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„Cell wall modulations in heavy metal stressed moss *Physcomitrium patens*“

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1. ABSTRACT

The thesis aims to give an overview of laboratory methods used for detecting ultrastructural changes in the cells of *Physcomitrium patens*, specifically the alterations in the cell wall of protonemata under copper and zinc stress. The cell wall can reflect changes in the environment by adjusting the amount of homogalacturonan, a component of pectin which has ability to bid heavy metals into its structure, immobilise them and thus protect its protoplast from harmful effects of the metals.

At the beginning, we monitored growth ability of 14 bryophyta species from three groups: mosses, liverworts and hornworts. Further experiments were carried out with *P. patens*, a model organism used in experiments for more than 80 years. This moss with simple development that grows only few types of tissues is easily handled and cultured vegetatively. We chose protonemal tissue for the experiment for its simple anatomy and direct contact with the medium and we aimed to propagate and harvest the tissue as much as possible.

Later, we followed protocols for the preparation of semi-thin resin sections and immunohistochemistry in order to prepare the tissue samples for visualisation under both light and confocal microscopes.

Mosses have been used as biomonitors of atmospheric pollution for their advantageous properties e.g., absence of cuticle or root system, absorption of nutrient with the whole body and large surface-to-weight ratio. Moss tissue load can give us valuable information about amount of heavy metals in the environment however the research under controlled laboratory conditions is lacking and the fact that each moss species reacts differently to the stress should be taken into consideration as well. Searching for a linear relationship between ultrastructural alterations and concentration of heavy metals in the environment is therefore crucial.

2. ZUSAMMENFASSUNG

Die vorliegende Masterarbeit soll einen Überblick über die Labormethoden geben, mit denen ultrastrukturelle Veränderungen in den Zellen des Mooses *Physcomitrium patens* nachgewiesen werden können. Der Schwerpunkt liegt auf Veränderungen in der Zellwand nach Kupfer- und Zinkstress. Die Zellwand kann Veränderungen in der Umwelt widerspiegeln, indem sie den Homogalacturonan-Gehalt anpasst. Homogalacturonan ist eine Komponente von Pektin, die die Fähigkeit hat, Schwermetalle in ihre Struktur einzubinden, sie zu immobilisieren und so die Zelle vor den schädlichen Auswirkungen der Metalle zu schützen.

Zu Beginn der Arbeit wurden 14 Moosarten aus den Gruppen der Laubmoose, Lebermoose und Hornmoose in Sterilkultur gezogen und deren Wachstumsfähigkeit auf kupfer- und zinkhältigen Medien untersucht. Weitere Experimente wurden mit dem Laubmoos *P. patens* durchgeführt, einem Modellorganismus, der seit mehr als 80 Jahren in wissenschaftlichen Versuchen verwendet wird. Dieses Moos besitzt nur wenige Gewebetypen, lässt sich leicht im Labor kultivieren und vegetativ vermehren. Wir wählten das Protonema-Gewebe für die Experimente der vorliegenden Arbeit wegen seiner einfachen Anatomie und seines direkten Kontakts mit dem Medium.

Anhand des Protonema-Gewebes erfolgte die Herstellung von semi-dünnen Harzschnitten für Immunhistochemie, um die Gewebeproben sowohl im konventionellen Lichtmikroskop als auch im Konfokalen Laser Scanning Mikroskop ultrastrukturell zu visualisieren.

Weil Moosen eine Kutikula oder ein differenziertes Wurzelsystem fehlt, findet die Aufnahme von Nährstoffen bei diesen Organismen über die gesamten Oberfläche statt. Daher werden Moose als Biomonitore für Luftverschmutzung und Metalltoxizität verwendet. Die Belastung des Moosgewebes liefert uns daher wertvolle Informationen über die Menge an Schwermetallen in der Umwelt. Allerdings gibt es noch wenige Untersuchungen zu dieser Thematik unter kontrollierten Laborbedingungen. Die Tatsache, dass jede Moosart unterschiedlich auf die Metallbelastung reagiert, sollte ebenfalls berücksichtigt werden. Die Suche nach einem Zusammenhang zwischen ultrastrukturellen Veränderungen und der Konzentration von Schwermetallen in der Umwelt ist daher von entscheidender Bedeutung.

3. INTRODUCTION

Heavy metals are released into the environment by both natural and anthropogenic processes. Heavy metal pollution has been a serious environmental problem caused mainly by human activities, for instance industry, agriculture, mining or metallurgical activities. Once heavy metals are released into the environment they may become part of food chains where they are difficult to metabolise and are likely to cause toxicity to living organisms (Olusegun et al. 2012).

On the other hand, there are heavy metals which are needed for proper functioning of the cells. In low concentrations these micronutrients (Zn, Cu, Fe, Mn, Co, Mo, Ni) are involved in enzymes and other active molecules important for expression of genes, biosynthesis of proteins and nucleic acids. These trace elements have involvement in plant growth, chlorophyll formations as well as metabolism of lipids and carbohydrates (Prasad and Hagemeyer 1999).

3.1 MOSSES AS BIOMONITORS

Some organisms can tolerate heavy metal stress to a certain degree without any physiological or morphological disruptions to their bodies. Such organisms are used as bioindicators and biomonitors. Bioindicators are organisms that provide qualitative information about the environment whereas biomonitors give quantitative information on the quality of the environment (Markert 2007). That means that biomonitors give information not only about the quantity of heavy metals in the air or soil but also their effect on the living systems. They can alter their structural and physiological properties.

The first initiative to monitor heavy metal particles in the air was introduced in 1968 by Rühling & Tyler. Sweden was the first country to measure the impact of heavy metal air pollution on Bryophyta. In 1991 the European moss biomonitoring network was set up and many European countries have joined the survey since then. The survey is carried out in 5-year intervals. Now the survey is coordinated by ICP Vegetation which is an international research programme that reports how the vegetation is affected by air pollution and the data of the survey are sent to Working Group on Effects (WGE) of the UNECE Convention on Long-Range Transboundary Air Pollution (LRTAP) (Harmens 2009; Harmens and Norris 2008).

The data collected are later analysed using geographic information system software which reconstructs geographic information system (GIS) maps. European moss survey provides information on 10 heavy metals: arsenic, cadmium, chromium, copper, iron, lead, mercury, nickel, vanadium and zinc. Since 2005 it has also given information on aluminium, antimony and nitrogen (Harmens, Norris, and Mills 2013).

To assess the health of the environment, bryophytes were introduced as potentially ideal biomonitoring systems for evaluation of heavy metal stress and as an alternative to instrumental techniques. What makes bryophytes ideal biomonitors is their ability to colonise metal contaminated environments or take up high amounts of heavy metals without any visible negative effects to their bodies. They absorb heavy metal trace elements with the whole organism, especially from the air. They lack a root system and cuticula layer which makes heavy metal access to cell wall easy. This ability is improved by their pronounced ion-exchange properties and large surface-to-weight ratio. Trace metal elements in the air can also be monitored by field receptor measurements. These are usually expensive and sampling is time-consuming because it is conducted on numerous sampling areas which need a lot more scientific equipment than to collect the moss (Stanković, Sabovljević, and Sabovljević 2018).

