil.

Naturally, iron occurs for example as Fe_3O_4 (Magnetit), Fe_2O_3 (Hämatit), FeCO_3 (Siderite) or FeS_2 (Pyrite).

3.1.7 Effects of heavy metals

Traces of heavy metals like the essential micronutrients (copper and zinc) as well as the non essential cadmium occur in most soils. Under normal conditions plants even have to mobilize the essential elements to meet their requirements. In the other case of highly elevated heavy metal content in the soil, plants have developed defense mechanisms to tolerate the surplus of micronutrients or non-essential metals which would lead to toxic effects. This mechanism has been described as metal homeostasis and is common to all higher plants (Krämer and Clemens, 2005).

Metal homeostasis describes the ability of an organism to maintain its physiological optimum of all elements. It is evident that metal homeostasis can only work within certain physiological limits. Under unusually high or low concentrations, metal homeostasis fails and the optimum cannot be obtained any longer. Therefore, effects of micronutrient deficiency or oversupply will follow.

For non-essential metals like cadmium no such plateau of deficiency- and toxicity symptoms was observed: Above a certain concentration the investigated organisms showed toxic symptoms (Shaw, 1990).

3.2 Strategies of heavy metal tolerance

In general, there are two main strategies to cope with elevated heavy metal levels: avoidance and tolerance.

3.2.1 Avoidance

After Tyler (1990), the avoidance strategy mainly consists in trying to prevent the entering of elevated amounts of heavy metals into the organism. This is a fundamental strategy of higher plants to resist heavy metal stress.

Plants show clear avoidance by *mycorrhiza*, a symbiosis with fungi, which filter the heavy metals or exude metal binding chelates into the rhizosphere (Klugh-Stewart and Cumming, 2009; Zheng et al., 2009). This immobilizes the heavy metals in the soil

and renders them unavailable to the plant. Another defense system is the cell wall, with its capacity to bind heavy metal ions on negatively charged pectin (Krzyszowska et al.).

For mosses none of these strategies are described: The “root” system of mosses is only rudimentary (rhizoid, see below) and genes for metal chelates have not been found in the *P. patens* genome (Rother et al., 2006). It is likely that the cell wall of mosses has similar tasks as in higher plants and it is a part of this thesis to investigate the deposition of heavy metals to the cell wall.

3.2.2 Tolerance

The strategy of tolerance allows heavy metals to enter the organism and it enables the organism to cope with elevated heavy metal concentrations within the body and cell. The membrane is considered to be the first living barrier of the cell, therefore its role in metal homeostasis is very important. Many plant metal transporters into the cytosol like ZNT1 (for zinc and cadmium); COPT1 (for copper); AtNramp1/3/4 (for iron and cadmium) and ZAT (for zinc, CAX2 for cadmium) from cytosol to vacuole as well as and RAN1 (for copper) from cytosol to golgi could be identified (Clemens, 2000).

Furthermore, chelation is considered to be important for metal homeostasis, as demonstrated by Schmidt et al. (1999) for copper in yeast cells and for phytochelatins in plant cells (Briat and Lebrun, 1999), metallothioneins, organic and amino acids, chaperones, metal trafficking and intracellular sequestration are all part of a complicated regulatory network for metal tolerance which is still not completely understood and under investigation (Clemens et al., 2002).

3.2.3 Accumulator, Indicator and Excluder

After Baker (1981), three groups of metallophytes are described depending on where the plant tries to immobilize a surplus of heavy metals. The groups are differed by their metal concentration in aerial plant parts.

The excluders try to maintain constant and low concentrations of the heavy metal in the aerial parts of the plant. There is retention of the heavy metal in the soil and root. However, this strategy only works up to a certain concentration of metals in the soil.

The indicators respond linearly to the heavy metal offered by the soil and can therefore be used as indicator plants.

Accumulator and hyperaccumulator plants are able to concentrate metals in aerial parts up to very high levels which may exceed the concentration in the soil up to 300-400 times.

3.3 Mosses

Mosses are a heterogenic group which is positioned in the kingdom of plants between the *Charophytes* and the *Tracheophytes*. They form their own division of the *Bryophyta* to which also liverworts and hornworts belong. Due to different systematical approaches, the liverworts and hornworts are sometimes considered as a division of their own. Simplified mosses are an evolutionary very old group of land plants. For further information on the position in the systematic tree please visit the following website (<http://ucjeps.berkeley.edu/TreeofLife/hyperbolic.php>) (Rensing et al., 2008).

Mosses are small plants (1 - 10 mm) and can be found ubiquitary, which means worldwide and in many ecological habitats. They are poikilohydric and may survive complete desiccation. However, they cannot regulate their water balance in the way higher plants do. In difference to most land plants with a dominant diploid phase in their lifecycle, the dominating vegetative phase of mosses is haploid. This makes them ideal models for genetic experiments, because due to the single set of chromosomes, mutations or transformations of the genome are immediately effective. Only during sporophyte production, mosses enter a short diploid phase (Bopp, 1981; Chopra and Kumra, 1988; Frahm, 2001).

During its lifecycle, the moss sporophyte produces haploid meiospores by meiosis. The spores germinate to filamentous haploid protonemata, separated into caulonemata ("roots") and chloronemata. The protonema is a precursor of the leafy haploid gametophyte which as in flowering plants produces the *Gametangium*, the sexual organs (*Antheridium* ♂ and *Archegonium* ♀). After fertilization, the diploid sporophyte ripens and again forms meiospores (Frahm, 2001).

Different to flowering plants, mosses do not possess real leaves, stems or roots. The leaflets are *phylloides*, the stems are *cauloides* and the root like structures are *rhizoides*. Although I am well aware of this fact, nonetheless for facile reading the term leaf or stem is sometimes used.

3.3.1 Mosses as Bio-indicators

Due to the special ability of some moss species to survive high levels of abiotic and biotic stress as well as to accumulate harmful pollutants in their biomass (e.g. heavy metals), they are used as bio-indicators. For example mosses are used to monitor the concentrations of the air pollutant SO₂ (Frahm, 1998) and also as indicators for environmental metal pollution (Aceto et al., 2003).

3.3.2 Mosses on heavy metal sites

Similar to other specialized plants mosses like *Mielichhoferia elongata*, *Pohlia drummondii* and *Scapania undulata* are known to grow on heavy metal polluted sites (Url, 1956). Some bryophytes are even considered to be copper accumulating and were described as "coppermosses" (Martensson and Berggren, 1954). The mosses from metal contaminated habitats were thoroughly tested (<http://www.cosmoss.org/>). Recently, the establishment of sterile cultures of *P. drummondii* and *M. elongata* was successful (Wernitznig, 2009; Wernitznig et al., 2009). So far, *Physcomitrella patens* is not known to occur on heavy metal polluted sites.

3.3.3 *Physcomitrella patens* (Hedw.)

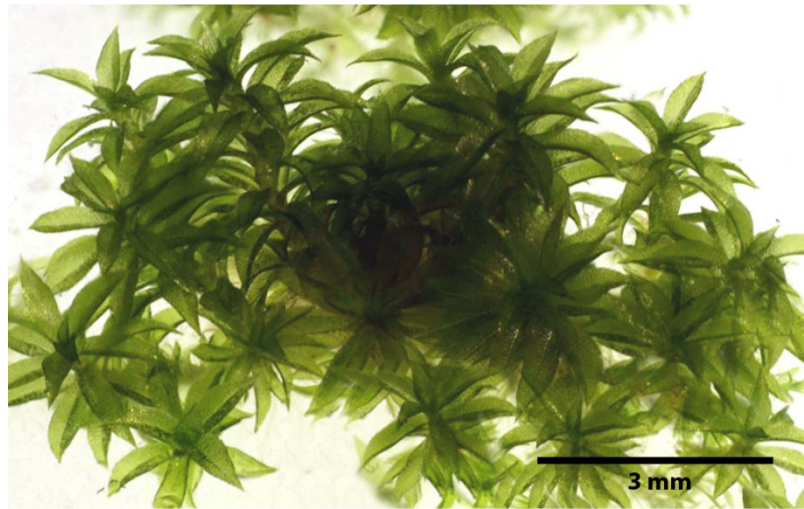


Fig. 1: Macroimage of *P. patens* grown *in vitro* on agar petri dishes

Physcomitrella patens (Funariaceae) was first described by Johann Hedwig (1730-1799). It is a member of the order of the Funariales which belong to the class of Bryopsida (Fig. 1).

P. patens is a non-vascular, multi-cellular land plant which is up to 5 mm high. Its natural habitats are argillaceous or silted soils mainly of dry-fallen river banks throughout Eurasia and northern America. It does not occur at higher altitudes (Frahm, 2003).

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 (Cove and Knight, 1993; Lang et al., 2008). The complete sequencing of the genome of *P. patens* even increased its importance as model organism (Reski and Cove, 2004; Rensing et al., 2008).

3.4 Aim of the work

The aim of this thesis was the investigation of the postulated high heavy metal tolerance of bryophytes in the model organism *P. patens* (Frahm, 2001). Although Reski and Cove (2004), also mentioned that *P. patens* is highly tolerant to abiotic stresses like low temperature, salinity, drought and even heavy metals no classic resistance test nor other physiological investigations have been performed so far. The exact resistance to specific heavy metals and metabolic responses of *P. patens* still need to be determined. Furthermore, the allocation and the possible storage sites of heavy metals within the cells or cell walls are still to be revealed.

Investigating these open questions, the following methods were used and the results discussed that are partially already presented or published (Sassmann et al., 2009; Sassmann et al., 2010).

4 Material and methods

4.1 Plant material

Physcomitrella patens (Hedw.) belongs to the family of Funariaceae which are part of the division of Bryophyta. The similarity to higher plant anatomy, simple morphology and the single layer leaflets as well as the role as model organism made *P. patens* the ideal subject of this study. Whole gametophytes (HPLC, EDX), leaves (TEM, EELS, CLSM) and protonema (CLSM) were used. The habitus of *P. patens* is shown in Fig.2.



Fig. 2: Image of the *P. patens* gametophyte

4.2 Cultivation and growth control

4.2.1 Media

Growth media were prepared after Benecke (1903) and modified according to Gang et al. (2003) to establish heavy metal free growth conditions on control plates. The medium contained 200 mg/l NH_4NO_3 , 100 mg/l $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 400 mg/l KH_2PO_4 and 100 mg/l $\text{CaCl}_2 \times 2\text{H}_2\text{O}$; the pH was adjusted to 5.8. Agar (0.8%; VWR, Leuven, Belgium) was added and the mixture was autoclaved before casting into sterile plastic petri dishes with a diameter of 9 cm (Greiner Inc, Austria), (Sassmann et al., 2010).

4.2.2 Cultivation

First *P. patens* plants were a kind gift of the Reski lab (www.plant-biotech.net) in Freiburg. The plants were grown and sub cultured regularly under sterile culture conditions. Using vegetative reproduction of the whole gametophyte, all investigations could be performed with closely related plant material. No sexual reproduction or spores were used.

Nine moss plants per petri dish (Fig. 3) were grown for at least 5 weeks on media in sterile *in-vitro* culture at an average temperature of 24°C on a 14-h day /10-h night regime. Average light intensity was $48 \mu\text{M s}^{-1} \text{m}^{-2}$.

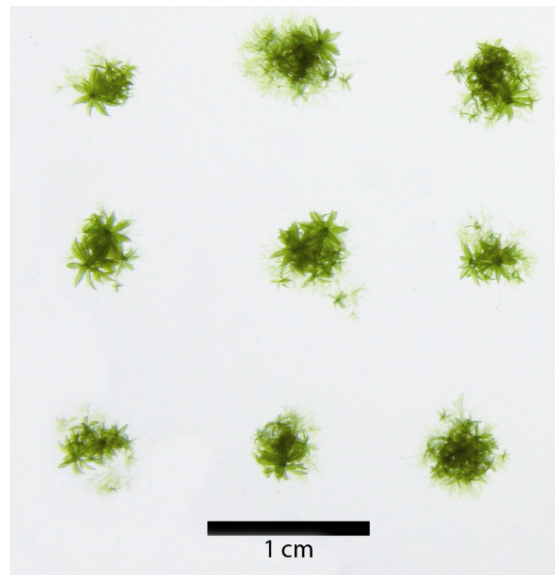


Fig. 3: Gametophytes grown on control media (nine per plate)

For cultivation with heavy metal spiked media, solutions of Cu-EDTA (copper-ethylenediaminetetraacetic acid), CuCl_2 , Zn-EDTA, ZnCl_2 and CdCl_2 were added from stocks. Final media concentrations were 100 mM, 10 mM, 1 mM and 100 μM (Cu-EDTA and Zn-EDTA), 5 mM, 1 mM and 100 μM (ZnCl_2), 1 mM and 100 μM (CuCl_2) and 5 μM , 1 μM and 0.1 μM (CdCl_2).

4.2.3 Growth control

For growth analyses, plantlets were photographed weekly over a period of 5 weeks. Macrographs of the petri dishes and a scale were taken with a Canon EOS 20D digital camera and a 28-80 mm zoom lens. Analysis was performed with Adobe Photoshop CS4.

The increase or decrease in growth was calculated in percent of the starting point using planimetry measurement. Under certain stress conditions, *P. patens* tended to form protonemata instead of normal plant development. A two dimensional approach would therefore lead to incorrect calculations, and widely differ from the actual biomass accumulations. In order to balance such distorted measurements, 10, 20 or 40 percent of the increase of growth were subtracted depending on the amount of protonemata built (>25, 25-50, or <50 percent, respectively) was subtracted.

By random selection of the total pool of 10 plates, a minimum of 5 plates were chosen for each heavy metal concentration and 9 plants from each plate were measured. That resulted in a total of at least 45 measurements per concentration. For control, 83 measurements of *P. Patens* grown on standard media were averaged. Approximately 720 plants were measured in 4200 measurements over 5 weeks. The mean values, standard error and confidential interval (Students t-test; $p < 0.10$) were calculated and plotted in a graph (Excel, Microsoft); (see also Sassmann et al., 2010).

4.3 Resistance

For resistance experiments, leaflets and whole gametophytes of *P. patens* were submersed in graded heavy metal solutions for 48 hours according to Url (1956). *P. patens* was exposed to Cu-EDTA, CuCl₂, Zn-EDTA, ZnCl₂ and CdCl₂ in concentrations ranging from 1 M to 10⁻⁹ M. In control experiments, bi-distilled water was used instead. After 48 h, the cells were investigated for plasmolysis in 0.8 M D-mannitol, followed by deplasmolysis in 0.3 M D-mannitol under the light microscope to reflect the viability of the cells. A survival rate of more than 50 percent was accounted as resistant (+), less than 50 percent as non-resistant (-). 50 percent living to 50 percent dead cells marked the lethal dose of 50 percent (LD50) displayed with (-+).

For resistance experiments and all other light microscopic documentation, an Olympus BX41 microscope with the objectives 10x (N.A. 0.85), 20x (N.A. 0.40), 40x (N.A. 0.64) and 60x oil (N.A. 1.25) with an attached digital camera (Color View III, Soft Imaging Systems, Olympus) was used. Using CellD (Olympus) software, the images were loaded into the computer.

4.4 Electron microscopy

4.4.1 Sample preparation (SEM, EDX)

For the scanning electron microscope (SEM) moss samples were cut and subsequently air dried, mounted on carbon stubs and carbon-coated. To avoid contamination great attention was paid that fresh moss samples had no contact with the heavy metal containing media.

4.4.2 Energy-dispersive X-ray spectroscopy (EDX)

EDX-analysis of heavy metal uptake was performed by X-ray microanalysis on a scanning electron microscope (Philipps XL 20) and data were collected with EDAX-Genesis software. This method is semiquantitative and heavy metal content is detected in relation to all other elements analyzed in the sample, in this case C, O, Na, Mg, P, S, Cl, Cd, K, Ca, Fe, Cu and Zn.

Analysis was performed at an acceleration voltage of 30 kV with working distance 12, tilt 15, take of angle 27.21, with a dead time of approximately 30 percent at 2500 x magnification over Lsec 100.

On each plant, between 5 and 10 regions were analyzed. In total, 644 analyses were performed. The main target were leaf cells except for 100 mM Zn-EDTA (protonema) but all moss tissues were investigated. Statistical analysis of the results was performed by Student's t test ($p < 0.05$; Microsoft Excel).

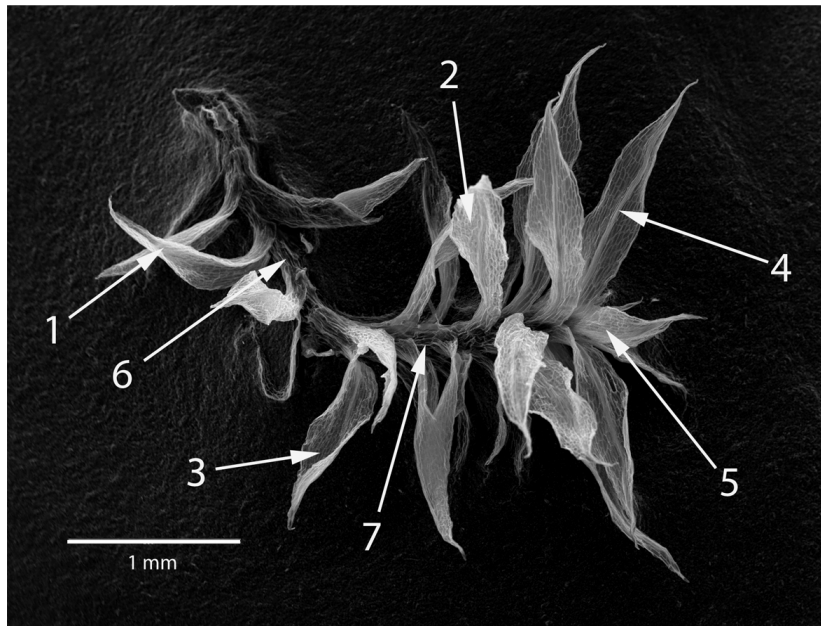


Fig. 4: SEM micrograph of a gametophyte; numbers indicate the regions of the EDX analyses, similar recordings were made for all samples. (picture presented by Sassmann et al. (2009) at the MC 2009 (Graz, Austria)).

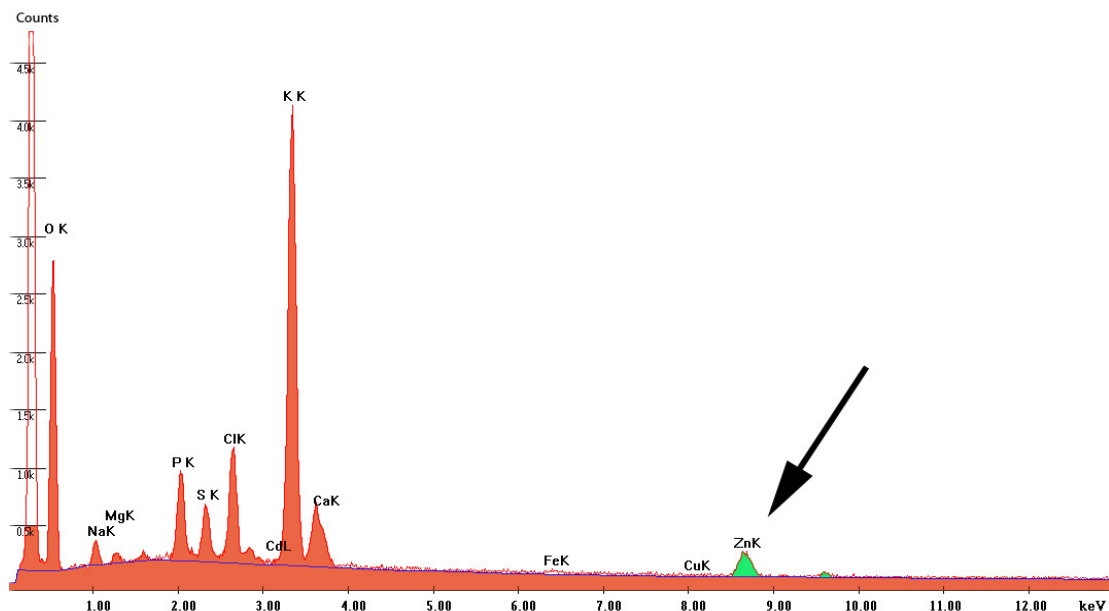


Fig. 5: Typical EDX-spectrum presenting a distinct Zn peak (arrow)

4.4.3 Sample preparation (TEM, EELS)

For sample preparations for electron energy loss spectroscopy (EELS), single leaves were cryofixed with a Leica EMPACT high pressure freezer by the kind help Prof. Dr. Ursula Lütz-Meindl and her group and in cooperation in Salzburg, Austria. Freeze substitution was performed by her group with 2 % OsO₄ and 0.05 % uranylacetate in acetone at – 80°C for 48 h as described in Meindl et al. (1992). The leaves were embedded in Agar Low Viscosity Resin (LV) (Agar Scientific) in petri dishes for ideal alignment of the samples.

The embedded probes were cut out of the thin petri dish resin and glued on resin blocks for ultra thin sectioning.

Blocks were trimmed and semi-thin sections were cut with glass knives. Semi-thin sections were stained with Toluidine blue (Tolonium chloride) to confirm the moss tissue within the sections.

Ultra-thin sections (25-50 nm) were performed with a diamond knife (Diatome) and mounted on hexagonal narrow mesh copper grids (Hex 700 Thin Bar, copper 3.05 mm; Agar Scientific).

4.4.4 Transmission electron microscopy (TEM)

Transmission electron microscopy was performed on a Zeiss CEM 902 at an acceleration voltage of 80 kV. Micrographs were taken by a SharpEye camera system and processed with Item 5.

4.4.5 Electron energy loss spectroscopy (EELS)

EELS analyses were performed in the lab of Prof. Dr. Ursula Lütz-Meindl in Salzburg (Austria) with a Leo 912 AB Transmission Electron Microscope. The microscope was operated at the specifications for EELS measurements. We used an acceleration voltage of 120 kV with an exposure time of 20 sec, with illumination angles from 1,25 to 2,5 mrad at a magnification from 25,000 x up to 80,000 x with a spectrum magnification of 200 x.

4.5 Confocal laser scanning microscopy (CLSM)

The heavy metal localization was also investigated by confocal laser scanning microscopy with a Leica DM-IRE 2, which is an inverted confocal microscope. The following objectives were used: HC PL FLUOTAR 10 x (N.A. 0.3) DRY, HC PL FLUOTAR 20x (N.A. 0.5) DRY, HCX PL APO 40x (N.A. 1.25) OIL, HCX PL APO 63x (N.A. 1.32) OIL. For better resolution images were recorded with line average 2 and average 4 meaning every point of the image was scanned eight times. Furthermore images were improved by average and maximum projections of image stacks consisting of 20 to 30 images per stack. For further details on confocal microscopy see also Hepler and Gunning (1998).

4.5.1 Sample preparation

CLSM-samples were prepared from living moss leaves or protonemata on microscopic glass slides. Cells were stained with Phen Green™ SK and FluoZin™-3 for one hour. After staining the samples were rinsed twice with bi-distilled water to remove residues of the dyes.

4.5.2 Fluorescent dyes

The used fluorescent dyes were a water soluble salt in the case of FluoZin-3 (Fig. 7) or a diacetate ester in the case of Phen Green™ SK (Fig. 6). Therefore high membrane permeability was expected.

A solution of 5 μM Phen Green™ SK was used to label Cu^{2+} within the moss cells. Its excitations maximum is at 507 nm with an emission maximum at 532 nm.

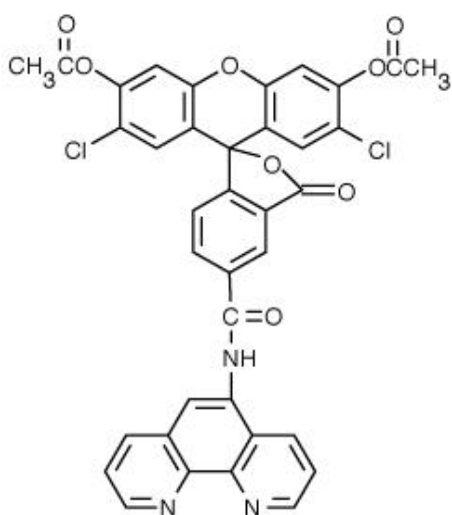


Fig. 6: Phen Green™ SK¹

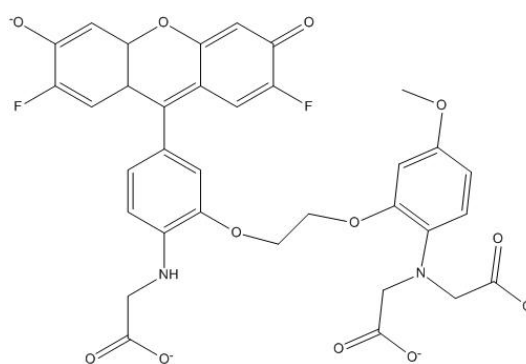


Fig. 7: FluoZin-3²

A solution of 5 μM FluoZin™-3 was used to label Zn^{2+} within the moss cells. With an excitation maximum at 493 nm, it was excited by an Ar-Ar/Krypton laser at 488 nm with an intensity of 40 percent and detected at 516 nm with a detection window of 20 nm for the dye and 50 nm for the auto fluorescence (see below).

¹ <http://www.invitrogen.com/site/us/en/home/support/Product-Technical-Resources/Product-Structures.-14313.html>

² http://www.nature.com/nchembio/journal/v5/n3/compound/nchembio.146_comp8.html

4.5.3 Auto fluorescence of *Physcomitrella patens*

Performing a wavelength-scan, autofluorescence of *P. patens* was analyzed in untreated control cells after excitation at 488 nm (Fig. 8). The scan was performed in 20 nm steps for a bandwidth from 495 nm to 750 nm. The scan resulted in the detection of autofluorescence of the chloroplast, as illustrated by a minor peak at 520 nm and a high autofluorescence peak ranging from 670 to 720 nm. No other cell components showed auto fluorescence at the used excitation wavelength of 488 nm.

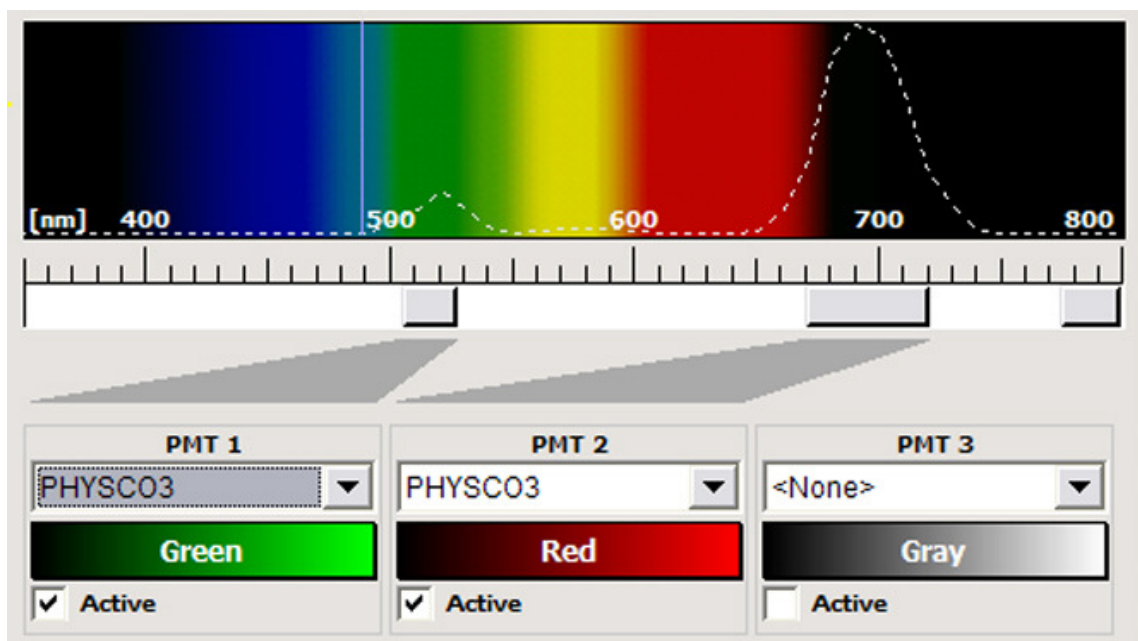


Fig. 8: Light spectrum with dotted line as an indicator of chloroplast auto fluorescence of *P. patens*; Blocks show detection ranges of two fluorescence detectors (PMT1 and PMT2)

4.6 High performance liquid chromatography (HPLC)

Detection of putative secondary metabolites was done by HPLC. The system used for HPLC was a Dionex Summit provided with a photodiode array detector (PDA) and a Famos auto-sampler. The column was a Phenomenex Synergi Max C12, 150×2 mm, 5µm particle size. The column oven was adjusted to 40°C, and the flow rate was 0.2 ml min⁻¹. The solvent was 1% acetic acid with equal parts of methanol (Chobot et al., 2009).

4.6.1 Sample preparation

The plant material of 4 growth plates (9x4 plants) was collected. Special attention was paid to avoid contact with the media. Mosses were immediately frozen by pouring liquid nitrogen over the plant material. While still in the frozen state, it was homogenized in a mortar and 100 ml of a mixture of methanol and concentrated acetic acid (99:1) were immediately added. The resulting solution was cooled to 4°C and stored for at least 24 hours.

Following the filtration of the sample with cotton (1x rinsed with methanol), the filtrated sample liquid was evaporated in a Rotovapor at 40°C. The remaining residues were dissolved in 1% acetic acid.

Separation of non lipophilic constituents in H₂O and lipophilic constituents in ethanol was performed with an Amberlite XAD-1180 column (15-20 g per column).

H₂O phase was collected and frozen at -20°C. It will later be used for gas chromatography. After collection of the ethanol phase, it was further concentrated by evaporation with a Rotovapor at 40°C. The residue was dissolved in absolute ethanol once more and transferred to brown glass flasks. After a second evaporation, the residue was stored at -20°C until final sample preparation.

Dry samples with a weight of approximately 10 µg were dissolved in 100 µl methanol and 0.1 % acetic acid (1:1), and further diluted to a total of 2.5 ml. Out of these, 1.5 ml were filled into microtiter flasks for final analysis.



Fig. 9: Diluted samples ready for analysis with HPLC

4.6.2 Benzoic acid

The main target of the HPLC investigations was benzoic acid (Fig. 10) which is the simplest aromatic carboxylic acid. It is considered to play an important role in general stress response metabolism (Senaratna et al., 2003).

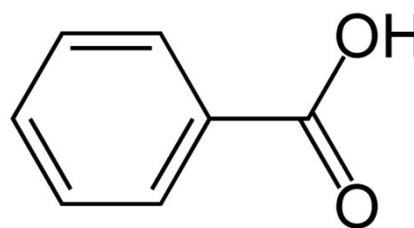


Fig. 10: Benzoic acid³

³ http://commons.wikimedia.org/wiki/File:Benzoic_acid.png

5 Results

5.1 Resistance

The ability of *P. patens* to resist heavy metal stress was estimated by a classical resistance test. Gametophytes of *P. patens* were submersed for 48 h in graded solution of Zn-EDTA, Cu-EDTA, ZnCl₂, CuCl₂, CdCl₂ and in water for control. Vitality was proven by following plasmolysis in 0.8 M mannitol as first step to prove vitality, subsequent deplasmolysis was realized in 0.3 M mannitol. Only living or surviving cells show plasmolysis and subsequent deplasmolysis (Fig. 12). *P. patens* survived high concentrations of up to 10⁻² M Zn-EDTA and 10⁻³ M Cu-EDTA solutions. In addition, the morphology of the cytoplasm and its organelles was screened for possible alterations due to heavy metal stress (Table 5-1).

5.1.1 Control

Control cells of *P. patens* (Fig. 11 and Fig. 12) show vital green chloroplasts and nearly 100 percent vitality rate.

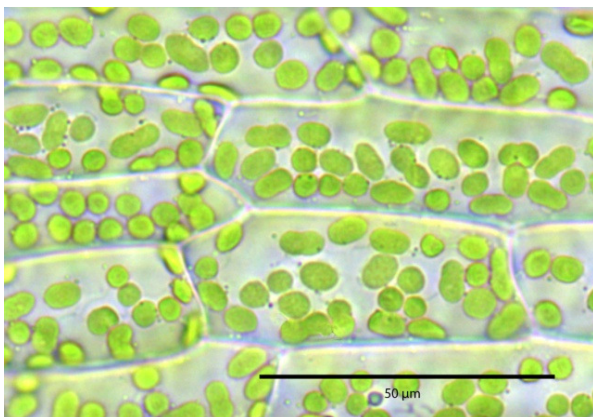


Fig. 11: Microscopic bright field image of control cells in H₂O

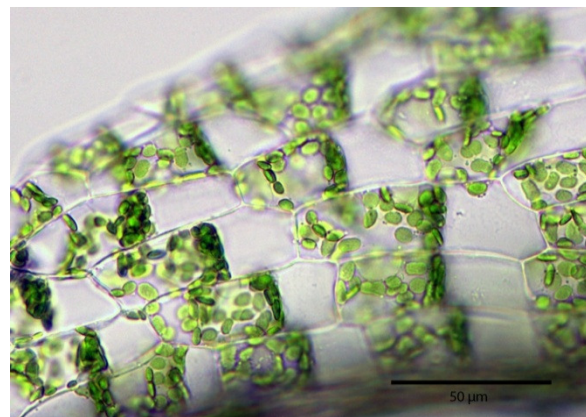


Fig. 12: Control (plasmolysed); bright field image

5.1.2 Zn-EDTA

At 10^{-1} M Zn-EDTA 50 percent (Fig.13) of the cells showed plasmolysis and subsequent deplasmolysis. 50 percent were registered dead or did not deplasmolyse. In these cells the cytoplasm was coagulated and chloroplasts if still visible had lost their green color. This result is suggesting a resistance of *P. patens* up to this concentration. After treatment with lower concentrations only vital, plasmolysed leaf cells of *P. patens* were observed (10^{-2} M Zn-EDTA; Fig. 14).

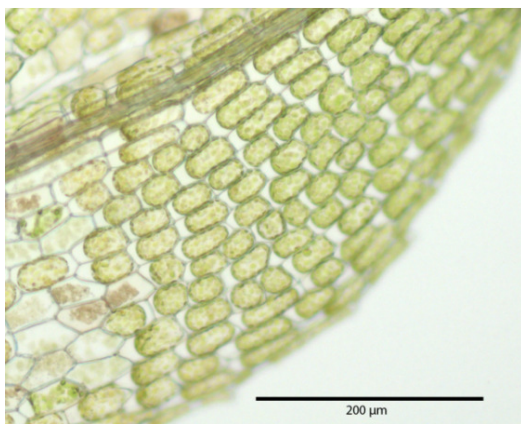


Fig. 13: Zn-EDTA 10^{-1} M (+, plasmolysis); bright field image

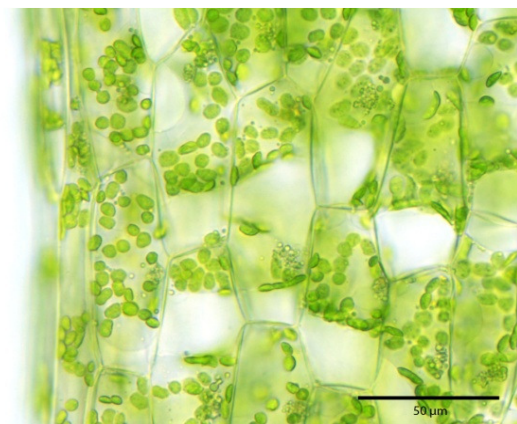


Fig. 14: Zn-EDTA 10^{-2} M (+, plasmolysis); bright field image

5.1.3 Cu-EDTA

Resistance to Cu-EDTA was one magnitude less than to Zn-EDTA, but else the pictures were similar. High resistance to a solution of 10^{-2} M Cu-EDTA over 48 h could be observed; up to 50 percent of the leave cells remained vital after this treatment. In cells unable to plasmolyse, the organelles maintained their form and chloroplasts kept their green color (Fig. 15). Almost all *P. patens* cells plasmolysed and remained vital after treatment with 10^{-4} Cu-EDTA (Fig. 16).

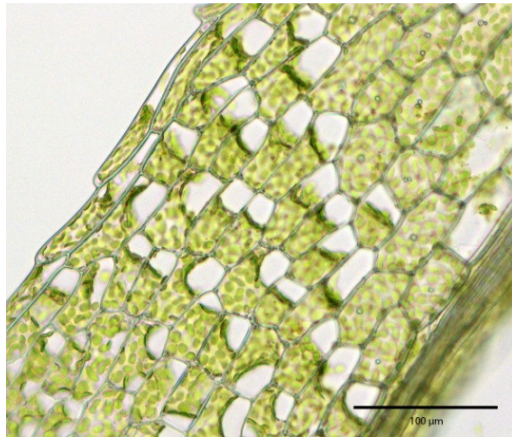


Fig. 15: Cu-EDTA 10^{-2} M (+, plasmolysis) ; bright field image

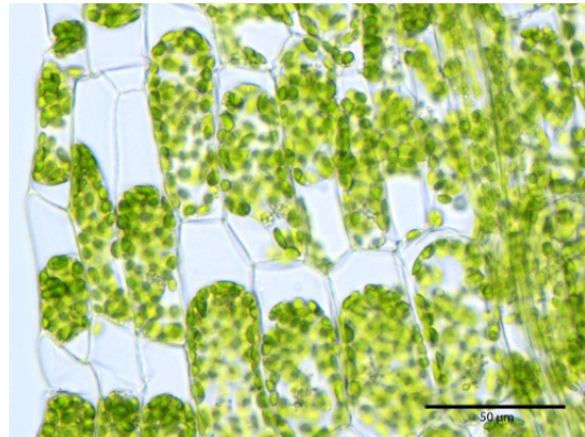


Fig. 16: Cu-EDTA 10^{-4} M (+, plasmolysis) ; bright field image

5.1.4 ZnCl_2

A high percentage of dead leaf cells was observed after exposure to 10^{-4} M ZnCl_2 , however outer leaf cells as well as parts of the gametophyte still showed plasmolysis (Fig. 17). In higher concentrations all cells were dead and chloroplasts had lost their green color. Lower concentrations of ZnCl_2 seemed to have no visible effects on the vitality of the cells (Fig. 18).

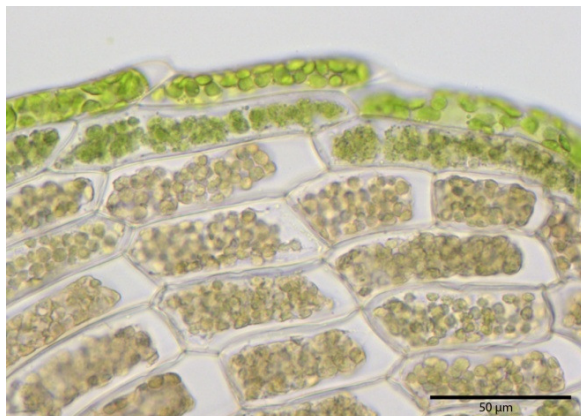


Fig. 17: ZnCl_2 10^{-4} M (-, plasmolysis); bright field image

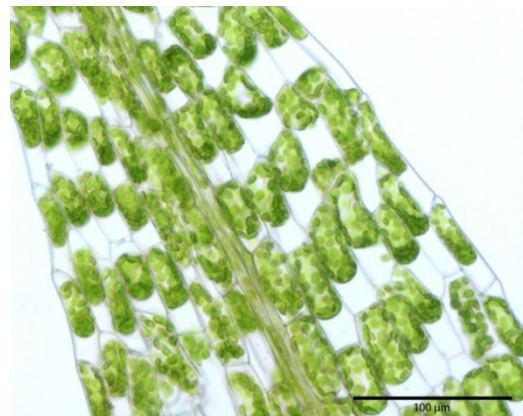


Fig. 18: ZnCl_2 10^{-7} M (+, plasmolysis); bright field image

5.1.5 CuCl_2

10^{-6} M CuCl_2 seemed to be the LD50 where approximately 50 percent of the cells had died (Fig. 19) and did not plasmolyse. In the next lower concentration of 10^{-7} M CuCl_2 this visible toxic effect was lost and nearly 100 percent showed plasmolysis and subsequent deplasmolysis (Fig. 20)

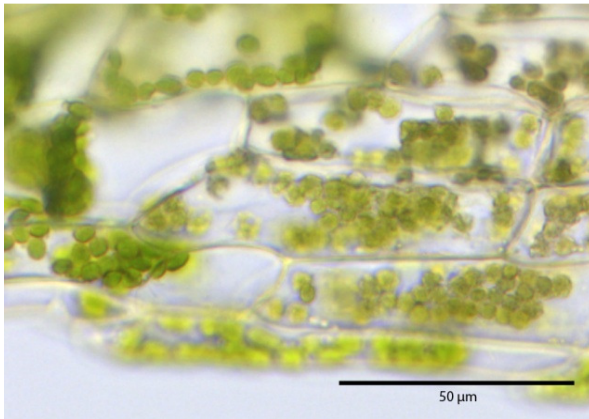


Fig. 19: CuCl_2 10^{-6} M (+-, plasmolysis); bright field image

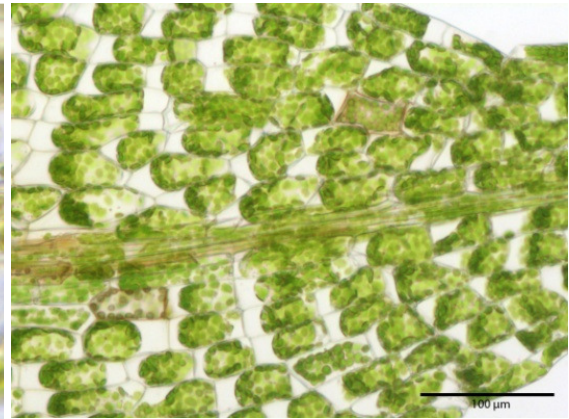


Fig. 20: CuCl_2 10^{-7} M (+, plasmolysis); bright field image

5.1.6 CdCl_2

In comparison to other heavy metals, CdCl_2 had the most toxic effect on *P. patens* cells; already the low concentrations of 10^{-7} M CdCl_2 resulted in 50 percent dead cells (Fig. 21). Interestingly lower concentrated CdCl_2 did not show any visual harmful effect (Fig. 22).



Fig. 21: CdCl_2 10^{-6} M same as 10^{-7} M (+-, plasmolysis); bright field image

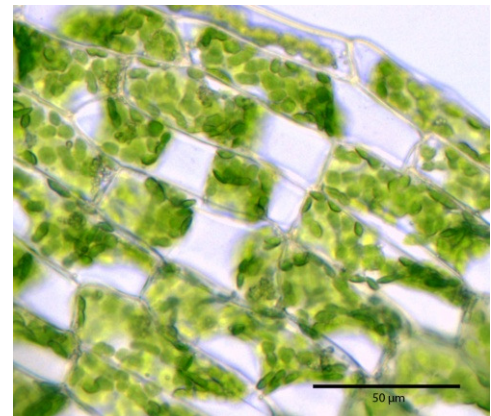


Fig. 22: CdCl_2 10^{-8} M (+, plasmolysis); bright field image

5.1.7 Summary

The resistance of *P. patens* is indicated by those concentrations where 100 percent of the cells survived. In summary, *P. patens* showed an unexpectedly high resistance in Zn-EDTA up to 10^{-2} M and in Cu-EDTA up to 10^{-3} M. More toxic effects of ZnCl_2 and CuCl_2 are indicated by the lower resistance (10^{-5} M and 10^{-7} M). The cells were less resistant to CdCl_2 (10^{-8} M). LD50 was 10^{-1} M for Zn-EDTA and from 10^{-1} to 10^{-2} M for

Cu-EDTA, from 10^{-3} to 10^{-4} M for ZnCl_2 . The two most harmful heavy metals had an LD50 of 10^{-6} M for CuCl_2 and from 10^{-6} to 10^{-7} M for CdCl_2 .

Media	Concentration of solutions										Control
	1M	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	
Zn-EDTA	-	-+	+	+	+	+	+	+	+	+	+
Cu-EDTA	-	-+	-+	+	+	+	+	+	+	+	+
ZnCl_2	-	-	-	-+	-+	+	+	+	+	+	+
CuCl_2	-	-	-	-	-	-	-+	+	+	+	+
CdCl_2	-	-	-	-	-	-	-+	-+	+	+	+

Table 5-1: Classic resistance results of *P. patens* (- = dead, -+ = 50% living, += living)

5.2 Cultivation and growth control

The aim of the cultivation tests was to find suitable, highest possible heavy metal concentrations for cultivating *P. patens*. Lower concentrations were therefore not used for cultivation.

Successful growth on media spiked with heavy metals could be observed up to concentrations of 10^{-1} M Cu-EDTA and Zn-EDTA. On ZnCl_2 and CuCl_2 the moss could be cultured up to a concentration of 10^{-3} M. *P. patens* plants could not be cultured on CdCl_2 in concentrations higher than 10^{-5} M.