There are two approaches of biomonitoring, passive and active. Passive technique involves collection of mosses in their natural habitats followed by subsequent analyses. This approach gives information about heavy metal pollution on site. The active approach uses so called moss bags made from nylon which are distributed in urban areas. The moss used for the bags is collected in unpolluted areas so that the heavy metal load can be compared before and after exposure to the polluted air in the cities where there is often little moss growing (Ares et al. 2012).

The downside of biomonitoring research is that the collections of the same moss species are often insufficient meaning that a collected sample contains more species mixed with different age, state of hydration or health. It is therefore very complicated to compare the results of the studies (Stanković, Sabovljević, and Sabovljević 2018).

In the present thesis, we picked two metals, copper and zinc. Since both metals are essential micronutrients and play important part in plant development and biology, they were chosen for this study. Copper in plants is involved in function of enzymes and is a key metal in

chlorophyll synthesis. It is responsible for plant metabolism of carbohydrates and proteins and plays a role in plant respiration. A normal range of copper in plant tissue is between 3 to 10 ppm. Plants are rather sensitive to copper toxicity and even a slight overabundance in their tissues can cause disturbances in growth and metabolism (A. Sabovljević et al. 2018; Willetts, Wong, and Kirst 2016).

Toxic effects of zinc, on the other hand, are not so common as with copper. Zinc is needed for formation of auxin in plants, transformation of starch to sugars, activation of enzymes for formation of certain proteins. It is involved in synthesis of chlorophyll and in its presence the plants can withstand low temperatures easily. The range of zinc in plant tissue is from 15 to 60 ppm. General symptoms of zinc toxicity include impaired growth of shoots, curling of leaves, chlorosis or necrosis of leave tips (Amalero et al. 2003; A. Sabovljević et al. 2018).

3.2 LIFE CYCLE OF BRYOPHYTES

Bryophytes comprise 3 major groups, mosses, liverworts and hornworts. There are around 215 species of hornworts, 7500 species of liverworts and the richest group mosses include around 10 000 species. (Shaw and Renzaglia 2004; Söderström et al. 2016). The life cycle is similar in all three 3 groups.

Bryophytes comprise of two generations, gametophyte and sporophyte. Fig.1 shows an example for mosses. In most bryophyta, the gametophyte is the dominant stage. The sporophyte is usually attached to the gametophyte to be provided with nutrition. Cell cycle begins with releasing haploid spores from a sporophyte capsule; the spore later germinates. Its spore wall can be ruptured by the growing protoplast. Two types of protonema filaments are formed, brown-reddish horizontal filamentous structures called caulonema and green protonema called chloronema. The leafy gametophore is initiated by formation of a bud mainly on caulonema which later grows by mitosis (Crandall-Stotler and Bartholomew-Began 2007; Strotbek, Krinninger, and Frank 2013).

Gametophores are composed of leaves usually one layer thick, shoot stems are typically consisting of thick-walled epidermal cells, thin-walled parenchyma cells, hydroids and leptoids which are part of stems conducting system, axillary hair and rhizoids. Archegonia, female sex organs, and antheridia, male sex organs, are formed at the tips of gametophores. In order to fertilise the egg, spermatozoids must be able to move towards the archegonia in a thin water

film. After fertilisation, a diploid zygote is formed from the fertilised egg and gives rise to hundreds of haploid spores in the spore capsule (Roberts, Roberts, and Haigler 2012; M. Sabovljević, Vujičić, and Sabovljević 2014; Strotbek, Krinninger, and Frank 2013).

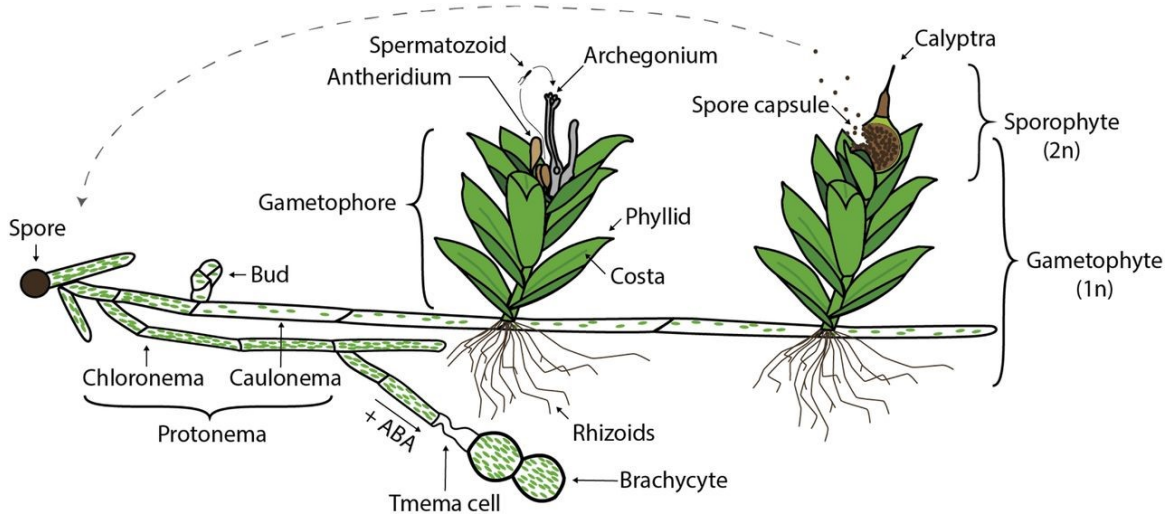


Fig.1 Life Cycle of *P. patens* (Strotbek, Krinninger, and Frank 2013)

Phycomitrium patens (Funariaceae) is a model organism for non-seed plants mainly used in evolutionary developmental biology due to its position in the phylogenetic tree, tendency for targeting of genes via homologous recombination and simple morphology. It is also used in reprogramming of stem cells because it can regenerate protoplast into whole organisms without any exogenous hormones as a response to wounding. It was the first non-seed plant to be fully sequenced (Rensing et al. 2020).

P. patens is an easy moss to work with. It does not require demanding maintenance or too much laboratory space and its cultivation is rather straightforward in sterile culture. Due to its small natural height and its anatomical simplicity the tissue can be handled effortlessly when microscoping because there is often no need for sectioning or dissection (Schaefer and Zrÿd 2001) (Rensing et al. 2020)

3.3 GENERAL CHARACTERISTICS OF CELL WALL

Bryophytes, as some lichens and vascular plants, can withstand harsh environmental conditions or heavy metal stress. In general, plants cannot move away from the stressful conditions and in order to deal with them, they had to evolve different strategies to either

tolerate or avoid the stress. To avoid the heavy metal stress means that organisms can protect themselves against heavy metals entering the protoplast by involving different structures or mechanisms. Tolerance of heavy metals is based either on neutralising heavy metals and their toxic effects in the protoplast or their removal out of the protoplast (Krzesłowska et al. 2009). In this respect, the moss cell wall plays a crucial role in heavy metal tolerance. It consists of cellulose, cross-linking glucans, pectin, glycoproteins, callose, lignin and possible cuticle in some mosses (Roberts, Roberts, and Haigler 2012).