When compared to classical resistance tests in liquid concentrations *P. patens* survived even higher heavy metal concentrations on growth medium (Table 5-2).

Media	Concentration in growth medium										Control
	1M	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	
Cu-EDTA	o	+	+	+	+	o	o	o	o	o	+
Zn-EDTA	o	+	+	+	+	o	o	o	o	o	+
ZnCl_2	o	-	-	+	+	o	o	o	o	o	+
ZnSO_4	o	-	-	+	+	o	o	o	o	o	+
CuCl_2	o	-	-	-	+	o	o	o	o	o	+
CdCl_2	o	-	-	-	-	+	+	o	o	o	+

Table 5-2: Heavy metal resistance on solid growth medium (+ = growth, - = no growth, o = not tested)

5.2.1 Copper

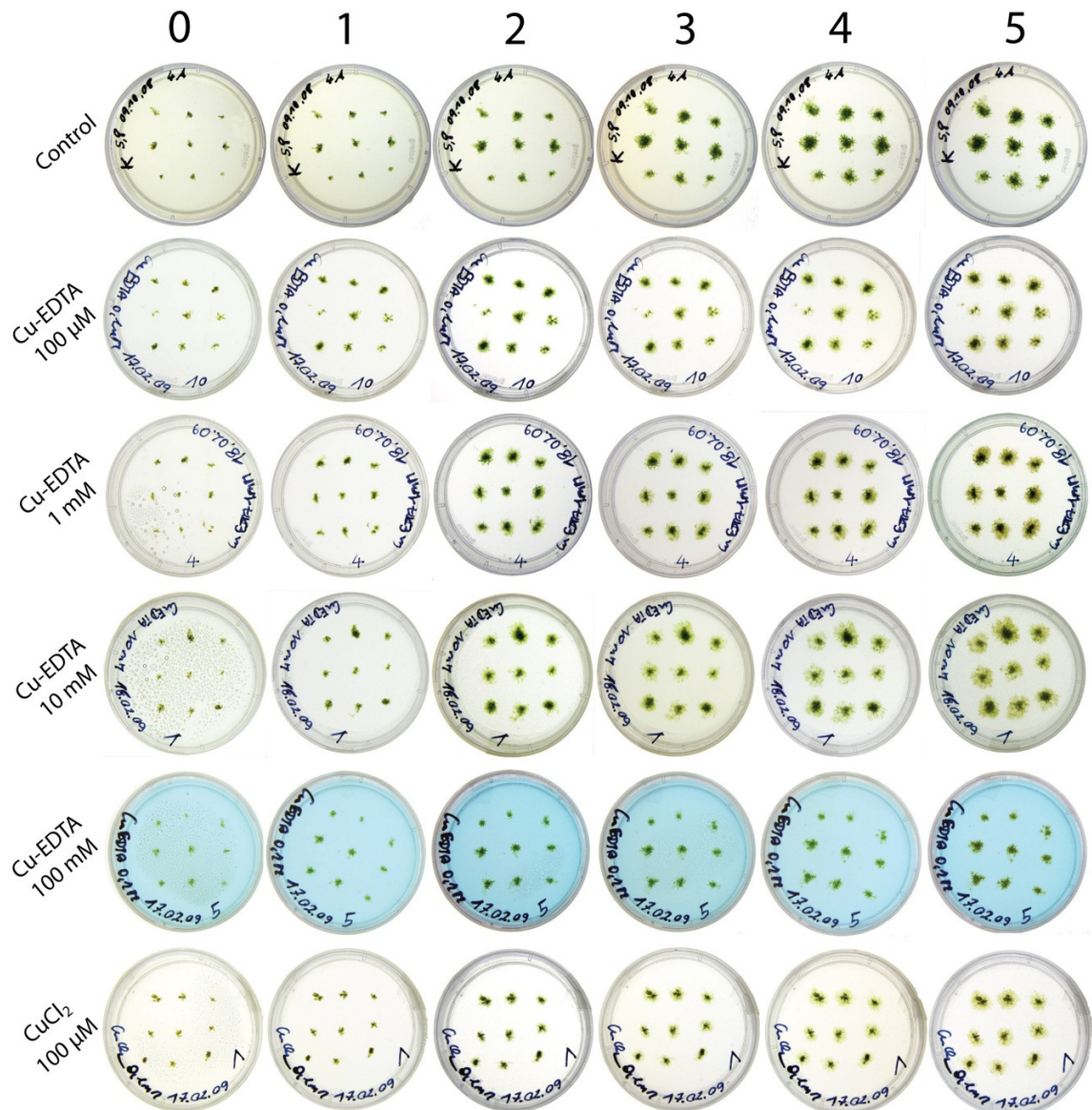


Fig. 23: Overview of copper grown *P. patens* during a test period of 5 weeks. First line (0-5) indicates week 0 to week 5.

Fig. 23 shows the starting point of cultivation at week 0 (left) to week 5 (right). All Cu-EDTA and CuCl₂ concentrations experienced an increase of growth over the period of 5 weeks. Control (top row) and Cu-EDTA 10 mM (4th row) showed the highest increase of biomass. Cu-EDTA 100 mM (5th row) and CuCl₂ 100 μM (bottom row) indicate decreased growth and a tendency to form more protonema than in control plants.

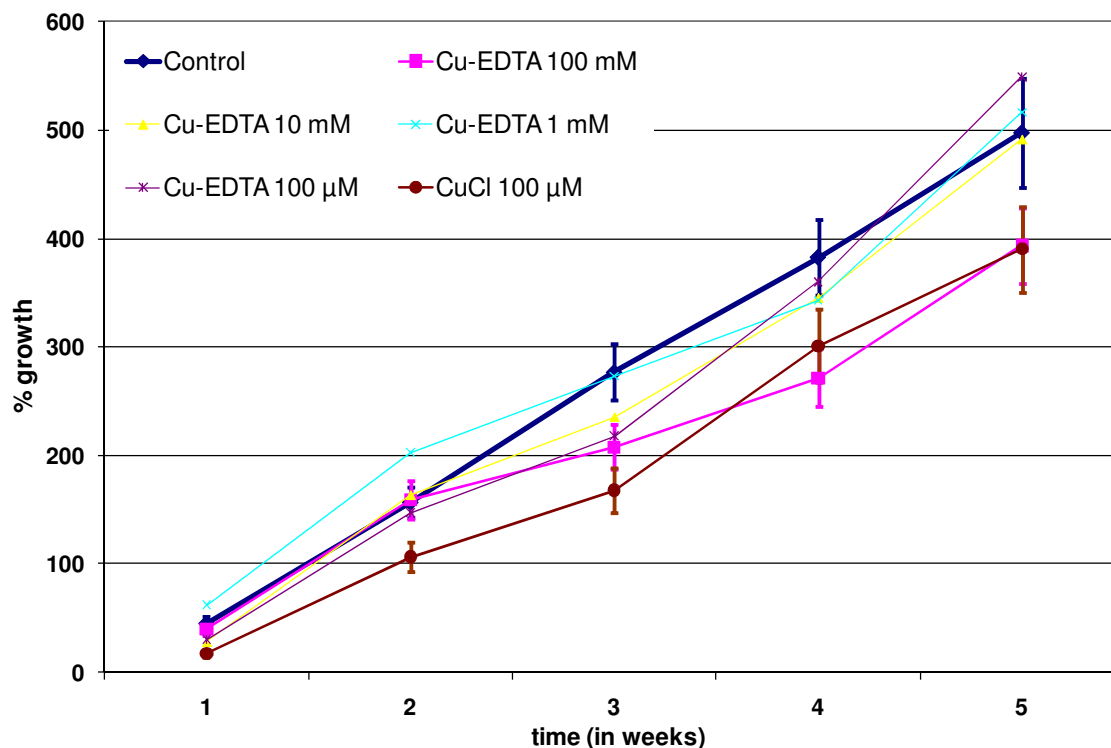


Fig. 24: Results of copper grown plants of *P. patens* in percent biomass accumulation from starting point ($p < 0.10$). First line (0-5) indicates week 0 to week 5.

On copper-enriched agar plates (100 μ M, 1 mM, 10 mM and 100 mM Cu-EDTA or 100 μ M CuCl₂, respectively), all investigated *P. patens* plants showed a constant increase of growth over a period of five weeks (Fig. 24).

A perfectly linear increase can be observed on control plates without copper. As compared to the control, only the highest Cu-EDTA concentration of 100 mM and CuCl₂ (100 mM) showed damaging effects and reduced growth. CuCl₂ had immediate harmful effects lasting for the whole period observed whereas the plants on Cu-EDTA 100 mM started off like the control until week 2 and only the long term exposure resulted in significantly reduced growth. Both, CuCl₂ (100 μ M) and Cu-EDTA 100 mM resulted in mean values of 393 percent augmentation after five weeks; control plants reached 497 percent within this time. Lower Cu-EDTA concentrations in the agar (1 mM and 10 mM) resulted in similar growth as in the control (516 and 491 percent). The two lowest Cu-EDTA concentrations of 100 μ M and 1 mM showed best growth, followed by the next higher concentration of 10 mM. At these three concentrations, copper stressed plants showed a higher variation in growth than on control medium:

beneficial effects at the start of the experiment, slightly harmful effects during week 3 and 4 and acceleration of growth at week 5 (at least for 1 mM and 100 μ M). After the second week, the biomass of all samples was at least doubled and within five weeks, even highly stressed plants grew 400 percent as compared to the start.

5.2.2 Zinc

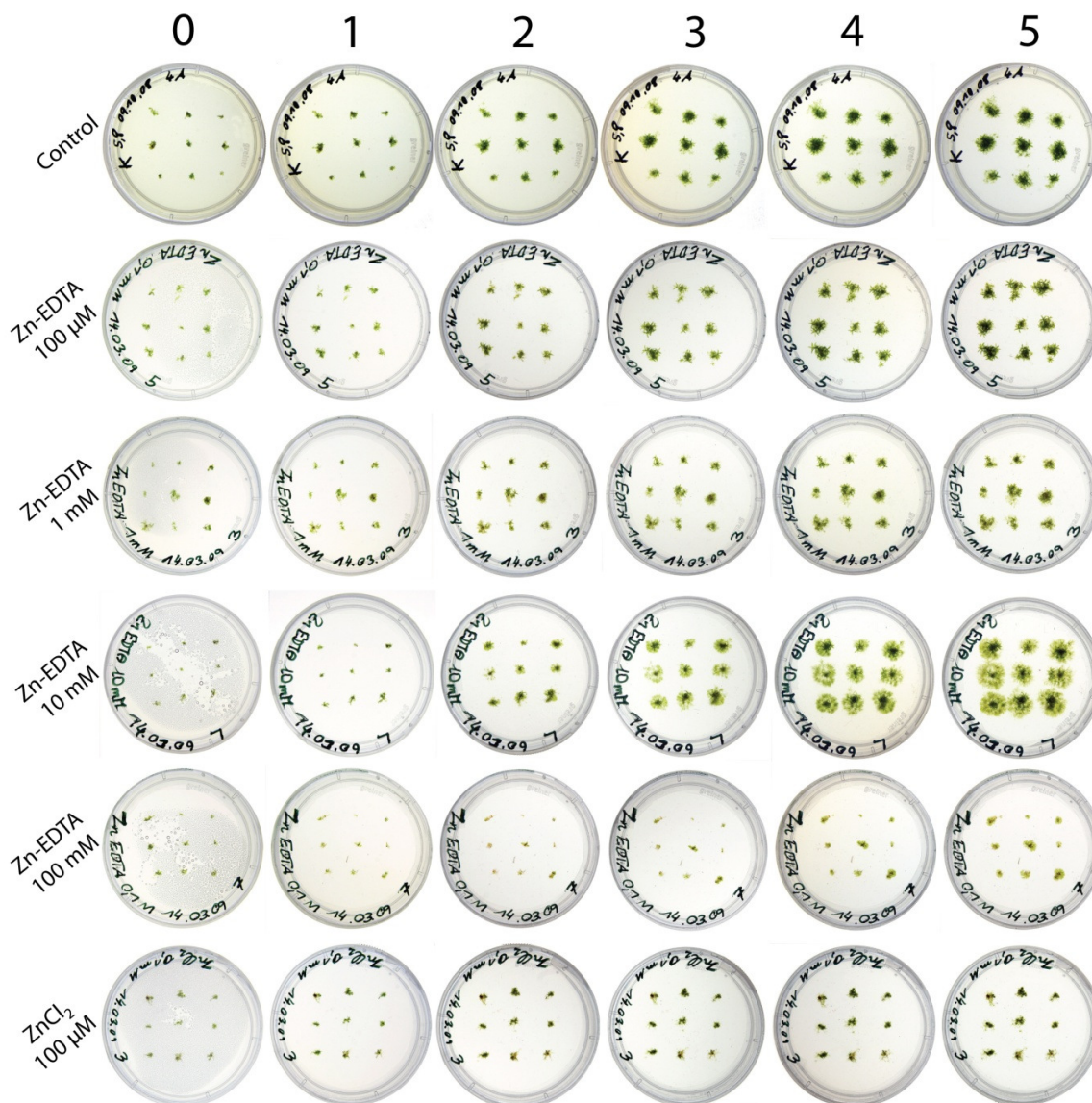


Fig. 25: Overview of zinc grown *P. patens* during a test period of 5 weeks. First line (0-5) indicates week 0 to week 5

All Zn-EDTA and ZnCl₂ concentrations show an increase of growth over 5 weeks. Zn-EDTA 10 mM (4th row) yielded the best growth. Zn-EDTA 100 mM and ZnCl₂ 5 mM

indicate decreased biomass accumulation when compared to control. On Zn-EDTA 100 mM abnormal growth of mainly protonemata was observed.

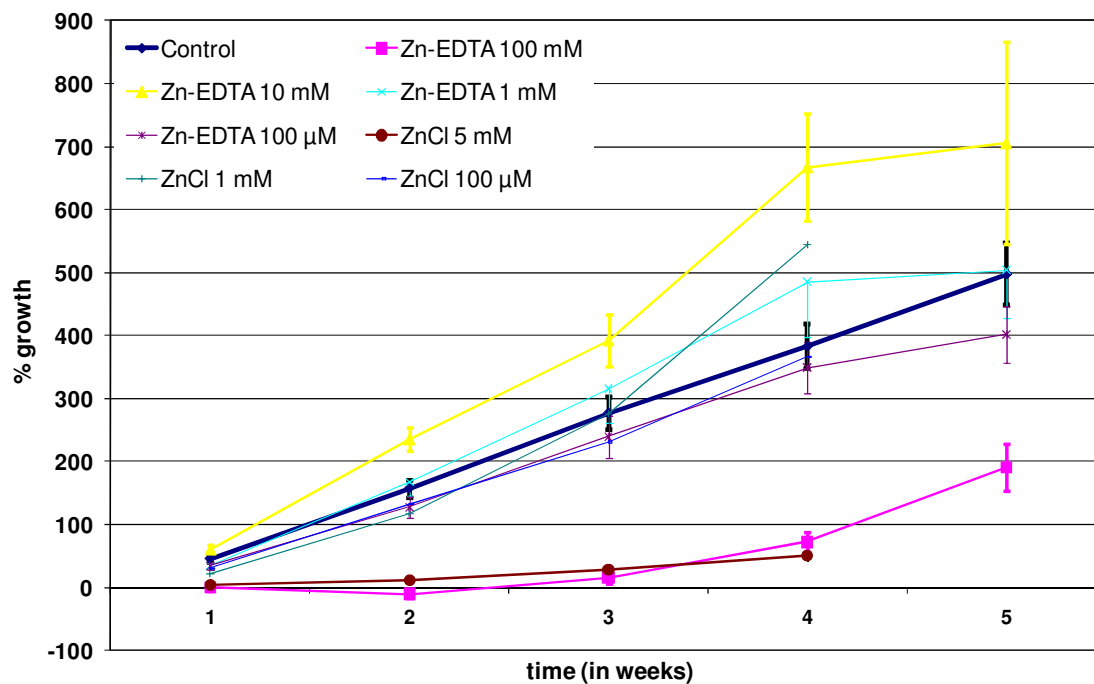


Fig. 26: Results of zinc grown plants of *P. patens* in percent biomass accumulation from starting point ($p < 0.10$)

On zinc-enriched agar plates (Zn-EDTA 100 μM, 1 mM, 10 mM and 100 mM or ZnCl₂, 5 mM, 1 mM and 100 μM respectively), not all investigated plants of *P. patens* showed a constant increase of growth over a period of five weeks. On Zn-EDTA 100 mM, a slight decrease of biomass was observed until week 2. Regardless of this first phase, the mosses increased in biomass to 190 percent of the starting point after 5 weeks. Similar results of drastically reduced growth compared to control or even a decrease in biomass could be observed for some plantlets on ZnCl₂ 5 mM. Mean values indicate slow but linear accumulation of biomass up to 72 percent. Concentrations of Zn-EDTA 100 μM, ZnCl₂ 100 μM and Zn-EDTA 1 mM displayed similar growth as controls: a total increase of biomass from 401 percent for Zn-EDTA 100 μM up to 503 percent for Zn-EDTA 1 mM and 366 percent for ZnCl₂ 100 μM in week 4. Best growth was exhibited by the mosses grown on Zn-EDTA 10 mM with an average biomass accumulation of 705 percent. Beneficial effects of the added Zn-EDTA could be observed throughout the whole growth period. However, in week 4 and 5 the mean values displayed a wide variation but a beneficial effect could still be observed in comparison to controls.

5.2.3 Cadmium

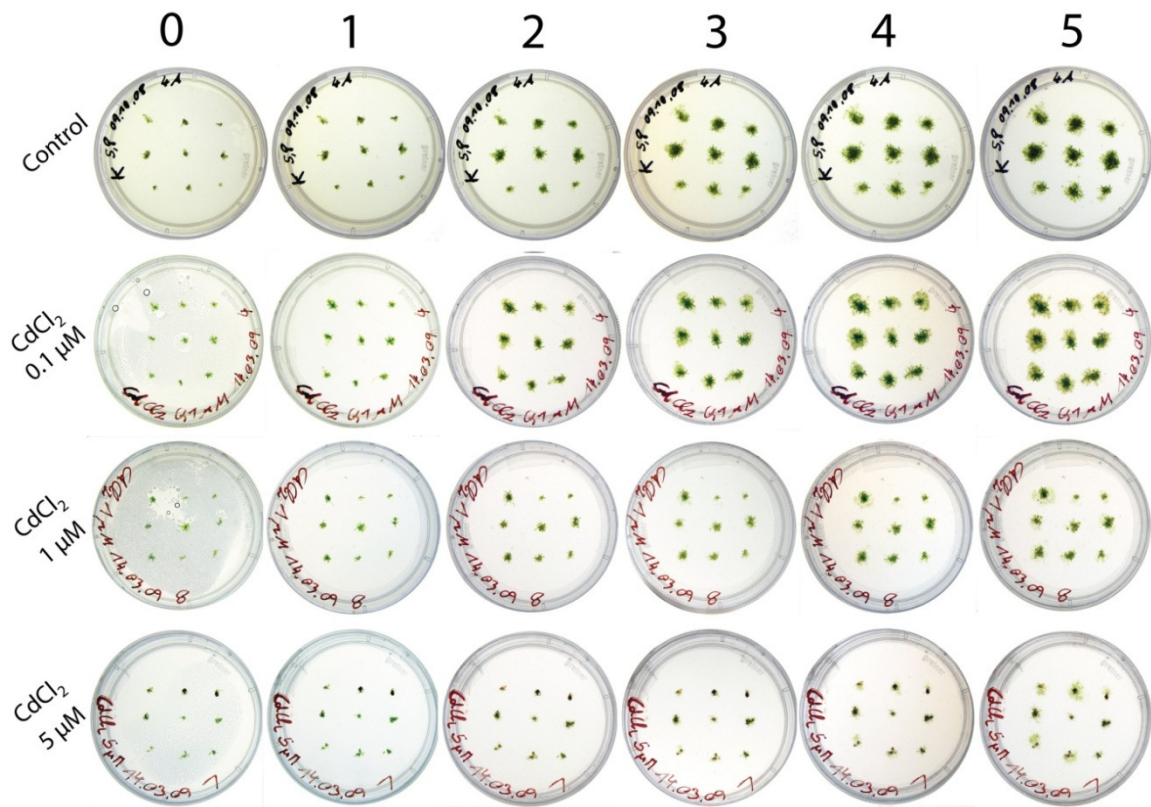


Fig. 27: Overview of cadmium grown *P. patens* during a test period of 5 weeks. First line (0-5) indicates week 0 to week 5.

Significant biomass accumulation was displayed by CdCl_2 0.1 μM (2nd row) when compared to control. Higher concentrations like CdCl_2 5 μM (bottom row) still induced growth, but mainly protonema developed whereas normal leaf development was inhibited; plantlets were clearly harmed.