Generally, the cell wall of plants has many mechanical and biochemical functions. It acts as a barrier against biotic impact and harsh ecological conditions, and it also gives strength to the cell. It is responsible for reorganisation of its components as well as signal transduction in response to pathogens. The cell wall is composed of polymers vowed into a meshwork with 3D architecture (Sarkar, Bosneaga, and Auer 2009). As the plant grows and develops the cell wall is formed by depositing series of layers beginning by middle lamella formed between two adjacent cells. Next, the primary cell wall (Fig. 2) is deposited which is found in all cells unlike the secondary cell wall found in wood cells or xylem. The deposition of secondary cell wall begins after the expansion of the primary cell wall has stopped or has been completed and it is formed between the cell membrane and primary wall. The secondary cell wall gives extra strength to the cell (Delmer and Stone 2012; Scheller and Ulvskov 2010).

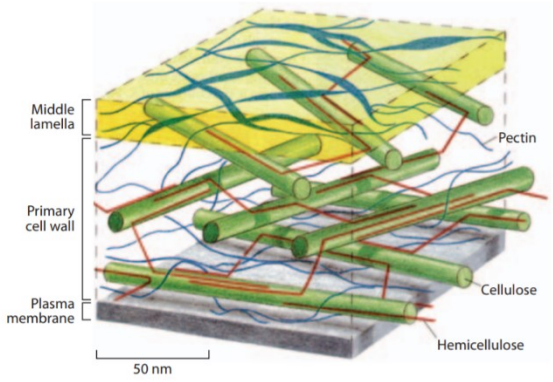


Fig.2 Model of primary cell wall (Scheller and Ulvskov 2010)

Bryophytes do not possess any vascular transport system in their tissue. They use simple diffusion and water pressure for transporting water in the organism therefore their cell wall is usually thin for an easy water access. It is not always possible to distinguish between primary and secondary walls in bryophytes.

The main constituent of the cell wall is cellulose which is an unbranched polymer consisting of (1→4) β-linked D-glucose units synthetized at the cell membrane and later released straight to the cell wall. This insoluble polymer forms parallel chains held together by hydrogen bonds and van der Waals forces into crystalline microfibrils which wrap around the cells (Delmer and Stone 2012). Another important component of the cell wall cross-linking glucans are soluble heterogenous group of polysaccharides with (1→4)β-linked sugar backbones which includes xyloglucans, mannans, xylans and mixed-linkage glucans. They do not form microfibrils because of their branched structure and modification by esterification. The role of cross-linking glycans is to tether adjacent cellulose microfibrils (Scheller and Ulvskov 2010).

Pectin in cell wall can bind divalent and trivalent metal ions, immobilise them and thus prevent them from entering the cell protoplast. They have a complex structure with four major polysaccharide domains: homogalacturonan, rhamnogalacturonan I, rhamnogalacturonan II and xylogalacturonan (Krzesłowska 2011). The main component of pectin in mosses and vascular plants is homogalacturonan (HGA). It is a linear polysaccharide formed by alpha-1,4-linked D-galacturonic acid and produced in the Golgi apparatus in a methyl-esterified form and later upon secretion into a primary cell wall it is de-esterified by enzyme called methylesterase. Elevated amounts of free carboxyl groups in HGA indicate higher ability to bind heavy metals. HGA in pectin consists of two polysaccharide backbones connected with Ca^{2+} ions linked to free carboxyl groups. This structure is called an egg box model (Fig. 3) and it gives pectin its gelatinous consistence (Herburger, Xin, and Holzinger 2019; Krzesłowska 2011). Under heavy metal stress, calcium ions can be replaced by divalent and trivalent ions where Pb^{2+} and Cu^{2+} are bound very strongly to pectin whereas there is a weaker bond by Zn^{2+} and Ni^{2+} ions (Krzesłowska 2011).

Common molecular tool for detection of pectin and its main domains in plant science is the use of monoclonal antibodies. They allow precise localisation of pectin domains within an intact wall structure and their interactions with other components (Willats, Knox, and Mikkelsen 2006).

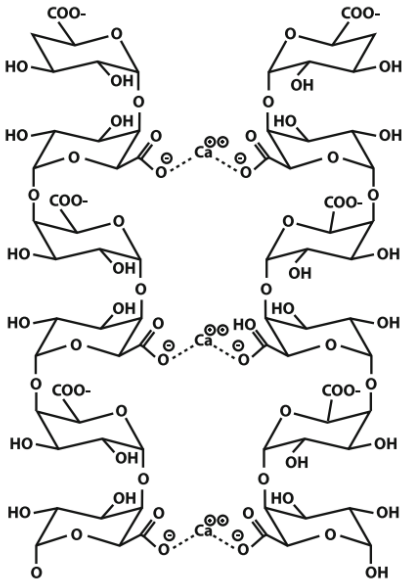


Fig 3. Egg-box structure in cell walls of higher plants (Krzesłowska 2011)

3.4 METHODS USED FOR INVESTIGATING HEAVY METAL STRESS

3.4.1 PHYSIOLOGICAL TESTS

The effect of heavy metal stress can be estimated by physiological parameters for example chlorophyll content, disrupted nitrogen metabolism or induced oxygen reactive species and by ultrastructural changes (Choudhury and Panda 2005). Heavy metal stress alters the shape of chloroplasts or thylakoid organisation and the stromal plastoglobules may gain different appearance. In some mosses there are also small vacuoles with precipitants, some lipid droplets or multivesicular bodies found in the protoplast. Some of these cell alterations are metal specific (Stanković, Sabovljević, and Sabovljević 2018).

The simplest method used for evaluation of heavy metal impact on the moss is the naked eye observation. Affected moss may manifest various signs of chlorosis, discoloration or impaired growth.

3.4.2 IMMUNOLABELLING TECHNIQUES

Immunohistochemistry is a biochemical method of staining tissue sections based on an interaction of antibodies and antigens. It is a popular tool for the identification and localisation of antigens in cells. Specific antibodies are supposed to attach to the antigen like a key fits

a lock (Griffith, 2014). Antigens possess specific molecules called epitopes which bound to a specific antibody. Antibodies on the other side own a special region called paratope where an epitope attaches.

There are two types of antibodies, monoclonal and polyclonal. The main difference is that monoclonal antibodies are produced by the same clone of lymphocyte B cells whereas polyclonal antibodies are made of different plasma B cells. Monoclonal antibodies react only with a unique epitope and polyclonal antibodies can react with various epitopes on the same antigen (Panawala 2017). The most common type of antibodies is the large immunoglobulin molecule IgG which consists of two types of polypeptide chains, light and heavy. Each IgG molecule has two light chains and two heavy chains and are linked by disulphide bridges. The antibody molecule is roughly Y-shaped, and it can be cleaved by proteases into three parts, two identical Fab fragments with antigen-binding activity and on Fc fragment (Janeway CA Jr, Travers P, Walport M 2001).

Antibodies can also be classified into two groups based on their ability to bind to either antigen´s epitope or another antibody. Primary antibodies can detect a target antigen directly. Secondary antibodies are used in immunohistochemistry for detection of the primary antibody and the amplification of signal. They are used in indirect immunolabelling techniques. Primary antibodies bind to antigens with their highly specific Fab fragments leaving the Fc fragment exposed to binding to secondary antibodies (Lipman et al. 2005).

In immunodetection techniques, secondary antibodies are coupled with gold beads (for electron microscopy techniques, see “immunogold-labelling” below) or fluorophores (fluorescent complexes, see “immune-fluorescence” below) for signal detection in the light/fluorescence microscope.

3.4.3 IMMUNOGOLD-LABELLING

It is a common staining method used in immunohistochemistry following similar steps as immunofluorescence techniques and nowadays applied in both transmission electron microscopy (TEM) and scanning electron microscopy (SEM). This technique provides detection and localisation of target markers in the cell with high resolution and in real-time. The principle of the method is based on antibody-antigen reactions where in general, the method is more focused on indirect binding of gold particles conjugated to secondary antibodies which are

attached to specific primary antibodies. In order to get a desirable outcome of immunogold-labelling it is critical to handle samples correctly which includes careful fixation and dehydration protocol and right selection of embedding media. The size of gold particles used as a probe can vary from 1 to 40μm. To detect more antigens simultaneously it is needed to select gold particles with different sizes and subsequently quantify them which could be very useful in comparative research. Gold is a very reliable material for its high electron density, it efficiently emits secondary electrons, biocompatibility and large surface area (Goldberg and Fišerová 2016; Murtey 2016).

3.4.4 IMMUNO-FLUORESCENCE

This technique allows detection and localisation of different antigens in cells and tissues by observing the fluorescence of the sample treated and stained with a fluorophore. Fluorophores are fluorescent dyes chemically conjugated to primary or secondary antibodies that help visualise the samples' real-time activity under fluorescent microscope. Fluorescent microscope has a source of light that excites specimen respectively fluorescent dyes in the sample followed by emitting of light with longer wavelength or lower energy. Fluorophores differ in their wavelengths and colours they emit. Widely used fluorophores are fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) (Rooks, M.G and Garrett, W.S 2017).

3.4.5 CONFOCAL LASER SCANNING MICROSCOPY

Confocal microscopy is an imaging technique usually applied to detect fluorescent markers. It uses a pinhole apparatus to eliminate out-of-focus signal. Fluorescence of a marker is excited at a focal point which is generated by a laser. The laser excites specific fluorophores only at special wavelengths thereby allowing the distinction of several markers within the same sample. The small but intensive excitation point is scanning over the sample in x-y and z directions thereby generating optical sections. This provides increased contrast and optical resolution of an image. Images are collected as stacks and reconstructed by a software as maximum projections or 3D-reconstructions. Possible disadvantages of confocal microscopy are the high costs compared to conventional light microscopy and possible problems when scanning thick tissues of specimen. As for all fluorescence microscopy techniques, another

downside is photobleaching and possible phototoxicity (Nwaneshiudu et al. 2012; Paddock 1999).

3.4.6 EMBEDDING IN EPOXY RESIN FOR HISTOLOGICAL STUDIES

In order to study the structure of samples below the resolution of a light/fluorescence microscope, thin to ultrathin sections need to be obtained. Such sections of less than 100 nm can be achieved by resin embedding which involves fixation steps, dehydration and resin polymerisation necessary for the later observation by EM or immuno-labelling.

4. AIM OF THE WORK

The aim of this thesis is to detect any ultrastructural changes in the cells under heavy metal stress with a focus on the cell wall of moss *Physcomitrium patens* (Funariaceae). We follow a hypothesis that some cell wall components are able to immobilise heavy metals and that the plants can protect themselves e.g., by increasing the amount of pectin within the cell wall. Specific pectin domains like demethylesterified homogalacturonan can be detected in the protonemata by immuno labelling-techniques and by cell wall thickening. Thus, the present thesis also gives an overview of some laboratory methods used for solving the cell biological questions above.

A total of 14 bryophyte species were cultivated on solid media in sterile culture. One of them, *P. patens,* grew also on the media spiked with copper and zinc solutions. The protonemata were preferred for this research for their single lined cells with a direct access and better contact with the medium.

5.MATERIALS AND METHODS

5.1 MOSS CULTIVATION

Moss transplants are grown on Petri plates filled with agar medium (see below) which provides all the necessary nutrients for the development. The moss is grown axenically meaning only one species of moss is present on the plate. The Petri plates are placed in a cultivator with programmed light intensity and temperature.

5.1.1 PREPARATION OF AGAR MEDIUM ($PpNH_4$)

The cultivation medium is prepared according to Bezanilla (2008) and a summary of ingredients is shown in Table 1. In brief, 400 ml of distilled water was added to 1 l sized glass beaker followed by 2 ml $MgSO_7H_20$ (500X) (SIGMA ALDRICH), 2 ml KH_2PO_4 (500X) (SIGMA ALDRICH), 2 ml $CaNO_3.4H_2O$ (500X) (SIGMA ALDRICH), 12,5 mg $FeSO_4.7H_20$ (AMRESCO 0387-500G), 1 ml Micro Elements (SIGMA ALDRICH), 2 ml Di-ammonium tartrate (500X) (SIGMA ALDRICH). The volume was brought up to 1 litre and mixed with a magnetic stirrer in the beaker. Then, 7 g of agar was weighed into five conical glass flasks (1,5 g each) and 200 ml of the prepared solution was added to each flask with agar. Metal spiked medium was prepared by adding 200 μl of 0,1 M $ZnSO_4$ or 0,1 M $CuSO_4$ into the prepared agar solution with a micropipette to reach the final concentration of 100 μmol. The flasks were loosely plugged with cotton wool, covered with aluminium foil and autoclave tape. The prepared flasks (Fig.4) were sterilised in the autoclave and later stored in the fridge. For moss cultures, the medium was heated in a microwave oven to liquify the agar solution and was poured warm into Petri dishes under strictly sterile conditions. The Petri dishes were sealed with parafilm and stored again in the fridge ready for moss cultivation.

Chemical substance	**Volume/Weight**
$MgSO_7H_20$ (500X) (SIGMA ALDRICH)	2 ml
KH_2PO_4 (500X) (SIGMA ALDRICH)	2 ml
$CaNO_3.4H_2O$ (500X) (SIGMA ALDRICH)	2 ml
$FeSO_4.7H_20$ (AMRESCO 0387-500G)	12,5 mg
Micro Elements (SIGMA ALDRICH)	1 ml
Di-ammonium tartrate (500X) (SIGMA ALDRICH)	2 ml
Agar (SIGMA ALDRICH)	7 g

Tab.1 Formula for preparation of 1000 ml of agar medium ($PpNH_4$)

Fig.4 Prepared agar medium in flasks before sterilisation

5.1.2 PROPAGATION AND GROWTH CONDITIONS

Small parts of 14 different moss species (Tab.2), all wild type, were placed in Petri dishes on solid control agar media under sterile conditions in the laminar flow cabinet. Only one species, *P. patens,* was cultivated, both, on control and on spiked medium with $ZnSO_4$ or $CuSO_4$. These propagated samples were grown in the cultivator (Conviron, Fig. 5) under constant temperature of 20 ˚C, humidity 60 %, day/night cycle 14 light/10 dark and monitored regularly. The moss plantlets of *P. patens* were propagated first after 3 weeks and later every week for a period of 2 months and they were later let grown in the cultivator for 7 months. The plantlets of other moss species were propagated for stock, checked and recorded regularly for a period of two months.