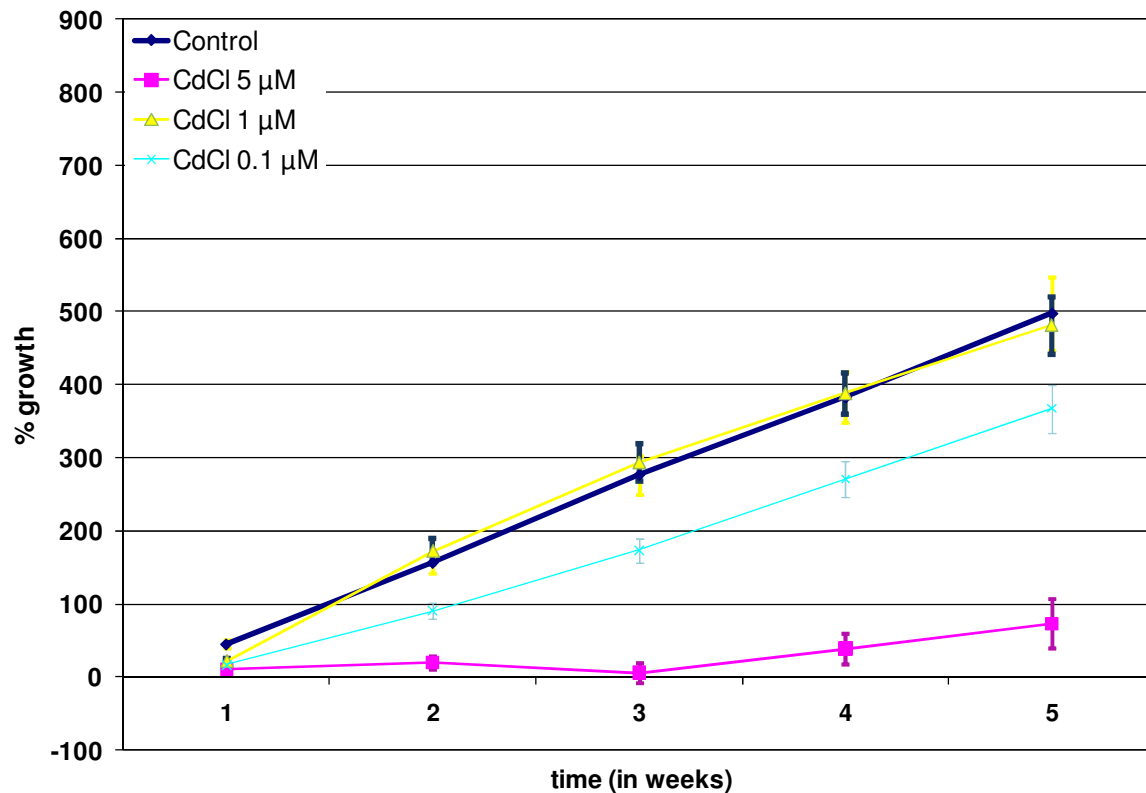


Fig. 28: Results of cadmium grown plants of *P. patens* in percent biomass accumulation from starting point ($p < 0.10$)

The cultivation on cadmium enriched plates (5 μM CdCl₂, 1 μM CdCl₂ and 0.1 μM CdCl₂) displayed the highly toxic effects of cadmium to *P. patens*. The concentrations of the cadmium spiked media are about 100 times lower than the concentrations used for copper and zinc plates. Still, drastically decreased growth rates and vitality were detected on plates with CdCl₂ 5 μM . Biomass accumulation of 100 percent could not be achieved during the test period of five weeks. Mosses were not able to form normal leafy gametophytes, only protonematal growth could be observed. Surprisingly, CdCl₂ 1 μM indicated very similar growth than control plates whereas the lower concentrated CdCl₂ 0.1 μM plates display slightly negative effects of the added CdCl₂. Biomass accumulation was about 20 percent lower than in control plates, with a peak of 367 percent in comparison to the starting point.

5.2.4 Combined results

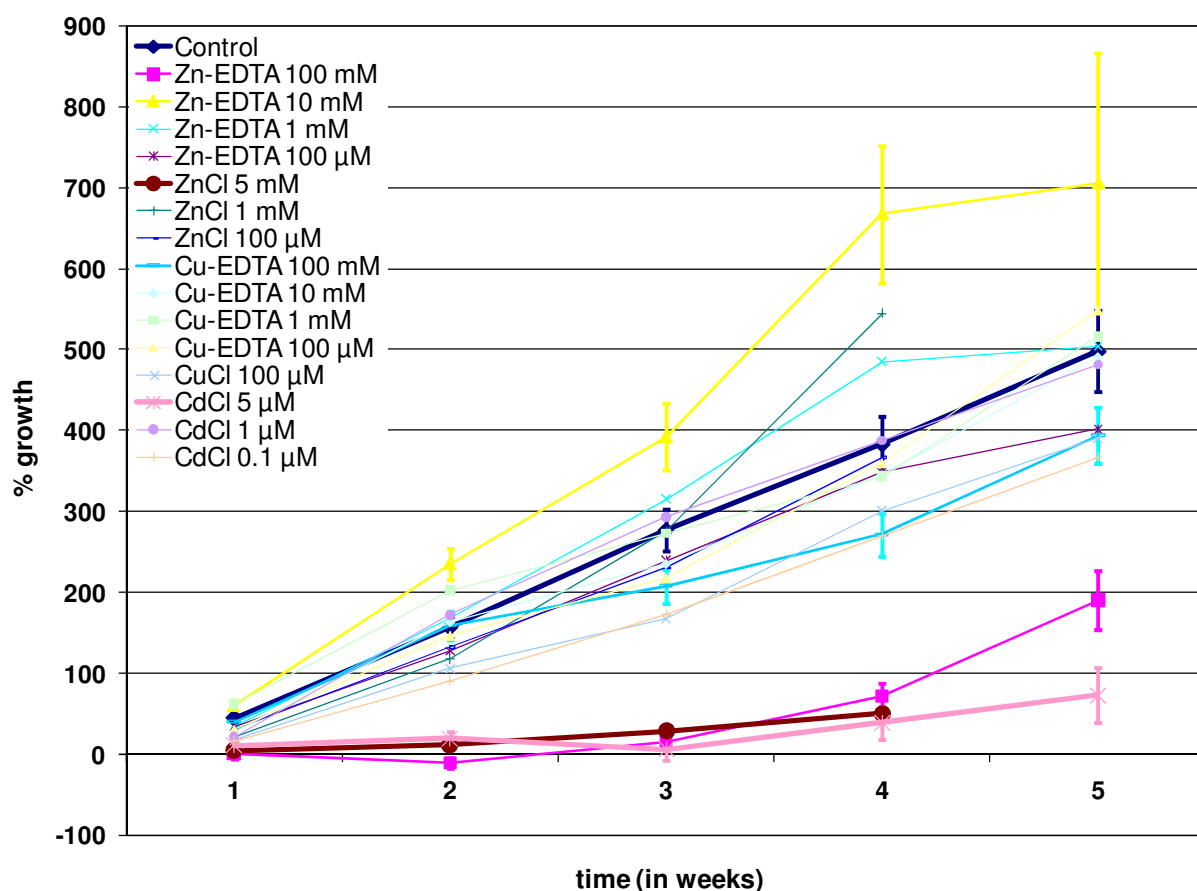


Fig. 29: Combined results of growth control ($p < 0.10$)

The results of all investigated heavy metal concentrations are summarized in 4 groups.

The first group is defined by a strongly decreased biomass accumulation and abnormally high protonemal growth. This reaction occurred at Zn-EDTA 100 mM, ZnCl₂ 5 mM and CdCl₂ 5 μM. These concentrations displayed slow growth or even decrease of biomass during the first 3 weeks of the experiment. After week 4, *P. patens* grown on Zn-EDTA 100 mM showed an unexpectedly high rise in biomass of up to 190 percent from the starting point. Interestingly this group includes both, EDTA and chloride spiked media.

The second group, formed by Cu-EDTA 100 mM, Zn-EDTA 100 μ M, CuCl₂ 100 μ M and CdCl₂ 0.1 μ M showed a less intense but still considerable decrease in growth. Moss plantlets did exhibit leaf development. The biomass accumulation has been only approximately 350 to 400 percent.

The third group or control group contained Cu-EDTA (10 mM, 1 mM, 100 μ M), CdCl₂ 1 μ M, Zn-EDTA 1 mM and ZnCl₂ 100 μ M. All these concentrations did not show a significant difference in biomass accumulation when compared to control. No phenotypical alterations to control could be observed. Like in control samples the biomass was approximately 500 percent after 5 weeks of cultivation.

The forth group contains Zn-EDTA 10 mM and ZnCl₂ 1 mM. These concentrations seemed to have beneficial effects on moss growth. While this effect can be observed over the full test period for Zn-EDTA 10 mM up to a biomass of 705 percent of the starting point, for ZnCl₂ 1 mM this beneficial effect is only present in week 4 at 544 percent biomass increase.

week	1	2	3	4	5
Control	45,07	156,69	276,61	382,85	497,56
Zn-EDTA 100 mM	0,60	-10,21	15,64	72,18	190,52
Zn-EDTA 10 mM	60,68	235,44	392,39	667,46	705,76
Zn-EDTA 1 mM	34,52	167,39	315,50	485,30	503,75
Zn-EDTA 100 μ M	34,36	127,25	239,67	349,10	401,59
ZnCl 5 mM	4,01	11,42	28,35	50,27	
ZnCl 1 mM	21,62	117,14	275,48	544,62	
ZnCl 100 μ M	30,46	132,52	230,61	366,39	
Cu-EDTA 100 mM	39,92	159,04	207,76	271,36	393,62
Cu-EDTA 10 mM	28,00	163,51	235,30	345,17	491,08
Cu-EDTA 1 mM	61,92	202,49	272,88	342,67	516,29
Cu-EDTA 100 μ M	30,25	147,03	218,01	360,00	549,11
CuCl 100 μ M	17,10	106,47	167,81	301,17	390,41
CdCl 5 μ M	11,01	19,51	5,74	38,70	72,88
CdCl 1 μ M	21,27	171,99	293,38	387,87	481,51
CdCl 0,1 μ M	16,68	90,82	173,12	270,48	367,17

Table 5-3: Results of growth control, absolute numbers of mean values.

5.3 Energy dispersive X-ray spectroscopy (EDX)

5.3.1 Copper

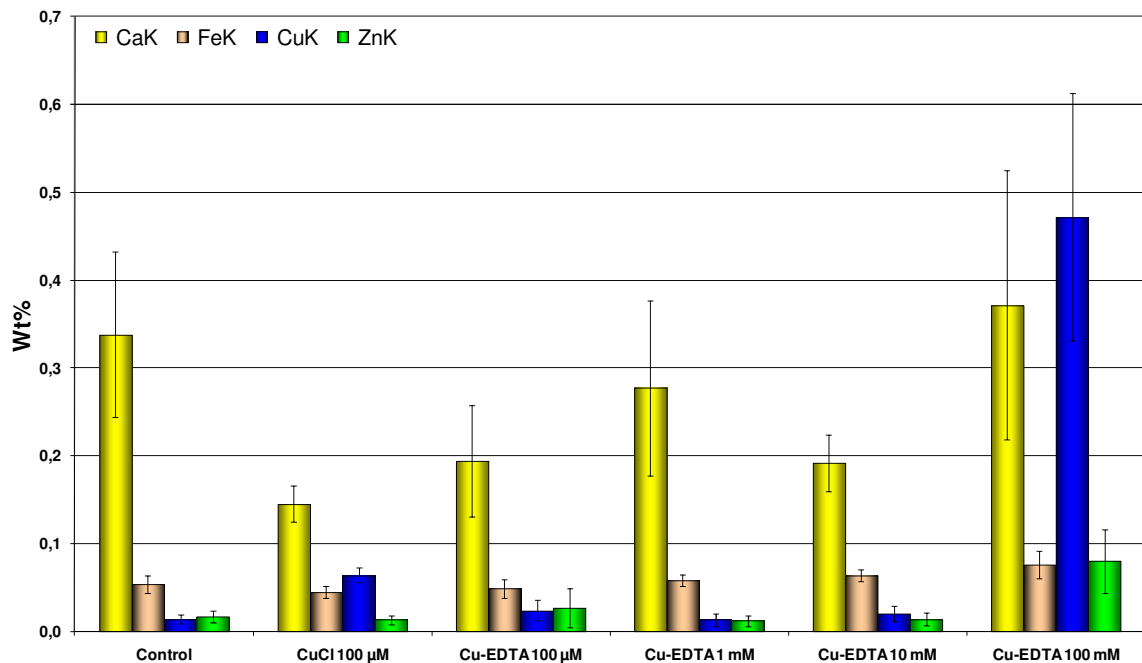


Fig. 30: EDX-Results of *P. patens* grown on graded CuCl₂ and Cu-EDTA enriched growth media. ($p < 0.05$)

The results of electron dispersive X-ray spectroscopy (EDX) of *P. patens* grown on graded Cu-EDTA and CuCl₂ concentrations are summarized in Fig. 30. EDX is a semi-quantitative method; therefore the term weight percent (Wt%) indicates the rates of the tested heavy metal in relation to all present elements of the leaf tissue. Plants from plates with 100 mM Cu-EDTA showed the highest copper uptake into leaf tissue. On Cu-EDTA concentrations from 100 µM to 10 mM, the EDX measurements indicated rather low, non significant copper uptake. Similar to the results of zinc (Fig. 31), plants grown on CuCl₂ 100 µM displayed higher uptake than plants on Cu-EDTA in the same concentration. Compared to control, CuCl₂ 100 µM, Cu-EDTA 100 µM and Cu-EDTA 100 mM, a significant uptake of copper was detected. Calcium was also measured and concentrations differed slightly in all samples. Interestingly, also zinc was elevated in Cu-EDTA 100 mM despite the fact that no zinc was added to the medium. Small zinc amounts near the detection limit were found in all samples.

5.3.2 Zinc

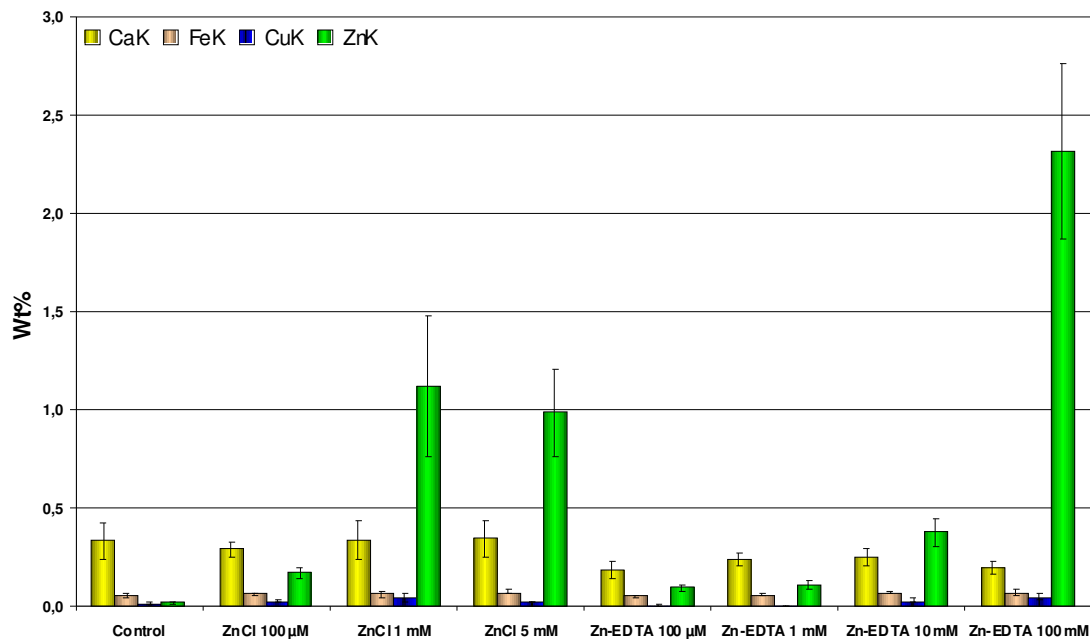


Fig. 31: –EDX-Results of *P. patens* grown on gradient ZnCl_2 and Zn-EDTA enriched growth media. ($p < 0.05$)

The uptake of zinc (Fig. 31) correlated with the amount of ZnCl_2 and Zn-EDTA offered in a graded manner. ZnCl_2 seemed to be more harmful to *P. patens* than Zn-EDTA. Similar to CuCl_2 (Fig. 30) a higher uptake of zinc was registered from ZnCl_2 -plates when compared to the same concentrations of Zn-EDTA. Zinc uptake from ZnCl_2 media reached its peak when plants were grown on plates containing between 1 and 5 mM ZnCl_2 . EDX measurements showed highest uptake of zinc from plates of Zn-EDTA 100 mM. However, it has to be considered that at this Zn-EDTA concentration *P. patens* did not develop any leaf; therefore aerial protonema was tested.

5.3.3 Cadmium

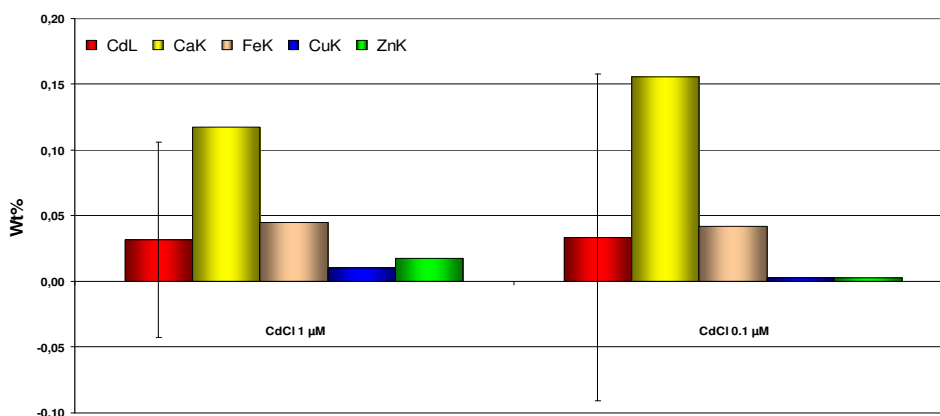


Fig. 32: EDX-Results of *P. patens* grown on graded CdCl_2 media. ($p < 0.05$)

Figure 32 shows the results of the EDX analysis of plants grown on CdCl_2 enriched media. Mosses could not survive on high concentrations of CdCl_2 . The results indicated that at a concentration within the detection limit for EDX, CdCl_2 is too toxic for *P. patens*. Although EDX we got values for cadmium using EDX, those results have to be treated carefully because they show a high confidence interval.

5.3.4 Combined results of all investigated heavy metals

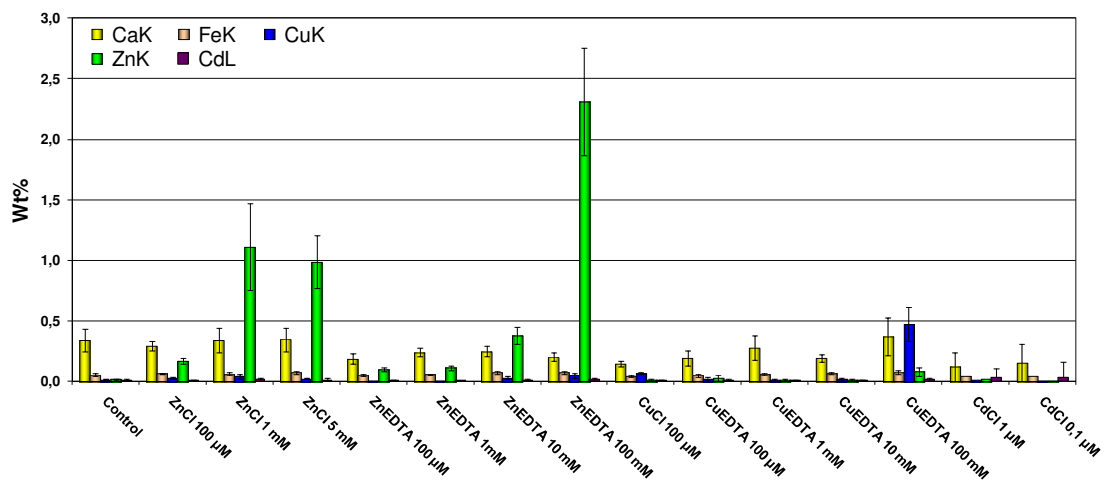


Fig. 33: Combined EDX-Results of all graded heavy metal enriched media. ($p < 0.05$)

Comparison of all used heavy metals revealed differences in zinc and copper enrichment of leafs. Even at the highest concentrated growth media of Cu-EDTA 100 mM, copper content of the leafs did not reach 50 percent of lower zinc-enriched media, e.g. ZnCl_2 1 mM. Sulfur content (data not shown) remained stable over all tested samples. Calcium, however, did vary in the tested samples.

5.4 Confocal laser scanning microscopy (CLSM)

To further localize heavy metals on the cellular level the heavy metal specific dyes Phen Green™ SK and FluoZin™-3, were used to detect copper and zinc. Two different types of tissue, leaf and protonema cells were investigated.

5.4.1 Tests with Phen Green™ SK

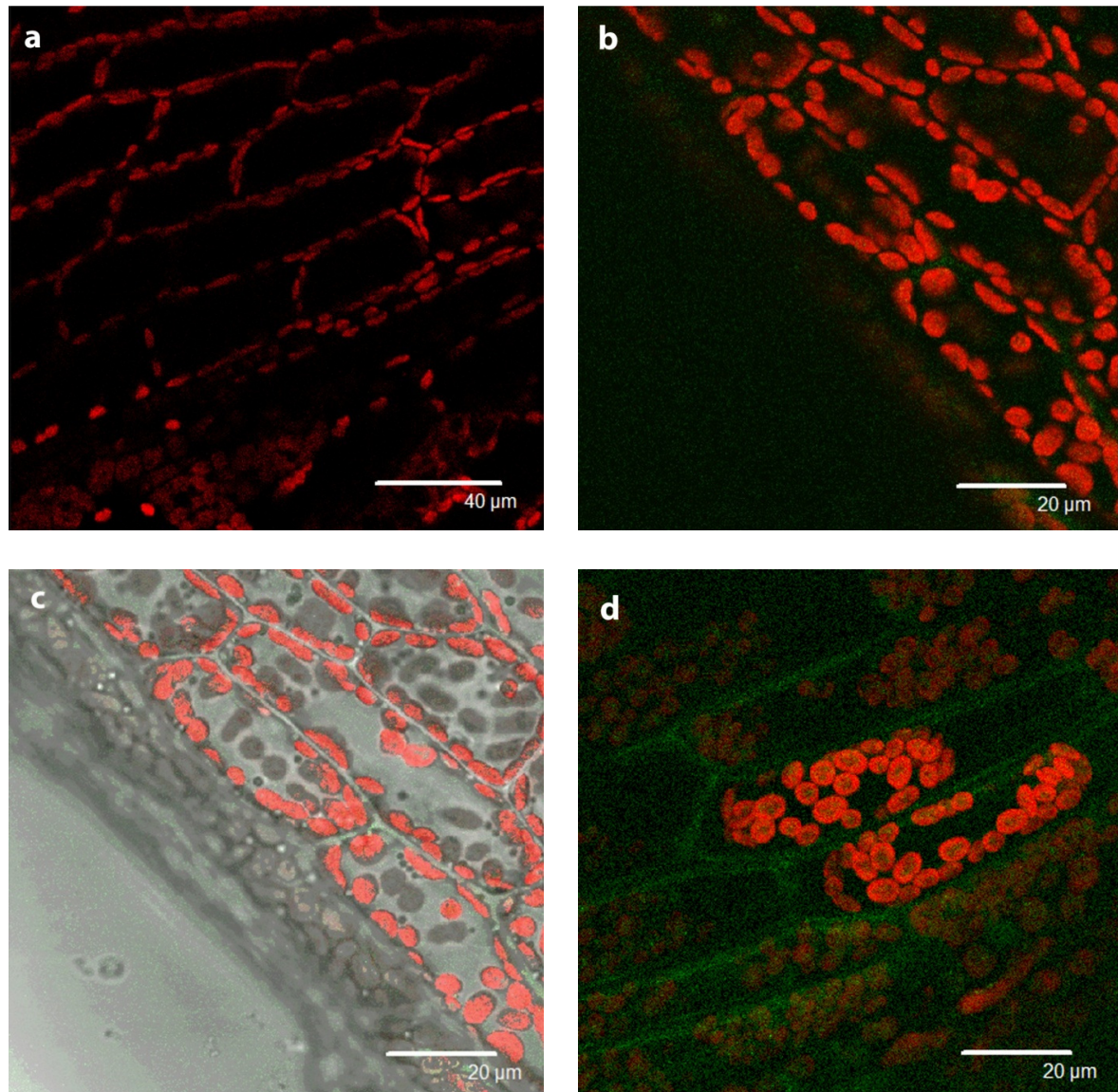


Fig. 34(a-d): Phen Green™ SK was added to leaf tissue cells from control (a)(fluorescence image), Cu-EDTA 100 mM (b [fluorescence image] and c [overlay of transmission and fluorescence image]) and Cu-EDTA 10 mM (d) (plasmolysed) plates.