All the above-mentioned moss species were propagated with sterile forceps. Small parts of leafy gametophore and protonemata were distributed with sterile forceps on the solid agar both control and spiked with the heavy metal solutions, sealed with Parafilm® and labelled. Only one moss species, *P. patens,* was also propagated by grinding with a tissue homogeniser Omni TM (Fig. 6). Larger chunks of leafy gametophore and protonemata of various cultures of *P. patens* were homogenised in a glass tube together with 2 ml of sterilized, distilled water. 0,5 ml of smoothly homogenised mix was then applied on agar plates with a micropipette and left to grow. The plates were regularly checked and left to grow over a period of 7 months.

Species	Family
Anthoceros formosae	Anthocerotaceae
Atrichum undulatum	Polytrichaceace
Concocephalum conicum	Concocehalaceae
Entosthodon hungaricus	Funariaceae
Funaria hygrometrica	Funariaceae
Henediella heimii	Pottiaceae
Hypnum cupressiforme	Hypnaceae
Lophocolea minor	Lophocoleaceae
Marchantia polymorpha	Marchantiaceae
Mielichhoferia elongata	Melichhoferiaceae
Pohlia drummondii	Melichhoferiaceae
Physcomitrium patens	Funariaceae
Podperaea krylovii	Hypnaceae
Scapania umbrosa	Scapaniaceae

Tab.2 List of propagated bryophytes

Fig.5 Moss cultivator (Conviron)

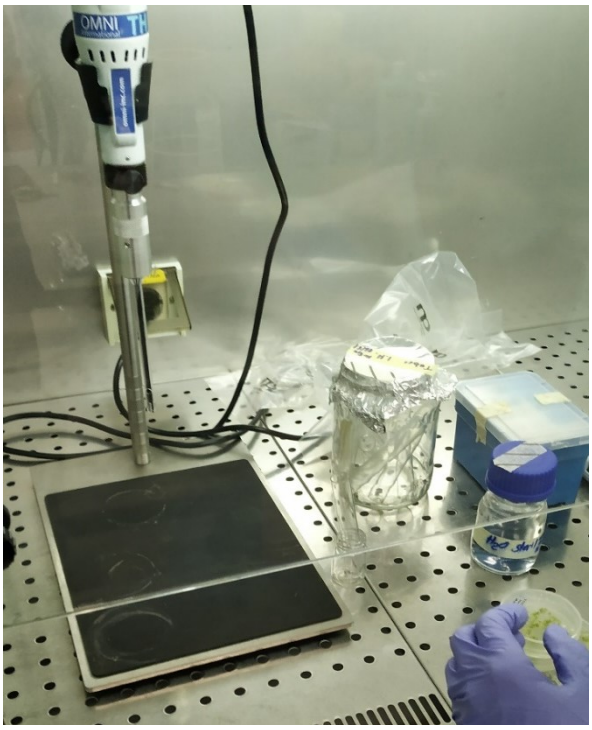


Fig.6 Tissue homogeniser setup

5.2 PREPARATION OF SEMITHIN SECTIONS FOR LIGHT MICROSCOPY

5.2.1 FIXATION PROTOCOL

First, 100 ml of 0.2 Mol buffer solution of sodium cacodylate ($C_2H_{12}AsNaO_5$) was prepared. To do this, 4,28 g of solid sodium cacodylate powder (AGAR SCIENTIFIC) was weighed in a glass bottle and diluted in 100 ml of distilled water. Next, 5% glutaraldehyde fixative ($C_5H_8O_2$) was prepared by diluting 25% glutaraldehyde solution (AGAR SCIENTIFIC) with distilled water. For both prepared solutions, sodium cacodylate and glutaraldehyde, pH was adjusted to 7.4 with NaOH or HCl (Fig. 7). Subsequently the solutions were mixed in the ratio 1:1. Fresh *P. patens* protonemata and were distributed into small glass veils with the use of forceps and immersed in the mixed fixative solution for 2 hours at room temperature. The veils were placed on the lab shaker. After 2 hours, the samples were washed 3 times in sodium cacodylate buffer which had been prepared from the original buffer solution by diluting it with distilled water in the ratio 1:1. The washing time intervals were 3 minutes, 5 minutes and 10 minutes while left on the lab shaker. The buffer was sucked out of the veils with a plastic pipette and disposed in the waste jar. All the steps were carried out in a fume hood. The unused solutions were labelled and placed in the fridge where they could have been stored for up to one month.

Fig.7 pH meter set up for calibrating pH of the fixative

5.2.2 OSMIFICATION

1,5 ml of osmium solution (H_8O_4Os) was added to the washed samples in the glass veils. They were then stored in the fridge overnight at 6 *°C.* In the morning, the samples were washed twice in distilled water for 10 minutes. All the waste from the osmium tetrahydrate solution had to be disposed in special waste containers.

5.2.3 DEHYDRATION

The samples were rinsed in graded concentration series of ethanol and acetone (Tab.3).

Concentration (percent)	Time (minutes)
30% Ethanol	5
30% Ethanol	10
70% Ethanol	3
70% Ethanol	5
100% Ethanol	3
100% Ethanol	5
100% Ethanol	7
100% Acetone	3
100% Acetone	5
100% Acetone	7

Tab.3 Dehydration steps with ethanol and acetone at room temperature

5.2.4 RESIN EMBEDDING

All the components of the resin were weighed in a plastic beaker and mixed with a magnetic stirrer. 6 ml of 100 % acetone was pipetted into a Greiner tube and subsequently 2 ml of the prepared resin was added in and vortexed on the mixer. The fixed protonemata were placed into the mixture in the Eppendorf tube for 60 minutes. After the treatment, the resin and acetone mixture were sucked out with a plastic pipette. This step was followed by another treatment in 3 ml resin and 3 ml 100 % acetone mix and the same protonemata were placed in the Eppendorf tube again for 60 minutes. Prior to the final embedding, the fixed protonemata were investigated under a stereo microscope and placed into an embedding mould filled with Agar Low Viscosity Resin (AGAR SCIENTIFIC). The filled embedding mould was put into the oven at temperature 60 °*C* for 3 days for polymerisation.

5.2.5 SECTIONNING OF THE SEMITHIN SECTIONS

The resin blocks were cut with the microtome (Leica EM UC7) using glass knives. The semithin sections 0,5 μm and 1 μm were taken from the knife with a special hair or a loop, placed in a water droplet on a glass slide, and dried on the heating plate at 80°C.