Low fluorescence could be detected in control cells (Fig. 34 a). Weak and unspecific fluorescence was found in cells grown on Cu-EDTA 100 mM (Fig. 34b,c). Even in plasmolysed cells grown on Cu-EDTA 10 mM only weak fluorescence of the cell wall

could be seen (Fig. 34 d). Phen Green™ SK is not completely Cu^{2+} specific and might also bind to other cations like Ca^{2+} . Our results were not explicit and indicate that the dye was not able to penetrate the cell wall or the plasma membrane. Further experiments will hopefully draw a clearer picture.

5.4.2 Tests with FluoZin™-3

FluoZin™-3 gave more reliable results and was more specific for Zn^{2+} than Phen Green™ SK was for Cu^{2+} . In order to get correctly labeled cells with FluoZin™-3, the washing process seemed to be a critical part of the preparation. If not done properly even under normal conditions, highly fluorescent background was predominant and these cells seem to have no reaction to the Zn^{2+} sensitive dye (Fig. 35). Another important fact is that only vital cells showed representative staining. Dead or dying cells were easily overstained (Fig. 36).

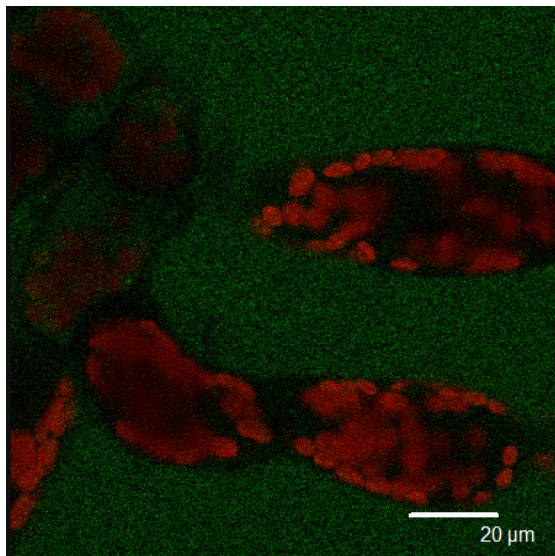


Fig. 35: Fluorescence image of protonema cells grown on Zn-EDTA 100 mM: stained without washing



Fig. 36: Fluorescence image of protonema cell grown on Zn-EDTA 100 mM, shortly after dying

Plant material from control and the graded zinc plates was tested.

Countenanced by the results of the first tests, *P. patens* was analyzed for increased Zn^{+2} levels with the heavy metal sensitive dye FluoZin™-3. Plants grown on control and different heavy metal enriched growth media were investigated and prepared under exactly the same calibration of the CLSM. Attention was paid to test only vital cells (see methods).

5.4.3 Control

Already the leaf tissue of control cells indicated the presence of very small amounts of zinc (green). High levels of auto fluorescence of the chloroplast are displayed in red (Fig. 37). An overlay of the transmission image with the two fluorescence channels showed the presence of non zinc containing light refracting vesicles, possibly lipid droplets (Fig. 38).

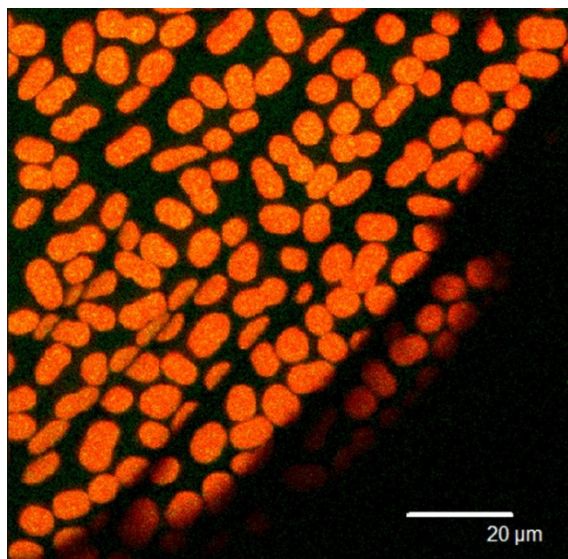


Fig. 37: Fluorescence of control taken by the CLSM

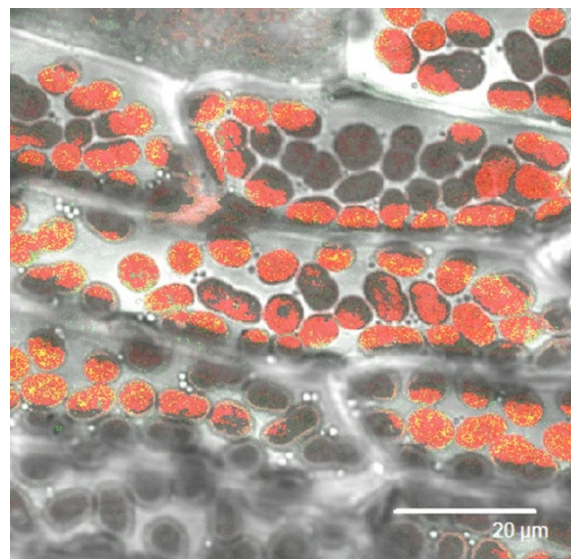


Fig. 38: Overlay of the transmission and fluorescence image of control

5.4.4 100 μ M to 1 mM Zn-EDTA

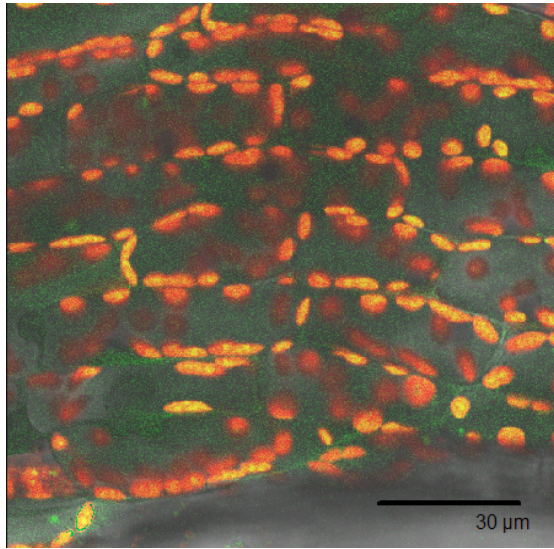


Fig. 39: Fluorescence of leaf tissue cells grown on Zn-EDTA 100 μ M

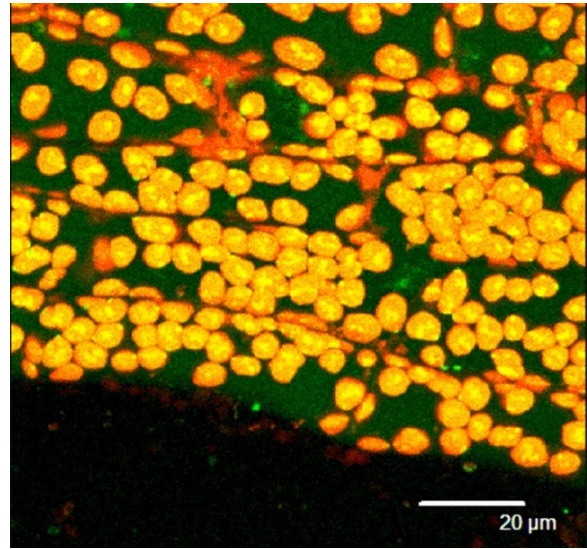


Fig. 40: Fluorescence of leaf tissue cells grown on Zn-EDTA 1 mM

The overlay-image of a *P. patens* leaf grown on Zn-EDTA 100 μ M showed enhanced fluorescence of FluoZin™-3. (Fig. 39) This result is evidenced by the increase of the labeling in cells from the higher 1 mM Zn-EDTA (Fig.40). This image is a maximum projection of 20 images.

5.4.5 1 mM Zn-EDTA to 10 mM Zn-EDTA to 100 mM Zn-EDTA

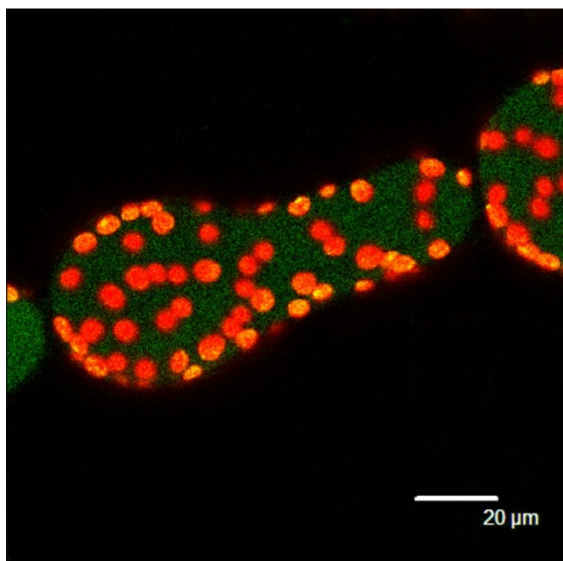


Fig. 41: Fluorescence of protonema cells grown on Zn-EDTA 1 mM

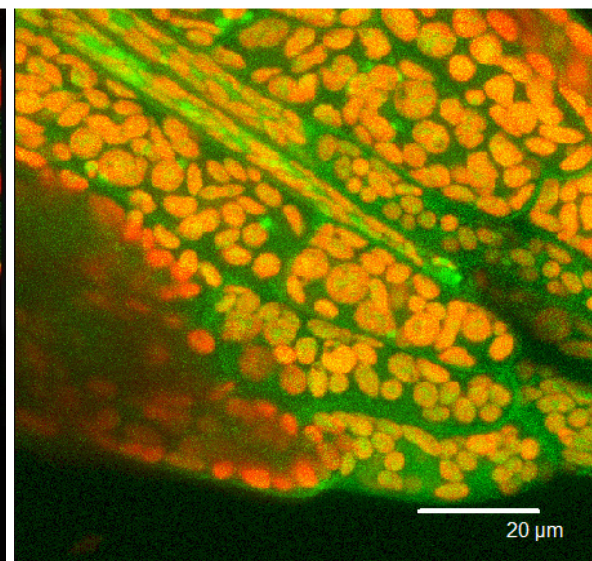


Fig. 42: Fluorescence of leaf tissue cells grown on Zn-EDTA 10 mM

To test for the rising cellular zinc levels according to the Zn-amount of the growth media, the cells of leaf and protonema from Zn-EDTA 1 mM, 10 mM and 100 mM were investigated (Fig. 41 – 44). Both protonema and leaf cells indicated an increase of fluorescence, relative, to the concentrations of zinc added to the growth media. However the cells and their organelles did vary in appearance and fluorescence within the same media-concentration. For example, chloroplasts tended to appear with yellow dots (Fig. 41) or completely yellow (Fig. 42) in plants grown on higher ZnCl₂ or Zn-EDTA concentrations. This fact is due to the overlay of the two recorded channels (green + red = yellow) and marks zinc containing auto fluorescent regions of the cell. In contrast also clear unchanged auto fluorescence could be seen in the vacuole of protonema tip cells from Zn-EDTA 100 mM plates (Fig. 44).

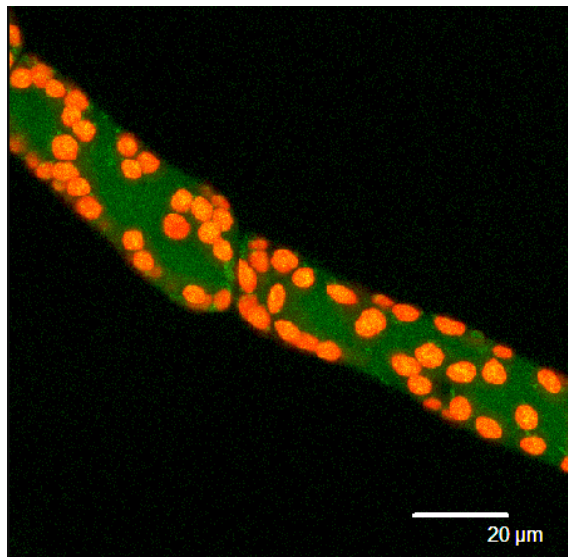


Fig. 43: Fluorescence of protonema cells grown on Zn-EDTA 100 mM

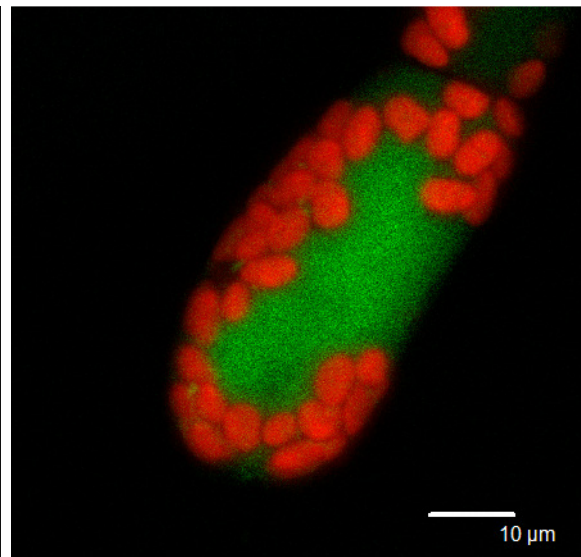


Fig. 44: Fluorescence of protonema tip cell grown on Zn-EDTA 100 mM

5.4.6 Localization of FluoZin™-3

Intracellular localization of fluorescently labeled zinc indicated a very low level of dispersive zinc in cell wall, vacuole, chloroplasts and cytoplasm of control cells (Fig. 45).

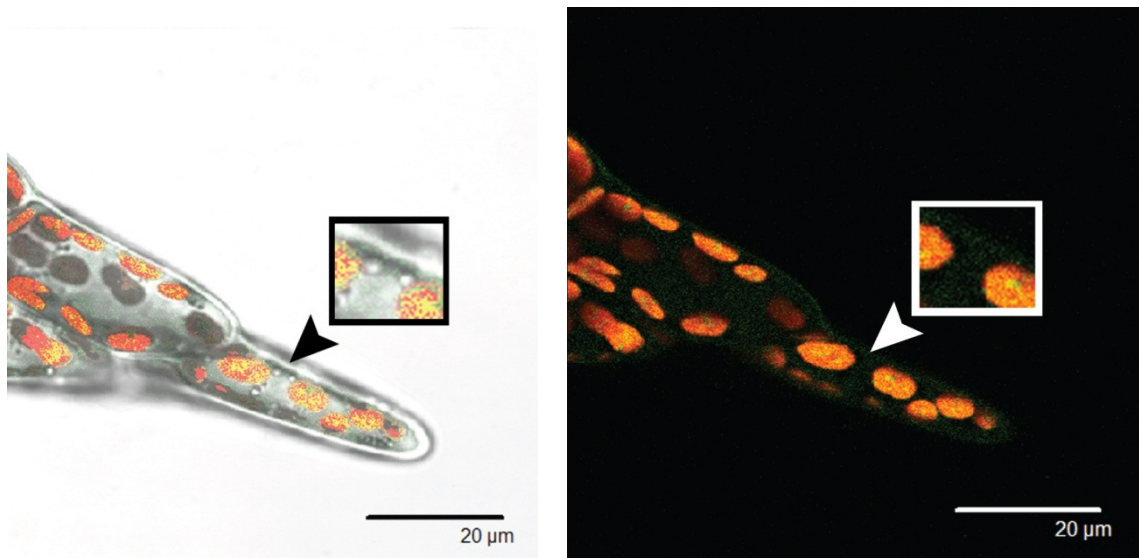


Fig. 45 (a-b): a) Overlay of a transmission and fluorescence image of control leaf tip cells; b) fluorescence image of control leaf tip cells; arrowheads point to non-zinc containing vesicles

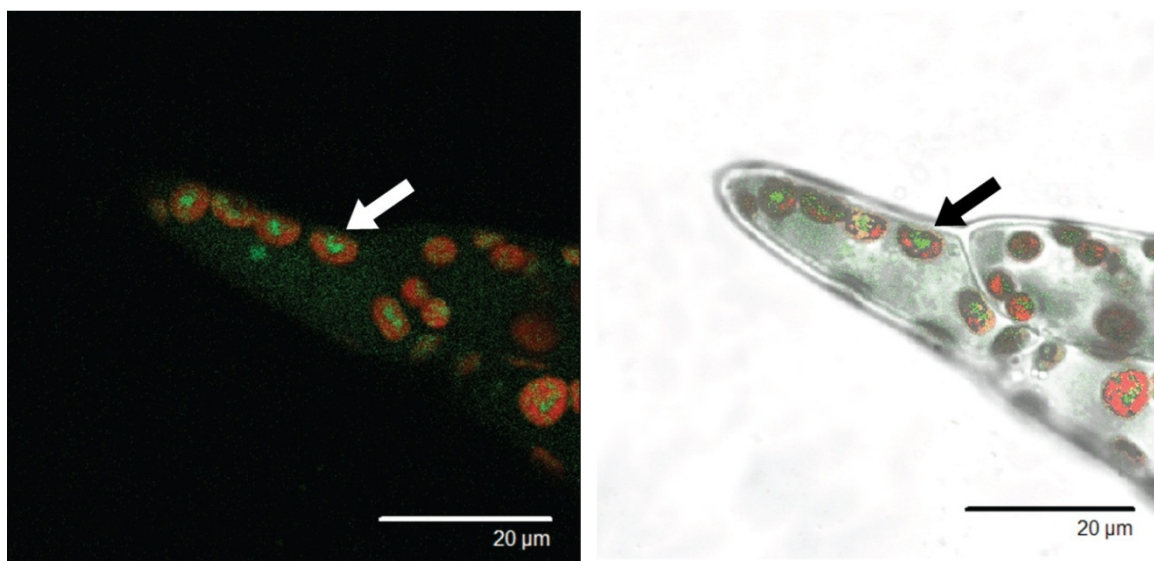


Fig. 46 (a-b): a) Fluorescence image of leaf tip cells grown on Zn-EDTA 100 µM; b) Overlay of transmission and fluorescence image of leaf tip cells grown on Zn-EDTA 100 µM; green dotted chloroplasts (arrows)

In contrast chloroplasts of heavy metal treated plants showed abnormal appearance of auto fluorescence. Arrows mark the “doughnut- shaped” zones of only green fluorescent parts within the chloroplast; high zinc and low chlorophyll levels in this

area can be presumed. Cell-walls did not show noticeable fluorescence. The vacuole and protoplasm indicated slightly elevated zinc contents.

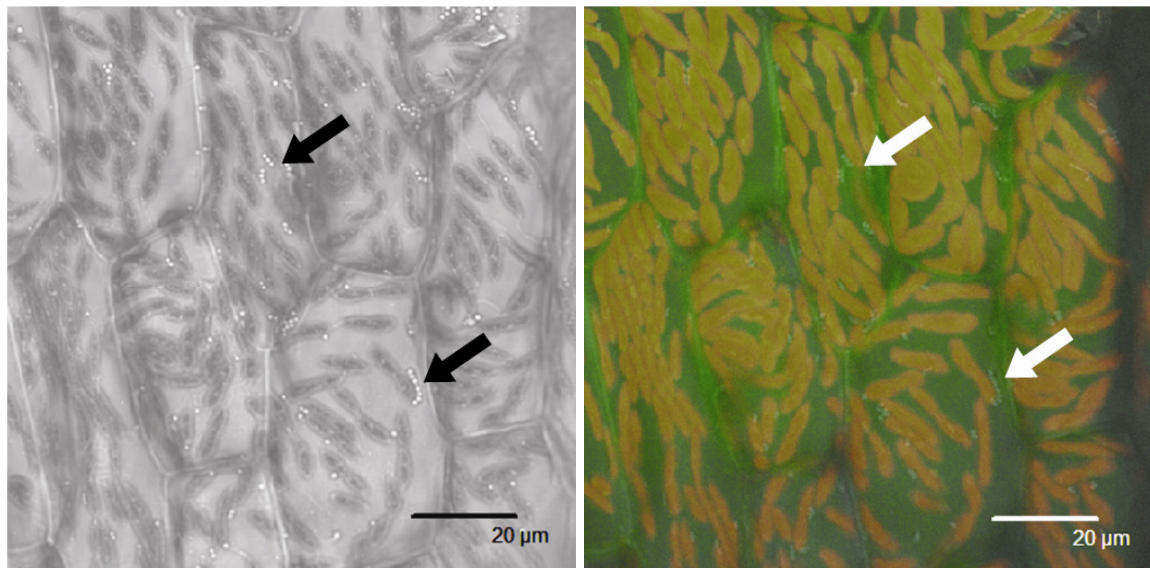


Fig. 47 (a-b): a) Transmission image of leaf cells grown on ZnCl_2 100; b) Fluorescence image of leaf cells grown on ZnCl_2 100 μM ; arrows mark zinc enriched vesicles

Fig. 47 shows the transmission mode image of vesicle-rich leaf cells from a plant grown on ZnCl_2 (black arrows). The fluorescence image confirms very high zinc levels in these organelles (white arrows). Maximum projection of a series of 20 images suggests zinc in the plasma membrane or bound to the cell wall.

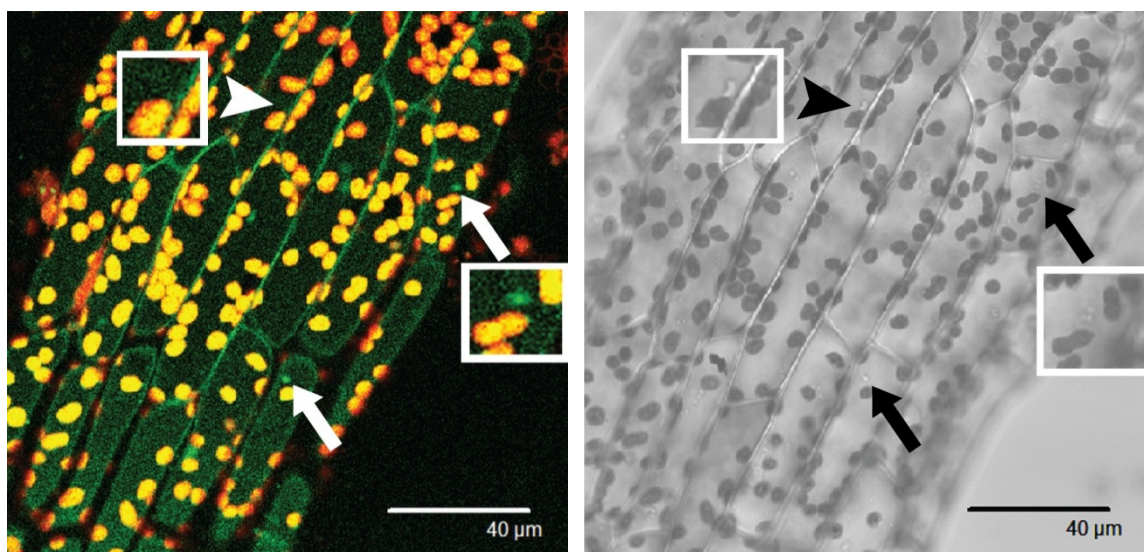


Fig. 48 (a-b): a) Fluorescence image of leaf cells grown on Zn-EDTA 1 mM; b) Transmission image of leaf cells grown on Zn-EDTA 1 mM; zinc (arrows) and non-zinc (arrowhead) containing vesicles

Leaf cells from plants grown on Zn-EDTA 1 mM revealed lipid droplet like vesicles in the transmission image. These vesicles are not obligatorily containing zinc; some of them were empty (arrow head) others showed high zinc concentrations (arrows).

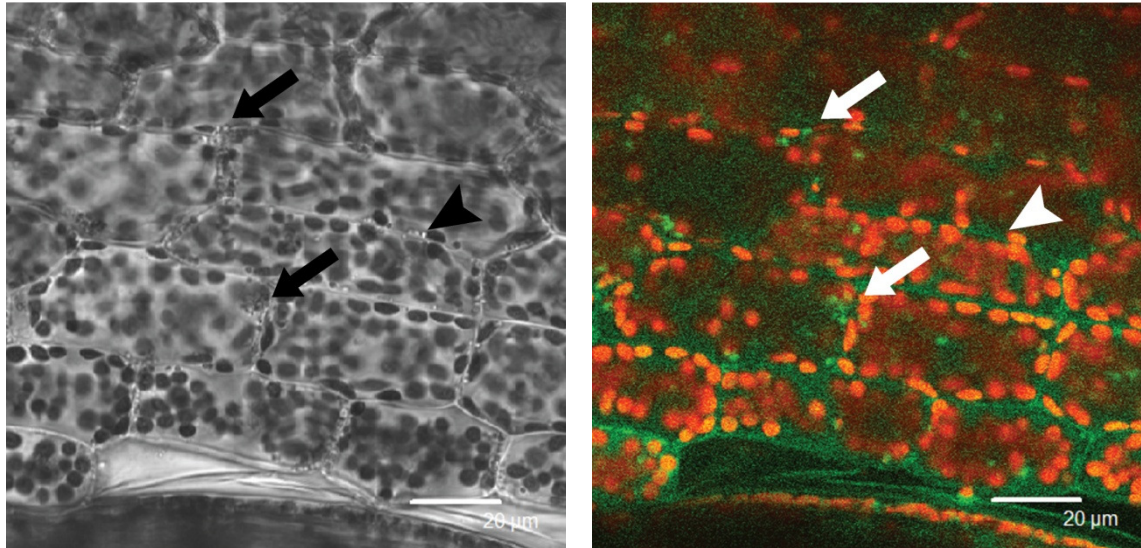


Fig. 49 (a-b): a) Transmission image of leaf cells grown on Zn-EDTA 10 mM; b) Fluorescence image of leaf cells grown on Zn-EDTA 10 mM; zinc (arrows) and non-zinc (arrowhead) containing vesicles

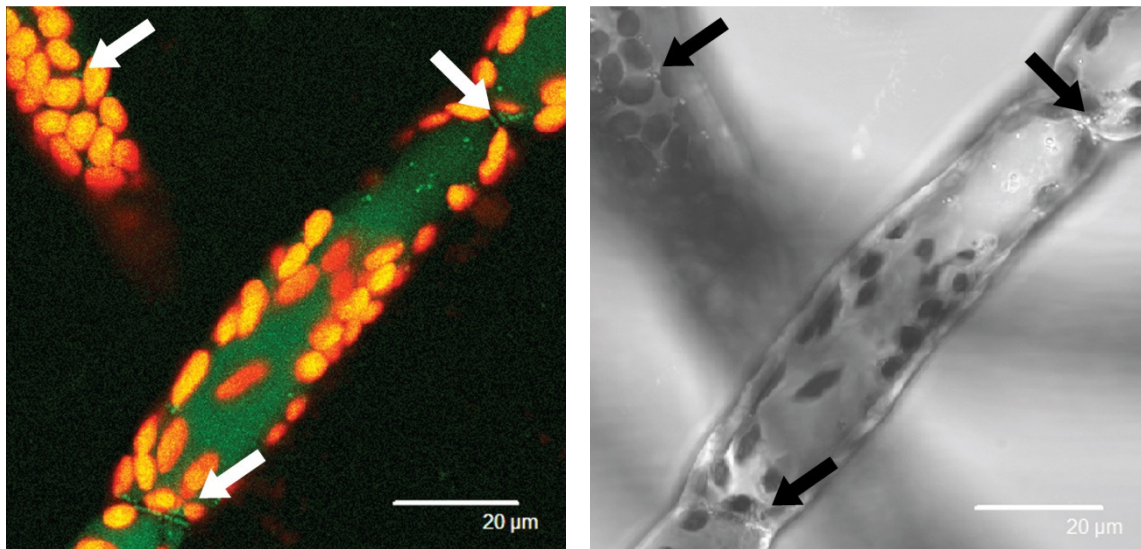


Fig. 50 (a-b): a) Fluorescence image of protonema cells grown on Zn-EDTA 100 mM; b) Transmission image of protonema cells grown on Zn-EDTA 100 mM; zinc (arrows) containing vesicles

The vacuoles of protonema cells grown on Zn-EDTA 100 mM displayed distinctive fluorescence, indicating strong zinc content. No detectable zinc appeared in the cell wall (Fig. 50). Aggregations of zinc (Fig. 49) as well as cell wall fluorescence could be observed in mosses grown on Zn-EDTA 10 mM. Zinc containing vesicles (arrows) as

well as non-zinc containing vesicles. (arrow head) could be confirmed in leaf and protonema cells for ZnCl_2 100 μM , Zn-EDTA 1 mM, 10 mM and 100 mM media.

5.4.7 Zinc localization after plasmolysis

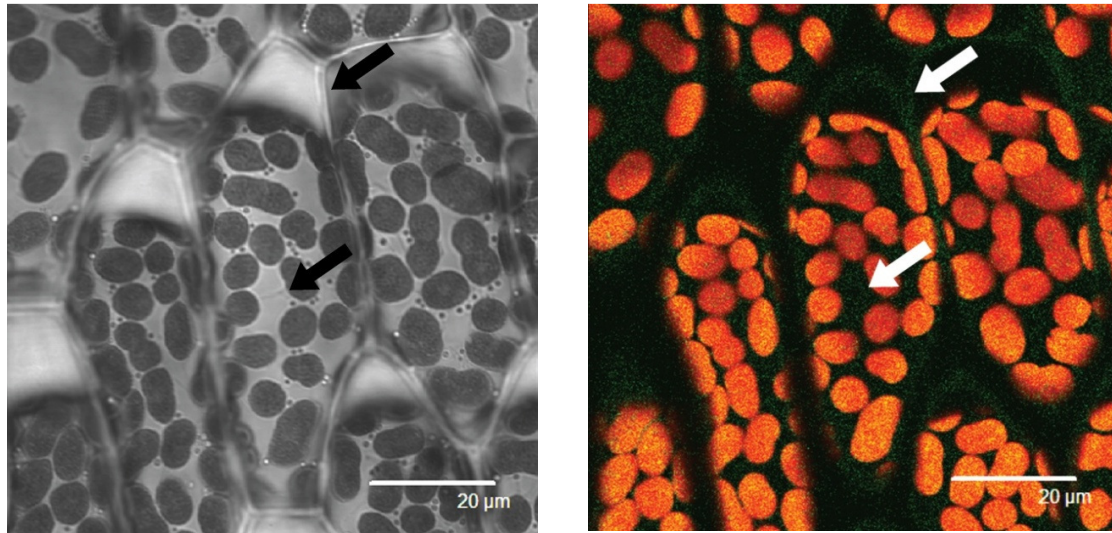


Fig. 51 (a-b): a) Transmission image of plasmolysed leaf cells grown on control; b) Fluorescence image of plasmolysed leaf cells grown on control plates; arrows mark cell wall and vacuole

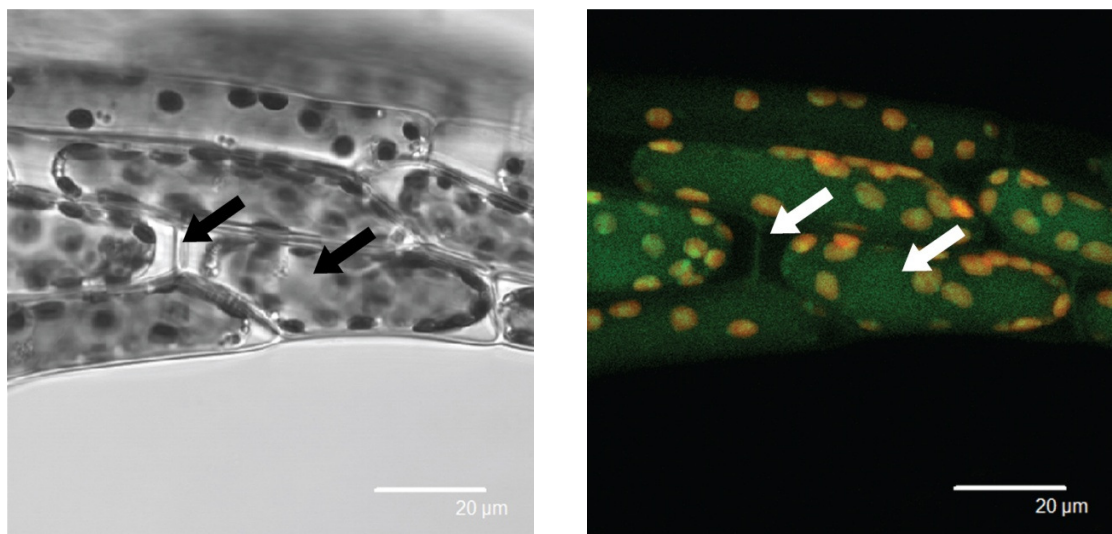


Fig. 52(a-b): a) Transmission image of plasmolysed leaf cells grown on Zn-EDTA 1 mM; b) Fluorescence image of plasmolysed leaf cells grown on Zn-EDTA 1 mM; arrows mark cell wall and vacuole

In order to determine zinc allocations within the plasma membrane or cell wall, moss cells were plasmolysed in 0.8 M mannitol after FluoZin™-3-staining. Control cells (Fig. 51a-b) did not show more than minimal fluorescence of the vacuole and cell wall (arrows). Plasmolysed cells from Zn-EDTA 1 mM plates (Fig. 52 a-b) illustrated zinc in the vacuole, the protoplast and, separated by the effects of plasmolysis, within

the cell wall (arrows). Staining in 0.8 M mannitol did not seem to affect the efficiency of FluoZin™-3 because the protoplast was normally stained and green dots of vesicles and chloroplasts could be observed as mentioned before.

5.5 Transmission electron microscopy (TEM)

TEM-Micrographs of leaf cells from control, Cu-EDTA 10 mM and Zn-EDTA 10 mM plates were taken as a preliminary test for electron energy loss spectroscopy (EELS). This was an attempt to find electron dense precipitations or organelle alterations which might indicate the effects or intracellular storage sites of zinc and copper.

5.5.1 Control

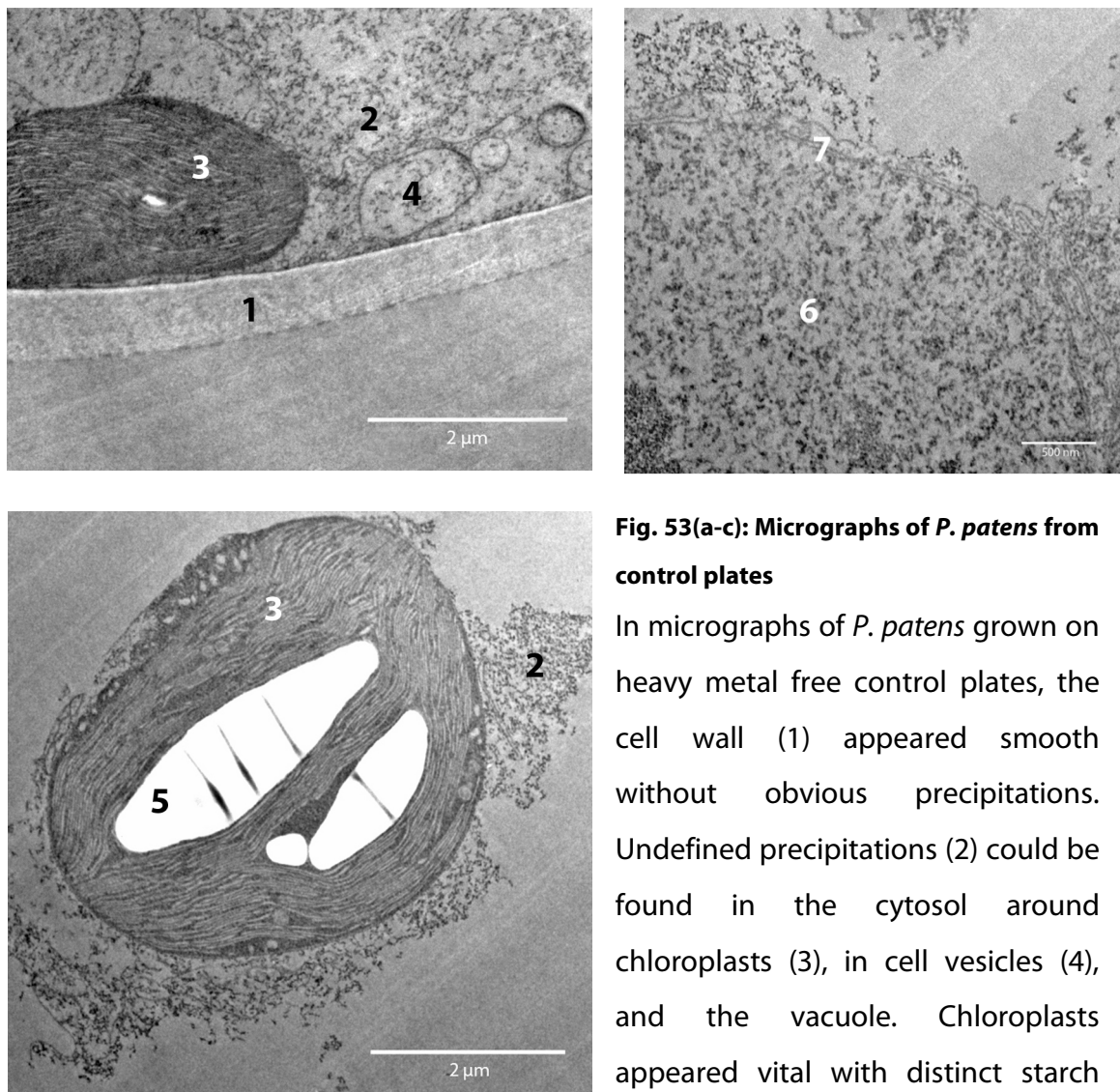


Fig. 53(a-c): Micrographs of *P. patens* from control plates

In micrographs of *P. patens* grown on heavy metal free control plates, the cell wall (1) appeared smooth without obvious precipitations. Undefined precipitations (2) could be found in the cytosol around chloroplasts (3), in cell vesicles (4), and the vacuole. Chloroplasts appeared vital with distinct starch grains (5). The nucleus (6) and the nuclear membrane (7) were normal. In general the preparation of the cells during TEM-preparation was excellent. This was particularly

obvious in the demonstration of fine membrane structures (e.g. chloroplasts, nuclear membrane).

5.5.2 10 mM Cu-EDTA

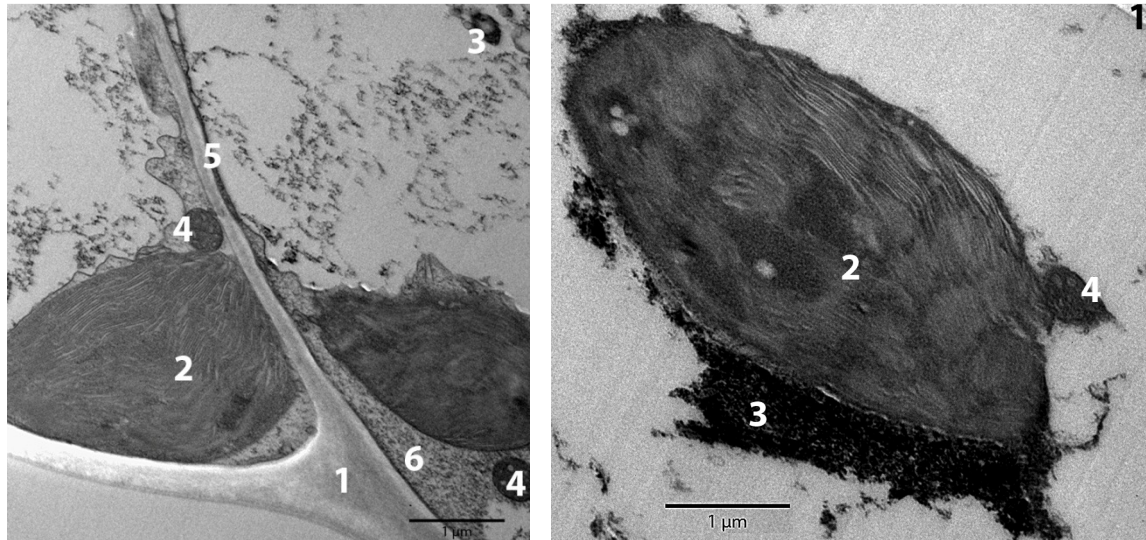


Fig. 54(a-b): Micrographs of *P. patens* cells grown on Cu-EDTA 10 mM media

TEM-micrographs (Fig. 54, a-b) of moss cells grown on Cu-EDTA 10 mM did not display any heavy metal precipitation within the cell wall (1). The appearance of chloroplasts (2) differed from the control; they seemed to be disintegrated and less starch grains were found. Considerable electron dense precipitations (3) could be observed near chloroplasts, in the vacuole and on the plasma membrane (5). Mitochondria (4) were frequently found close to chloroplasts. Also the cytoplasm (6) appeared relatively electron dense.

5.5.3 10 mM Zn-EDTA

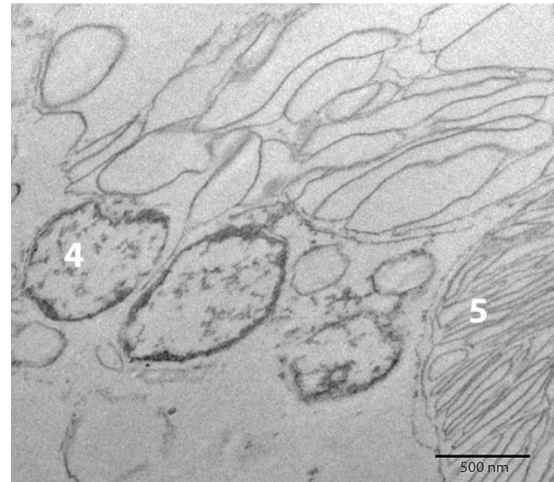
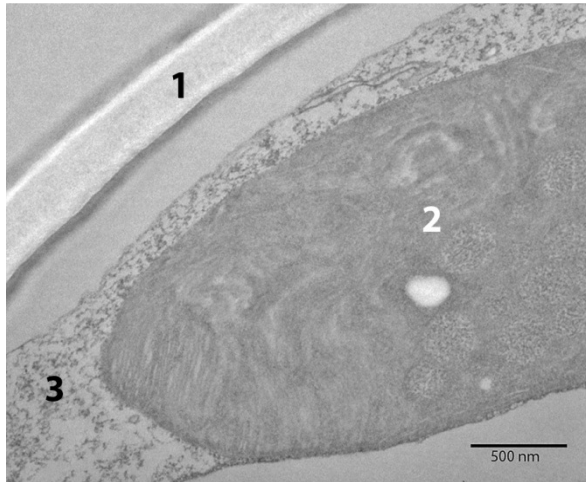
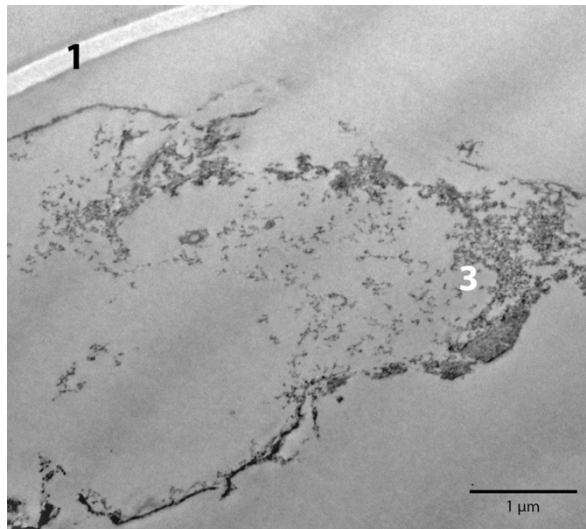


Fig. 55 (a-c): Micrographs of cells grown on Zn-EDTA 10 mM media



Leaf cells from Zn-EDTA 10 mM plates had similar chloroplast appearance (2) as cells grown on Cu-EDTA 10 mM. Small starch grains could be observed. Some precipitations could be found in the vacuole (3) and adjacent to the membrane of vesicles (4) but not in or bound to the cell wall (1). The loose

membrane stacks (5), might indicate the disintegration of this cell.

5.6 Electron energy loss spectroscopy (EELS)

Electron energy loss spectroscopy (EELS) was performed with the kind help Prof. Dr. Ursula Lütz-Meindl and her group at the University of Salzburg. Different cells of *P. patens* grown on control, Cu-EDTA 10 mM and Zn-EDTA 10 mM were investigated in a first set of experiments. Further investigations are currently underway.