5.2.6 STAINING AND POLYMERISATION OF THE SEMITHIN SECTIONS

The dried sections were stained with a 0.1 % toluidine blue in 2.5 % borax solution for about 30 seconds and rinsed with ddH2O. The stained sections were inspected under the light microscope to check the quality of the staining and the position of the sections. Then, a small drop of Agar Low Viscosity Resin (AGAR SCIENTIFIC) was applied on the dried and stained sections. A coverslip was put on and the samples placed in the oven at 60 °*C* for 3 days.

5.3 PREPARATION AND IMMUNOFLUORESCENCE STAINING OF GAMETOPHORES

5.3.1 FIXATION

Plantlets of *P. patens* were placed into Eppendorf tubes and fixed in 1 ml of 4 % Paraformaldehyde (PFA) in PM5E buffer (here, include the chemicals and concentrations of this buffer) for 60 minutes. After the fixation, the plantlets were rinsed in PM5E buffer three times for 5 minutes each.

5.3.2 CELL WALL DIGESTION

For cell wall digestion, the samples were transferred into a solution of 1% cellulase and 1% driselase in PM5E buffer for 60 minutes. After this enzyme treatment, the plantlets were again rinsed in PM5E buffer 3 times, for 5 minutes.

5.3.3 MOUNTING ON COVERSLIPS AND PERMEABILISATION WITH TRITON X

Clean coverslips were coated with Poly-L-lysine and left to dry for a few minutes. The plantlets were carefully placed on these coverslips and left until almost completely dry so that they stick to the coverslip. Then, membrane permeabilization was done with Triton X 0,2% (SIGMA ALDRICH) in PM5E buffer for 40 minutes. Later, Triton X solution was drained, and the samples rinsed with PM5E buffer. The plantlets were covered with Phosphate Buffered Saline (PBS) solution for 10 minutes.

5.3.4 APPLICATION OF PRIMARY ANTIBODIES

After carefully draining the PBS solution, primary antibodies were applied. We used monoclonal antibodies to homogalacturonan JIM 7 and LM 20 (PlantProbes, http://www.plantprobes.net/index.php) which can recognise only methyl esters but not un-esterified homogalacturonan. There were some coverslips prepared as controls without any primary antibodies. The samples on the coverslips were placed in a Petri dish with moist tissue to prevent drying (“moist chamber”) and incubated over night at room temperature.

5.3.5 APPLICATION OF SECONDARY ANTIBODIES

The following morning, all the coverslips were rinsed in PBS 3 times for 5 minutes. The secondary antibody was applied, and the samples placed in the moist chamber (Petri dish) and covered with tin foil. We used a Goat Anti-rat antibody and Rabbit Anti-mouse antibody (SIGMA ALDRICH) with a FITC-tag. Some samples did not receive secondary antibody to serve as controls. The Petri dish was placed in the incubator at 37 °C for 2 hours. The samples were rinsed in PBS again for 10 min and later 3 times in distilled water.

5.4 MICROSCOPY

5.4.1 SAMPLE INVESTIGATION WITH LIGHT AND CONFOCAL MICROSCOPE

The semithin sections were investigated with a light microscope (Nikon Eclipse Ni; Fig.8) using 20x, 40x, 100x oil immersion lens. Pictures were taken with a digital camera (Nikon) and software (NIS elements).

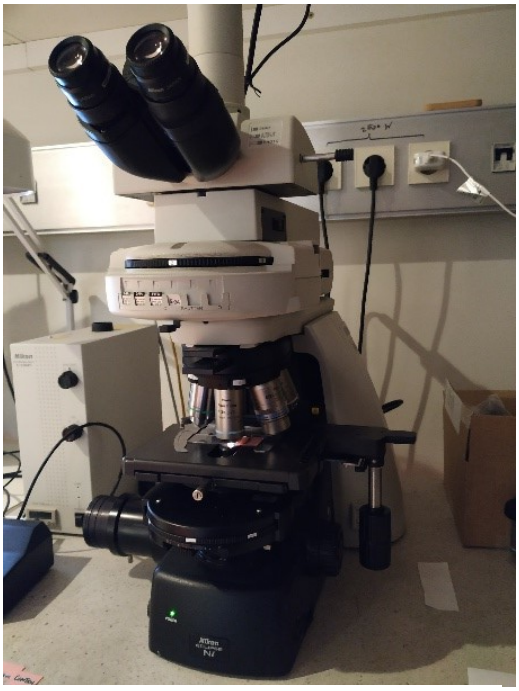


Fig.8 Light microscope Nikon Eclipse Ni.

Then the samples were placed on glass slides with a drop of water and observed in a confocal laser scanning microscope (Leica TCS SP5 X) using the 40x water immersion lens (Fig.9).

5.4.2 SETTINGS OF CONFOCAL MICROSCOPE

We used recommended settings for optimal detection of the FITC signal. In brief, the argon laser was at 488 nm (excitation wavelength), set at 30%. The emission wavelength was set to collect the FITC signal at 530nm. The transmitted light detector was also selected to collect a corresponding bright field image.

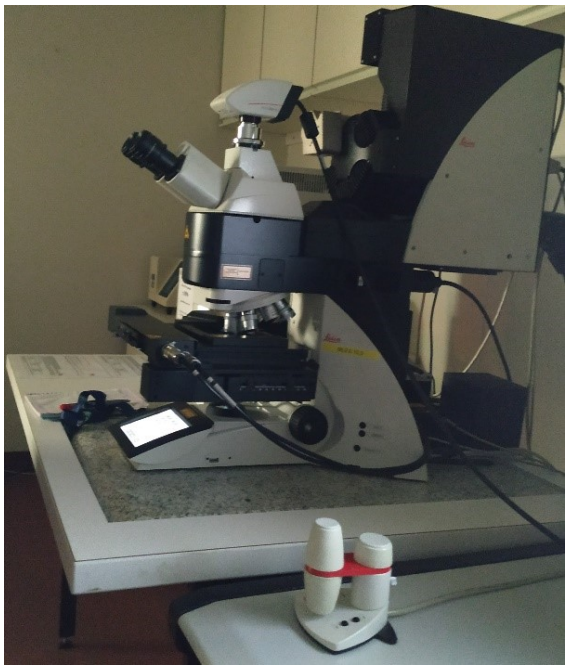


Fig.9 Confocal laser scanning microscope Leica TCS SP5 X

5.5 LIMITATIONS OF THIS WORK

Due to time and lab access restrictions caused by the COV-19 pandemic, the samples growth pattern could not be followed and recorded for longer time periods. Also, immunolabelling was not performed as planned. We intended to investigate both types of homogalacturonan, un-esterified and methyl esterified, grown on control and metal media but only managed to analyse some samples with primary antibodies detecting methyl esterified homogalacturonan. Similarly, we carried out microtome slicing of the control samples only although resin blocks were prepared from all the samples grown on the metal media.

6. RESULTS

6.1 CULTIVATION OF 14 MOSS SPECIES GROWN ON CONTROL MEDIA

To learn propagation techniques and overall handling of mosses, preparation of the growth media and their sterilisation, we started with cultivation of 14 different bryophyte species. Later in the study, we focused mainly on *P. patens* where we investigated how heavy metal stress affects pectin in the cell wall.