5.6.1 Control

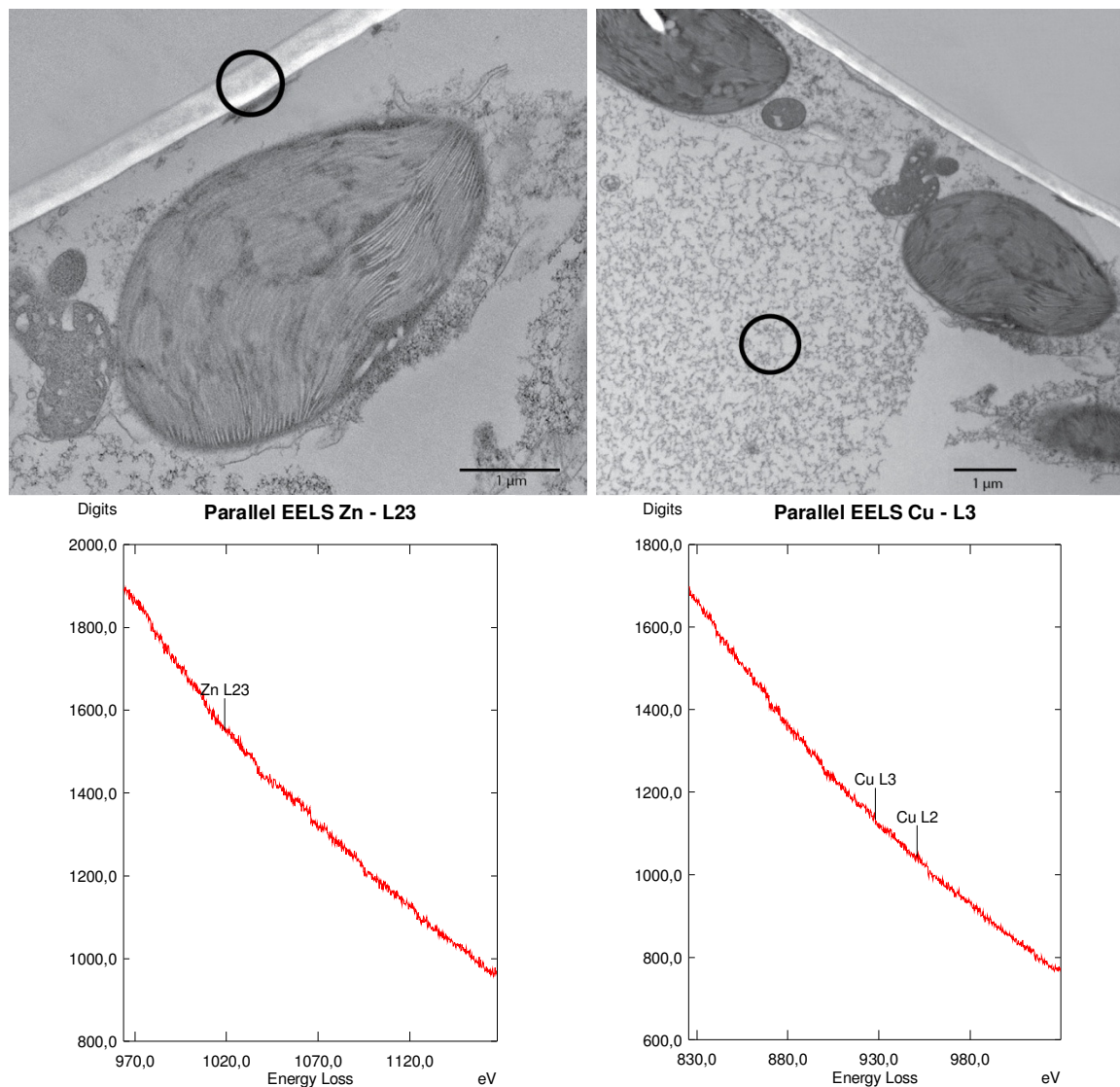


Fig. 56: Micrographs and EELS-spectra of control cells, circles mark the measured zones.

P. patens control cells did not show any presence of zinc or copper in neither the cell wall nor in the vacuole. Although these regions appeared electron dense, they did not contain any of the targeted heavy metals, as indicated by the EELS-spectra above (Fig. 56).

5.6.2 10 mM Cu-EDTA

Here, four micrographs were stitched to an overview to illustrate the zones of measurement within the moss cells grown on Cu-EDTA 10 mM. The circles mark non copper containing test sites, the hexagons mark copper containing sites.

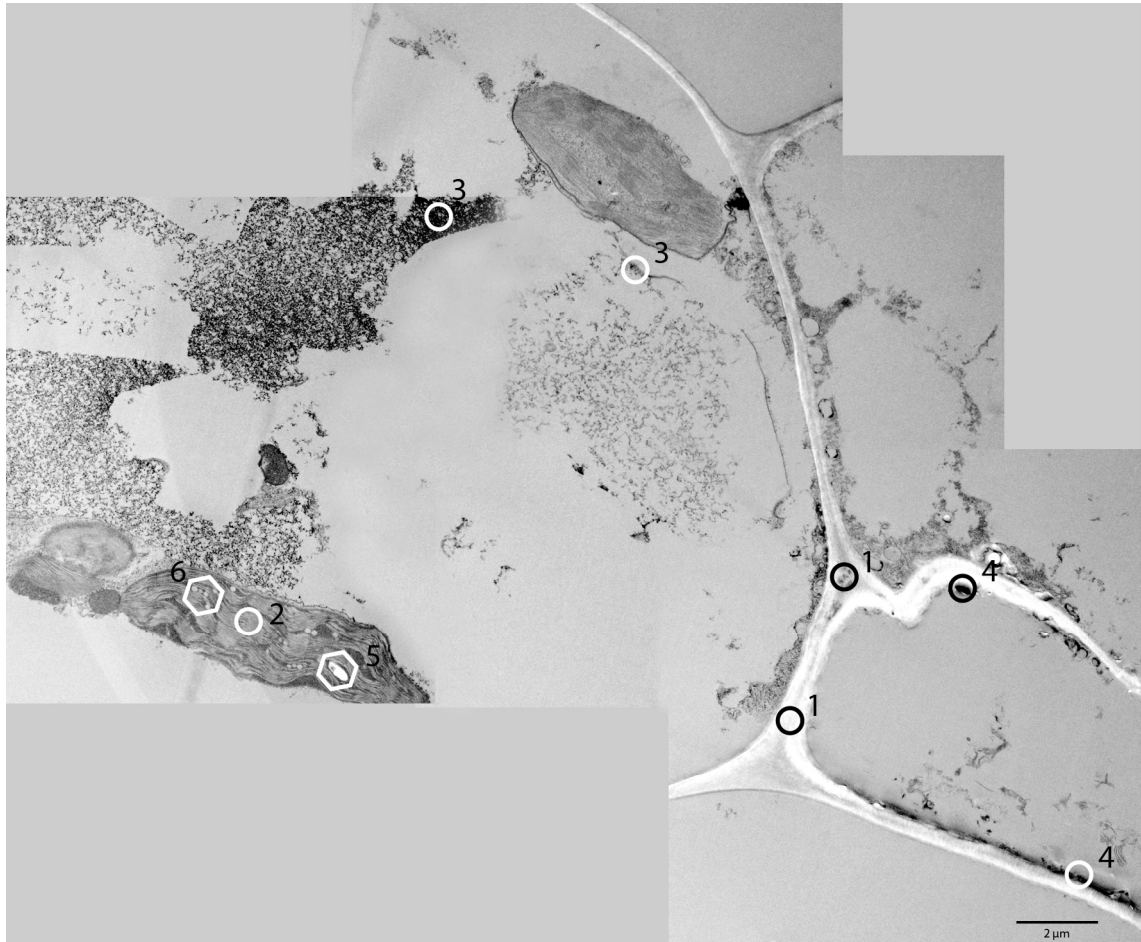


Fig. 57: Assembled micrographs of cells grown on Cu-EDTA 10 mM, circles mark the non-copper containing measured zones and hexagons the copper containing ones.

In Fig. 57 surprisingly dark electron dense precipitations in the cell wall (1), the chloroplast (2), the vacuole (3) and vesicles (4) did not show any copper peak in EELS-spectra. However in starch grains (5) and vesicular chloroplast bodies (6) elevated copper values could be found (Fig. 58 a-b).

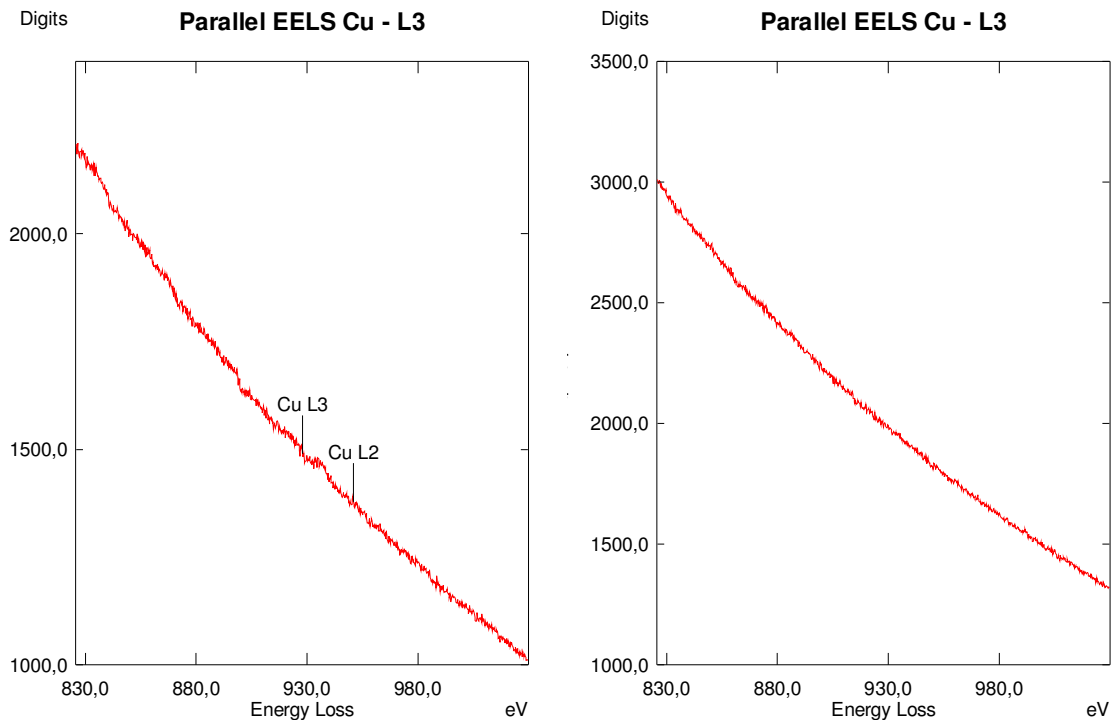


Fig. 58 (a-b): EELS spectra, left with copper peak, right with no copper peak

In the EELS-spectrum (Fig. 58a) of a starch grain (5), a small peak was observed between CuL3 and CuL2. The EELS-spectrum (Fig. 58b) of cell wall precipitations (1) did not result in any peak between CuL3 and CuL2.

5.6.3 10 mM Zn-EDTA

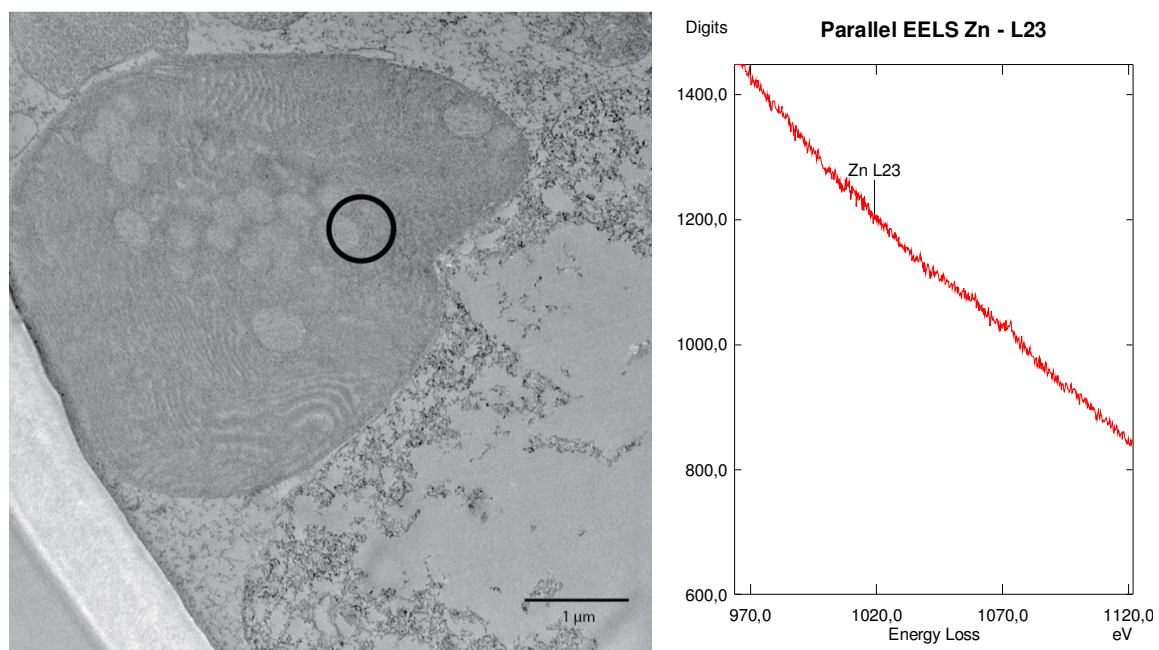


Fig. 59(a-b): a) Micrograph of a cell from Zn-EDTA 10 mM with chloroplast, circle marks the measured zone; b) Spectrum of zinc measurement

The chloroplast of cells from Zn-EDTA 10 mM media often showed circular vesicle-like inclusions and no starch grains. The chloroplast in this micrograph was situated near the cell wall; some dark precipitations were visible in the vacuole. The point of measurement is marked with a circle.

Small zinc peaks could be observed by EELS analysis of cells grown on Zn-EDTA 10 mM media. Like for copper, chloroplasts seem to enrich zinc within plastoglobuli (Fig. 59) but on the other hand, zinc could also be found in precipitation surrounding the chloroplast (Fig. 60 a). As shown in Fig. 60 b, an overlay of the EELS curves from cytoplasmic precipitation (red) and a control area nearby (resin only, green) showed a clear difference in zinc content.

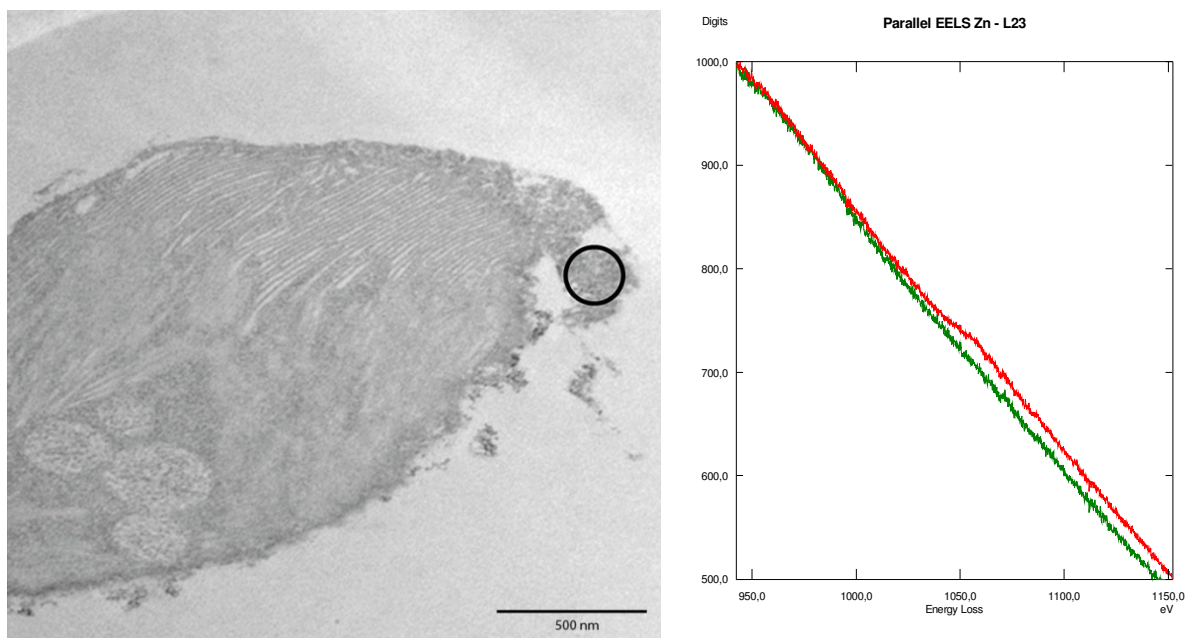


Fig. 60(a-b): a) Chloroplast with surrounding precipitation; the circle displays the test area from which in the red curve (b) results, as control the green curve was taken on a nearby area that contained only epoxin-resin

5.7 High performance liquid chromatography (HPLC)

HPLC was used to determine the possible increase of stress- induced secondary metabolites. Apart from the plants grown on heavy metal plates, two controls from different cultivation sites were investigated. Control_{KS} (blue) was grown under cooled conditions and control_{REG} (black) was grown on shelves with slightly higher temperature. The HPLC peak representing benzoic acid reached 1090 mAu for temperature stressed control_{REG} and is higher than for plants grown under normal cultivation temperature of 24°C (control_{KS}; n700 mAu).

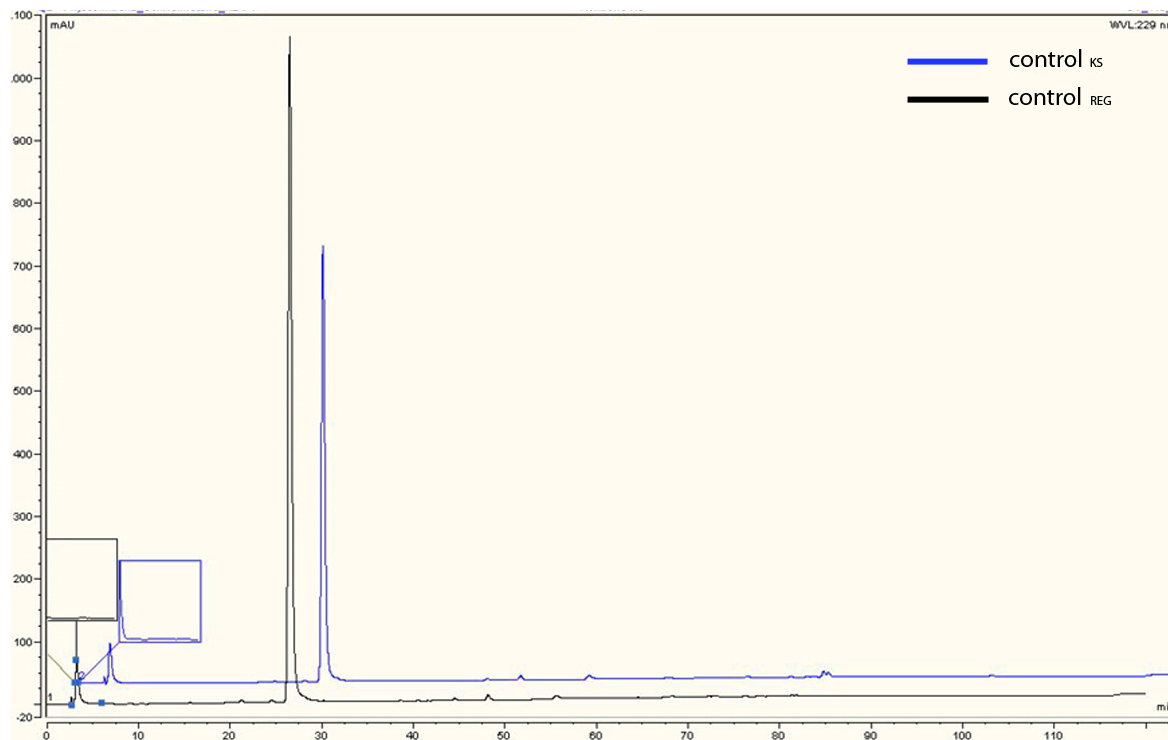


Fig. 61: HPLC-results; control_{KS} (blue) and temperature stressed control_{REG} (black)

HPLC analysis of *P. patens* from different heavy metal spiked media confirmed the expected stress response of mosses. Elevated levels of secondary metabolites – here benzoic acid – were detected in plants that were exposed to higher levels of copper and zinc. Fig. 62 shows a comparison of control_{KS} (black), Cu-EDTA 100 mM (blue), 10 mM (violet), 100 μ M (red) and ZnCl₂ 100 μ M (green). Cu-EDTA 100 mM and 10 mM displayed elevated levels of benzoic acid which is a common metabolite in stress

response. Surprisingly lower concentrations of Cu-EDTA 100 μ M and ZnCl₂ 100 μ M did not even reach the control values.

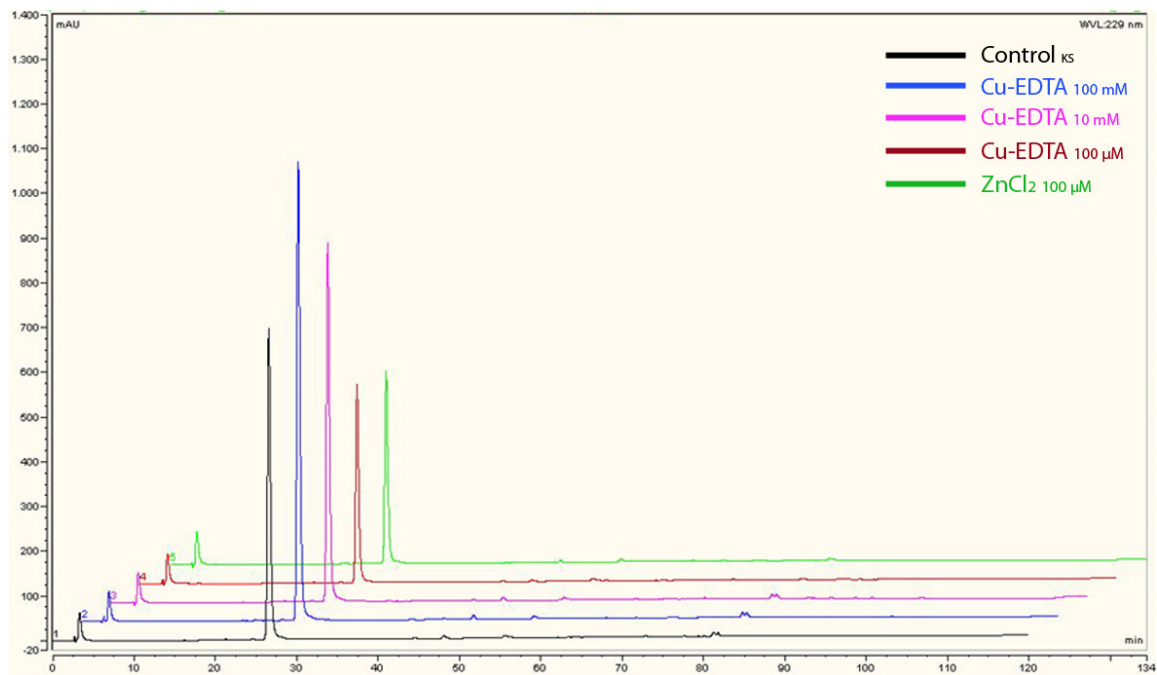


Fig. 62: HPLC-results for benzoic acid of plants from control_{KS} (black), Cu-EDTA 100 mM (blue), Cu-EDTA 10 mM (violet) and Cu-EDTA 100 μ M (red) and ZnCl₂ 100 μ M (green)

Higher concentrations of Zn-EDTA displayed the same metabolic response in *P. patens* than higher Cu-EDTA levels. Plants from Zn-EDTA 100 μ M (violet) and ZnCl₂ 100 μ M (brown) plates gave lower benzoic acid contents, similar/even less than control_{KS}(black).

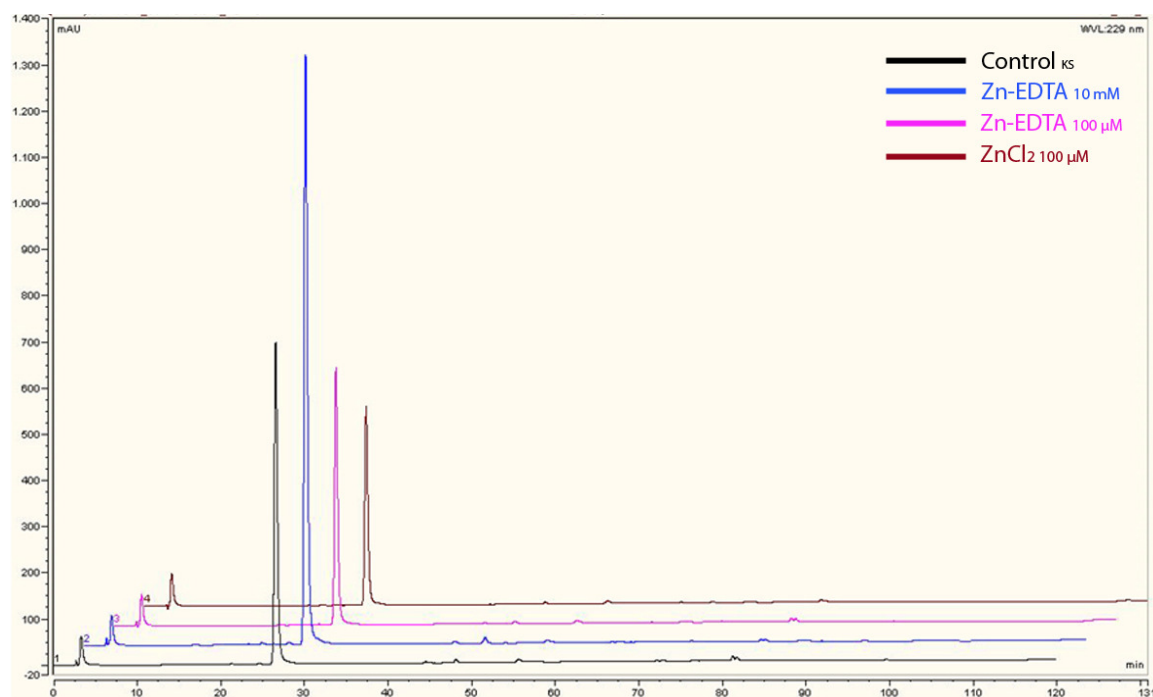


Fig. 63: HPLC-results for benzoic acid of plants from control_{KS} (black), Zn-EDTA 10 mM (blue), Zn-EDTA 100 μM (violet) and ZnCl₂ 100 μM (brown)

Comparison of control_{KS} (black) with CdCl₂ 0.1 μM (blue) and Fe-EDTA 1 mM surprisingly indicated no metabolic response to the offered heavy metals (Fig. 64). As in lower concentrations of Cu-EDTA, CuCl₂, Zn-EDTA and ZnCl₂ it seems that these plants were even less stressed or less active than control plants.

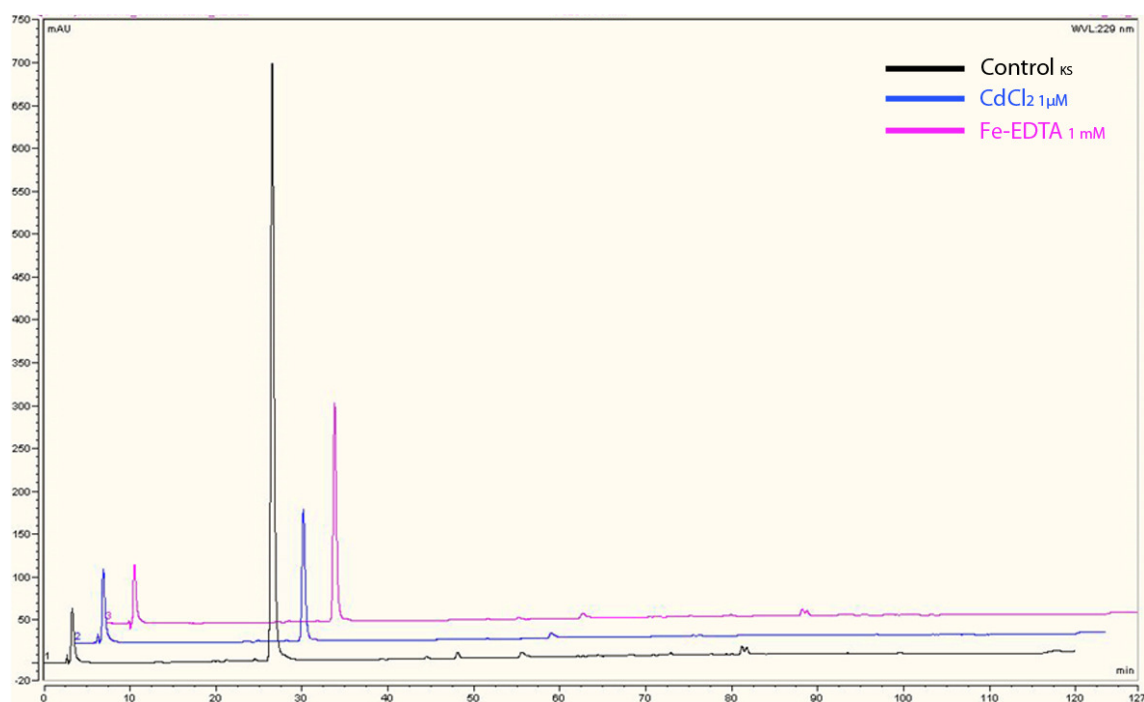


Fig. 64: HPLC-results for benzoic acid of plants from control_{KS} (black), CdCl₂ 0.1 μM (blue) and Fe-EDTA 1 mM (violet)

In summary, the results of HPLC-analysis indicated a correlation of benzoic acid concentration within the cells according to the amount of heavy metal offered to the mosses. Furthermore, these results showed that benzoic acid accumulation is a general stress response, since elevated temperature in control_{REG} had a similar effect as heavy metal induced stress.

6 Discussion

6.1 Resistance

The results showed that the “sensitive” *Physcomitrella patens* tolerates high heavy metal concentrations. The resistance test confirmed heavy metal toxicity in the order: $\text{CdCl}_2 \geq \text{CuCl}_2 > \text{ZnCl}_2 > \text{Cu-EDTA} > \text{Zn-EDTA}$ (Tyler, 1990). With the exception of cadmium which this author described to be less toxic than copper my results for *P. patens* match the results of Tyler (1990).

Early studies of Kaho (1933) and Url (1956) suggested that the application of heavy metal salts is least harmful to the cells when applied as sulphates rather than chlorides or nitrates. In our studies, however, we decided to use, chloride and EDTA bound to the heavy metals. This was due the fact that sulphates – I have used CuSO_4 in preliminary experiments (data not shown) – *P. patens* survived only up to 100 μM . Furthermore, metal chelating agents like EDTA are widely used in phytoremediation to enhance metal uptake and accumulation in plants (Alkorta et al., 2004). Finally, at the pH used, high concentrations of CuSO_4 could not be dissolved properly.

For Cu-EDTA, *P. patens* tolerates the same high levels as the known “copper moss” *Mielichhoferia elongata*. Already Url (1956), showed high CuSO_4 -tolerance (up to 500 mM) for *M. elongata* in resistance experiments and reported that mosses from non-metal polluted habitats, i.e. *Mnium affine* or *Madotheca platyphylla*, survived up to exceptionally high concentrations of over 500 mM CuSO_4 . In these resistance tests, the mosses displayed “dead zones” at lower concentrations (between 500 μM and 50 mM) where no living cells could be observed (Url, 1956). A formation of heavy metal precipitates on the plasma membrane as protective layer for the living cell was suggested by the author. However, other moss species like *Funaria hygrometrica* or *Mnium undulatum* reacted very sensitively to CuSO_4 and died already at low concentrations of 5 μM (Url 1956). *P. patens* tolerated the high heavy metal levels without showing “dead zones” in the resistance experiments of 48 hrs direct exposure.

According to Frahm (2001), mosses are described to be highly resistant to heavy metals. My data supports this generally high resistance of bryophytes to abiotic stress factors.

6.2 Cultivation and growth control

When I compared the LD50 of *P. patens* submersed in the heavy metal solutions with plants grown on heavy metal spiked agar plates, I found a difference in resistance. 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. Here a determination of "growth" was necessary. After Chiariello et al. (1989) it is a change in size, mass, form or number. In this work the size of the moss plants was measured and then compared to the starting size.

Media	Comparison of resistance on solid media and in liquid solution										
	1M	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹	C
Cu-EDTA (agar)	o	+	+	+	+	o	o	o	o	o	+
Cu-EDTA (solution)	-	++	++	+	+	+	+	+	+	+	+
Zn-EDTA (agar)	o	+	+	+	+	o	o	o	o	o	+
Zn-EDTA (solution)	-	++	+	+	+	+	+	+	+	+	+
ZnCl ₂ (agar)	o	-	-	+	+	o	o	o	o	o	+
ZnCl ₂ (solution)	-	-	-	++	++	+	+	+	+	+	+
CuCl ₂ (agar)	o	-	-	-	+	o	o	o	o	o	+
CuCl ₂ (solution)	-	-	-	-	-	-	++	+	+	+	+
CdCl ₂ (agar)	o	-	-	-	-	+	+	o	o	o	+
CdCl ₂ (solution)	-	-	-	-	-	-	++	++	+	+	+

Table 6-1: Combined table of the classic resistance results (- = dead, ++ = 50% living, += living), and on solid growth media (+ = growth, - = no growth, o = not tested) of *P. patens*.

At the first glance the classical resistance results and the resistance on solid growth media seem to be similar. A closer look reveals a difference in the resistance, for example *P. patens* was able to grow over a period of 5 weeks on heavy metal spiked growth media up to a concentration of 10⁻¹ Cu-EDTA, whereas the LD50 was 10⁻² after only 48 hours.

In general, *P. patens* was able to grow on heavy metal enriched agar plates with concentrations that were ten times higher than the concentrations used in the

classical resistance tests. The fact that unlike the heavy metal defence mechanisms in roots of higher plants (Baker, 1981), bryophytes do not possess real roots, the whole bryophyte tissue is able to take up ions (Tyler, 1990). Submersed in heavy metal solutions as in the classical resistance experiments, the whole gametophyte is exposed to the heavy metal while only a small part of the gametophyte has agar contact when cultivated.

Nonetheless, the moss gametophytes took up heavy metals on metal-spiked plates in a concentrations dependent manner. This is astonishing because *P. patens* has no vascular tissue.

In long term experiments on graded heavy metal plates combined with measurements of biomass accumulation the effects of the used heavy metals and their anions were tested. In low heavy metal concentrations, growth was similar to control and even showed a slightly positive effect compared to control. Here, the exceptions were cadmium concentrations (of CdCl_2) from 0.1 μM to 5 μM because even in these very low concentrations the described harmful effect of cadmium could be registered (Wang et al., 2009). High concentrations of Zn-EDTA 100 mM and the rather low ZnCl_2 5 mM resulted in drastically reduced growth, whereas Zn-EDTA 10 mM performed best biomass accumulation and supported the results of lower plasmatic toxicity of zinc (Tyler, 1990).

Interestingly *P. patens* did display very well the expected high tolerance of copper (Frahm, 2001); in low Cu-EDTA levels biomass accumulation even showed a slightly positive effect and elevated growth rates when compared to controls without copper. Again, this positive effect at low Cu-EDTA concentrations will need further characterisation in a future project.

In either way, the high variation of growth even on control plates was challenging as mentioned also by (Bijelovic et al., 2004). Elevating the number of measured plants could solve this problem to a certain limit but it has to be considered that genetic variation within populations as well as differentiation between populations as a strategy of mosses for heavy metal tolerance (Shaw, 1988) may influence these resistance tests. In this study, only closely related plant material was used and

attention was paid to sub-cultivate plantlets of approximately the same size to minimise this effect.

Detoxification of the excessive heavy metals by exclusion or deposition at internal storage sites could be the reason for the high tolerance of *P. patens*. Phytochelatins are considered to play a key role in metal homeostasis and final disposal in the vacuole (Cobbett, 2000; Krämer and Clemens, 2005). However, *P. patens* seems to lack phytochelatins suggesting different mechanisms of metal detoxification (Rother et al., 2006).

6.3 Electron energy dispersive x-ray microanalysis (EDX)

Electron energy dispersive x-ray microanalysis (EDX) is a semiquantitative method which analyses all elements with an atomic number above ten (potassium) and gives their relative amount within the probe. Therefore the analysis of the element composition is always in relation to all other elements contained in the sample. Due to physical limitations of the method (Kiss, 1987), analysis of single cells or intracellular localization of elements was not possible, but whole tissues had been investigated.

The results of EDX showed high zinc uptake up to an average of 2.43 Wt% in plants grown on Zn-EDTA 100 mM. Interestingly compared to Zn-EDTA much lower concentrations of ZnCl₂ 1 mM reached up to 1.11 Wt%. Higher concentrations of ZnCl₂ did not result in higher accumulation. There seems to be a peak for zinc uptake ranging from 1 to 5 mM ZnCl₂. No such plateau could be observed for Zn-EDTA. It seems that ZnCl₂ is more available for the mosses in lower concentrations. This effect still needs to be investigated.

EDTA is used as a fertilizer for plants which are able to mobilize micronutrients by root exudates. Mosses, however, are unable to produce root exudates. Nevertheless, the high zinc content of the moss biomass grown on high concentrations of Zn-EDTA 100 mM indicates that Zn-EDTA is available for the plantlets.

At first glance copper uptake of *P. patens* seems to be very low in comparison to the uptake of zinc. Maximum copper content was registered in Cu-EDTA 100 mM at 0.47

Wt%. This copper uptake of *P. patens* is higher than the analyzed content for the “coppermoss” *Mielichhoferia elongata* (0.33 Wt%) but significantly lower than for *P. drummondii* (0.88 Wt%), when both grown under the same culture conditions. AAS-measurements of the used growth media indicate plant availability of the used heavy metals (Hus, 2008; Wernitznig, 2009). The lower copper uptake compared to zinc and the lack of copper uptake at lower copper concentrations might indicate that *P. patens* can avoid a surplus of copper. These mechanisms of homeostasis and are still poorly understood and are not yet described for *P. patens* (Krämer and Clemens, 2005). Furthermore the EDX-analysis could not detect any elevated levels of sulfur in any concentration as described by (Rother et al., 2006), this might be also due to the limits of detection.

Cadmium was lethal for *P. patens* at very low concentrations. At the cultivated concentrations, no cadmium uptake could be observed, it seems that cadmium is already lethal beneath the detection limit of the EDX, and other ways to determine Cd content of the cells have to be used.

6.4 Confocal laser scanning microscope (CLSM)

The results of the EDX-analysis demonstrated a remarkable zinc uptake. For further investigations on cellular level *P. patens* cells were stained with fluorescent heavy metal tracer dyes and analysed with the CLSM.

Preliminary tests with Phen Green SK indicated that it was not specific enough for copper and it could not penetrate the cell wall of *P. patens*, although Phen Green has been used to detect copper in higher plants from heavy metal sites (Shingles et al., 2004). When analyzing FluoZin-3 staining in the confocal scanning laser microscope in order to visualize the uptake of zinc, attention was paid to keep the same adjustments for all samples so that changes of fluorescence intensity could be correlated with the cellular amounts of zinc. As expected from the results of EDX-data, rising fluorescence and therefore rising zinc content in the cells could be observed 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. The differentiation of fluorescence between the cell wall and the protoplast was achieved by increasing the space between them by plasmolysis. By contrast, the fluorescent vesicles could be clearly distinguished from the surrounding cytosol. These vesicles do not obligatorily contain zinc as displayed in the results. Furthermore, “doughnut” shaped chloroplasts could be observed in low zinc concentrations regardless of the anion. There seems to be an active transport mechanism for zinc into the vacuole by these vesicles or they function as zinc deposits isolated from the cytoplasm. However, transport by vesicles is not described for heavy metals and these mechanisms are still to be revealed (Krämer and Clemens, 2005).

6.5 Transmission electron microscopy (TEM) and electron energy loss spectroscopy (EELS)

6.5.1 TEM

Transmission electron microscopy (TEM) revealed electron dense precipitations in the vacuole and near the cell walls. Small electron dense vesicles could also be observed. Furthermore, a high amount of starch grains in the chloroplasts could be observed in cells grown on control medium. Interestingly, with rising concentrations of heavy metal in the growth media (up to 1 mM for EDTA), chloroplasts showed less and smaller starch grains and at very high heavy metal levels (from 10 to 100 mM for EDTA) no starch grains at all could be found. This observation might indicate that with rising heavy metal level the photosynthetic potential of the moss and therefore its capability to form starch as an energy reserve is defected. However, it has to be considered that the number of samples for TEM was rather low and therefore we cannot exclude that this observation is specific to the investigated individuals. In this respect, it will be interesting to screen all the sections that have been made already and to measure the photosynthetic activity of the stressed and non-stressed mosses.

6.5.2 EELS

Preparation of samples for transmission electron microscopy is time consuming and has to be specific to the aim of the work and the used material. In this case ultrathin sections, (as described in methods), were needed for electron energy loss spectroscopy. The preparation of these samples emerged to be quite a challenge. Even with experienced advice of the group of Prof. Lütz-Meindl (University of Salzburg), perfect cuts for analysis could not be obtained. A common problem was breaking of the ultrathin sections. This was particularly challenging because the fine cracks between cell wall and embedding resin could not be observed in semi-thin sections.

The analysis with electron energy loss spectroscopy was started with control and two heavy metal concentrations (Zn-EDTA 10 mM and Cu-EDTA 10 mM). The results showed no detectable zinc or copper in cells grown on control plates. Analyses of cells grown on Cu-EDTA 10 mM indicated copper in the chloroplasts. Surprisingly, the analysis of electron dense precipitations in the vacuole and inclusions of the cell wall did not show an apparent copper peak. Similar results were observed for cells grown on Zn-EDTA 10 mM. Here, also the chloroplasts and precipitations surrounding the chloroplast showed small zinc peaks.

However, these results of EELS had preliminary test character and still need to be confirmed by enhanced preparation techniques and a representative number of analyzed samples.

6.6 High performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) was performed in the laboratory of the department of Chemical Ecology and Ecosystem Research (University of Vienna) with the kind help of Prof. Dr. Franz Hadacek. The results for benzoic acid showed an increase according to the level of the offered heavy metals. These experiments clearly showed a stress response to the offered heavy metals. Benzoic acid is discussed as a precursor metabolite for salicylic acid which is believed to be a secondary metabolite for general stress tolerance (Senaratna et al., 2003). Those studies used mainly higher crop plants like *Lycopersicum esculentum*, *Phaseolus vulgaris* and *Nicotiana benthamiana*. Molecular studies on the moss *P. patens* indicate that it has a similar stress response as higher plants (Machuka et al., 1999). We therefore decided to search for benzoic acid and to correlate its quantity with the heavy metal treatment.

7 Conclusion and future aspects

The moss *Physcomitrella patens* is an established model organism. Its genome is fully sequenced and as it is used in broad investigations in different fields from molecular to physiological studies. It will be interesting to see further outcomes on heavy metal homeostasis at the molecular, genetic, as well as structural level. To broaden the view, ecological aspects and genetic variation have to be taken into account. We hope to see further, interesting outcomes by future comparison of mosses from natural habitats with *P. patens*. For example, a similar study on *Funaria hygrometrica*, a member of the same family (*Funariaceae*) as *P. patens*, compared the population of mosses from a mining site and an unpolluted site (Basile et al., 1994). Only minimal effects of plants from the population originating from the mining site could be observed when grown on lead and zinc spiked media. By contrast, severe structural and morphogenetic alterations occurred with plants from the unpolluted site. The authors suggested cell wall and vacuole compartmentation of lead and zinc to account for higher tolerance in the adapted samples.

It remains to be determined if the above effect is also the case for copper or it is restricted to certain metals like lead or zinc. Furthermore, effects of different anions of the used heavy metals should be investigated more closely.

As my first results of metabolites indicated a stress response by benzoic acid, it would be interesting to further analyze the metabolic stress response of *P. patens* to heavy metal stress in order to find other, stress-responsive metabolites.

Another interesting effect is time. The results of the present work showed an accumulation on heavy metals with time. However, some plants or algae have developed mechanism to discard toxic waste by crystallizations or exocytose. (personal communication, Ursula Lütz-Meindl). In long term treatments, such organisms would accumulate less metal on the cellular level than by shorter heavy metal application.

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Curriculum Vitae

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Higher Education:

10/02-07/04: University of Vienna, study of Biology,
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07/04-: Study for Second Degree:

Major Field of study:

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- Cell Physiology
- Heavy metal stress

Diploma thesis:

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Adjournment of Academic and Higher Education:

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