14 different bryophyte species were re-cultivated, and their growth pattern followed over tíme. The selected species were from the three big bryophyte groups: hornworths (*Anthoceros formosae*), liverworths (*Conocephalum conicum*, *Lophocolea minor, Marchantia polymorpha, Scapania umbrosa*) and mosses (*Atrichum undulatum, Enthostodon hungaricus, Funaria hygrometrica, Henediella heimii, Hypnum cupressiforme, Mielichhoferia elongata, Physcomitrium patens, Podperaea krylovii, Pohlia durmmondii*). The results were recorded for moss plantlets of similar age (between 2-3 weeks) cultivated on control media. In general, the growth pattern was similar for all 14 species (Fig.10 – Fig.23), however *Enthostodon hungaricus*, *Funaria hygrometrica* and *Physcomitrium patens* (Fig.13, Fig.14, Fig.20) formed most protonemata, larger colonies and they did not develop infection like other moss species that got repeatedly contaminated after recultivations.

Anthoceros formosae, Lophocolea minor and *Mielichhoferia elongata* were the most difficult mosses to establish in culture. Even after repeated cultivation, the mosses did not prosper. They formed only little shoots and got reinfected (Fig.10, Fig.17, Fig.19).

A lower rate of protonemata formation was also detected in *Henediella heimii* yet this moss formed a colony with higher diameter but as other mosses it was heavily contaminated (Fig.15).

A general growth pattern could not be linked confidently to one of the major bryophyte groups however three species of bryophyta (*Enthostodon hungaricus*, *Funaria hygrometrica* and *Physcomitrium patens*) were the most resilient and produced higher yield than other bryophyte groups. To follow a general growth pattern on a bryophyte group level it would be necessary to cultivate more species in liverworts and hornworts groups.

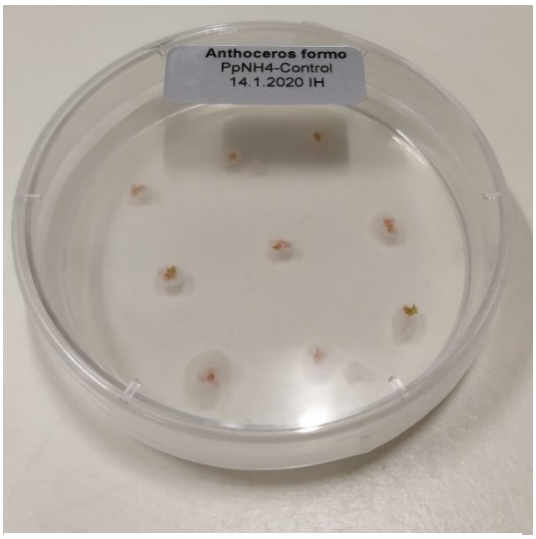


Fig.10 *Anthoceros formosae*

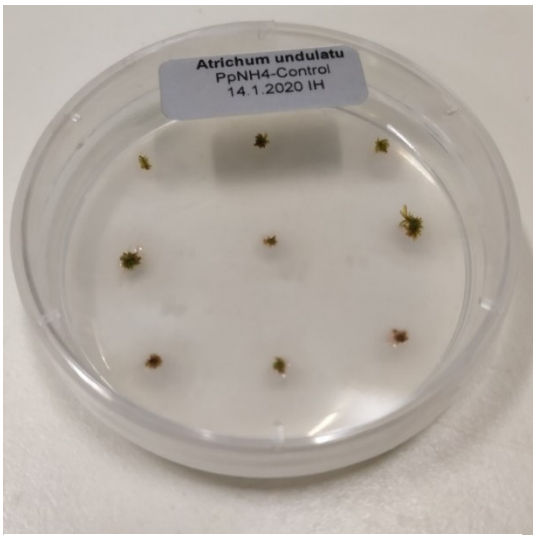


Fig.11 *Atrichum undulatum*

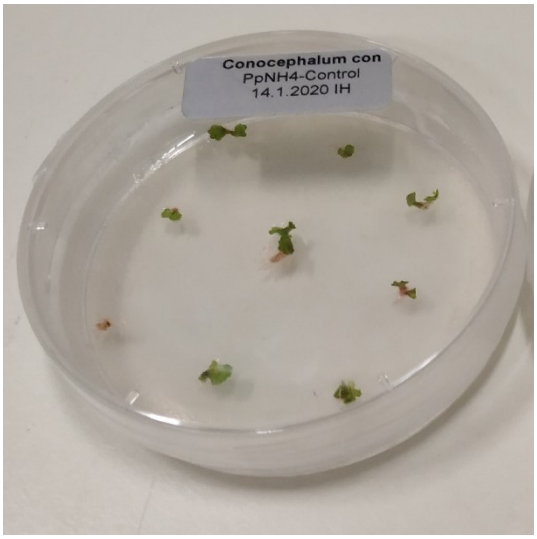


Fig.12 *Conocephalum conicum*

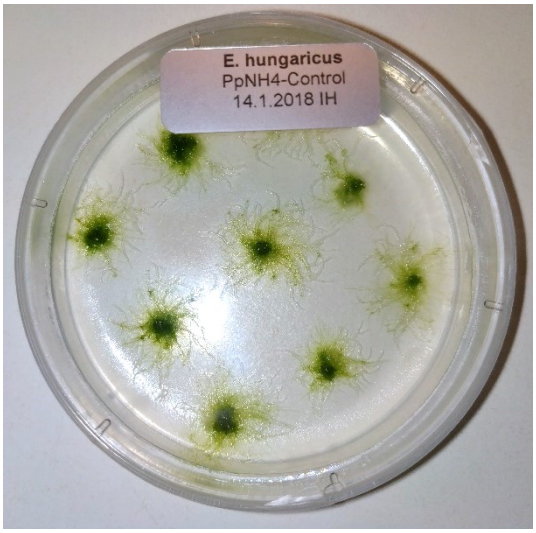


Fig.13 *Enthostodon hungaricus*

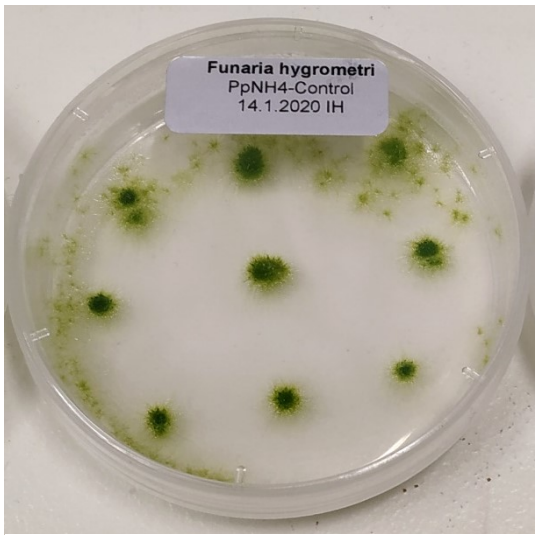


Fig.14 *Funaria hygrometrica*

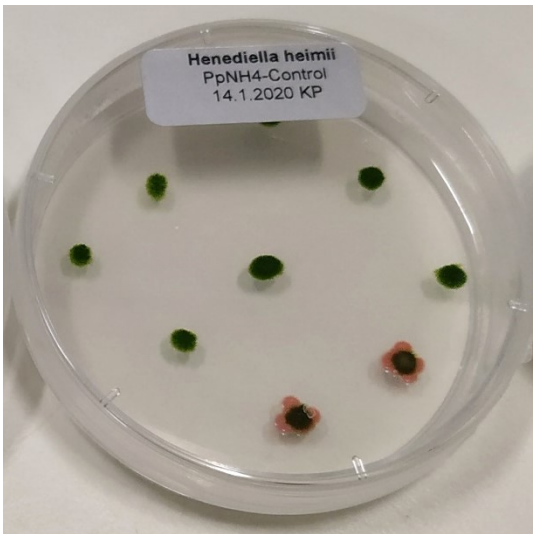


Fig.15 *Henediella heimii*

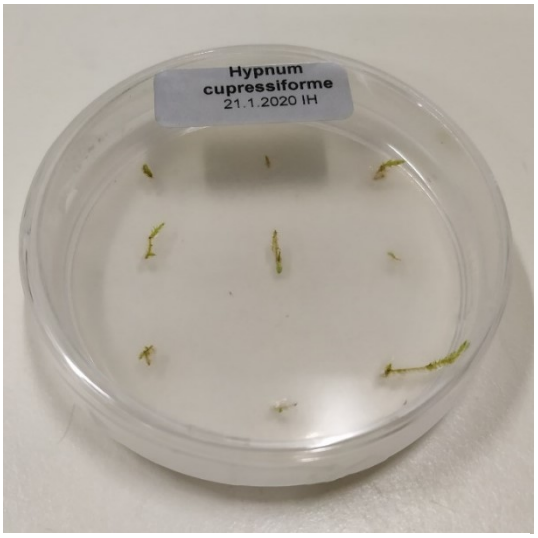


Fig.16 *Hypnum cupressiforme*

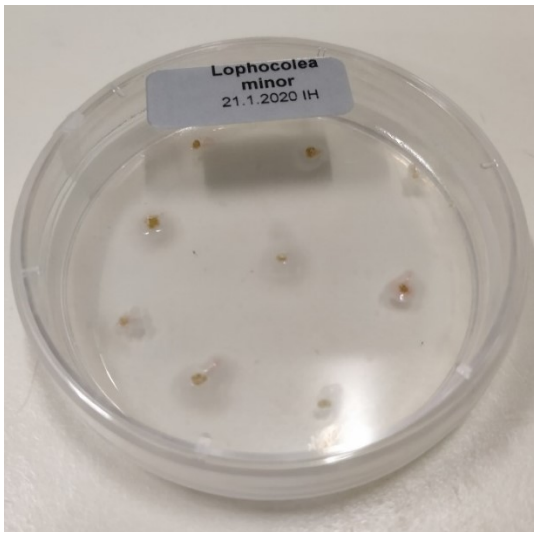


Fig.17 *Lophocolea minor*

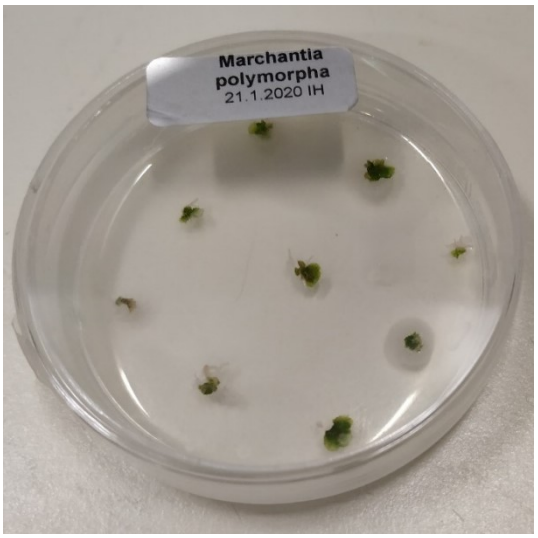


Fig.18 *Marchantia polymorha*

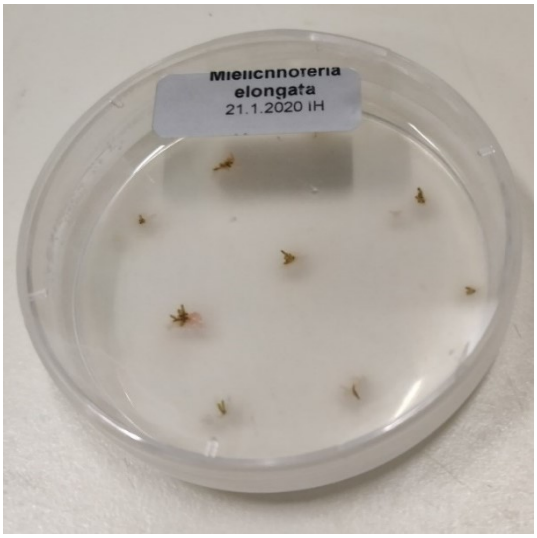


Fig.19 *Mielichhoferia elongata*

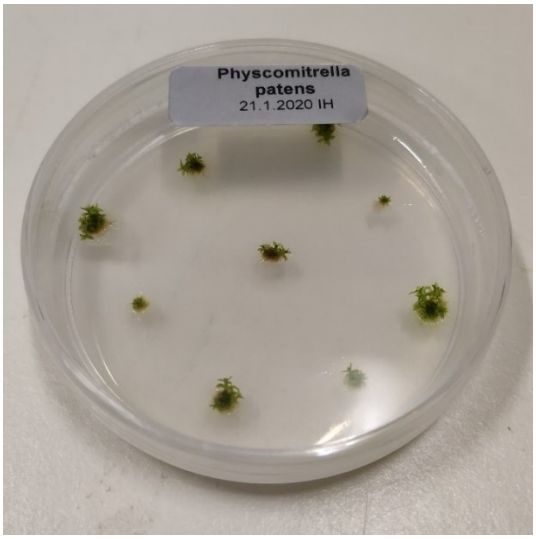


Fig.20 *Physcomitrium patens*

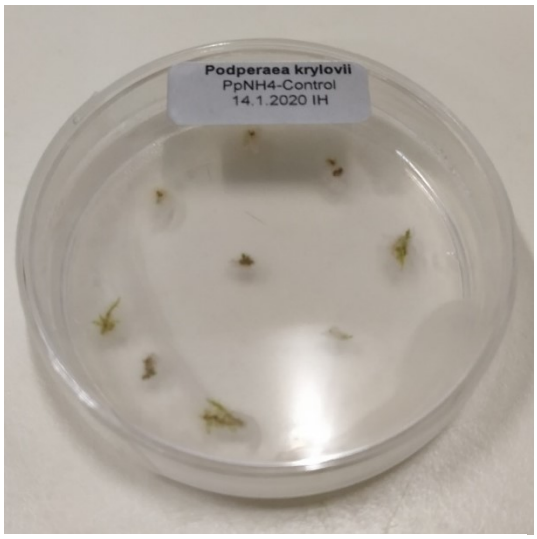


Fig.21 *Podperaea krylovii*

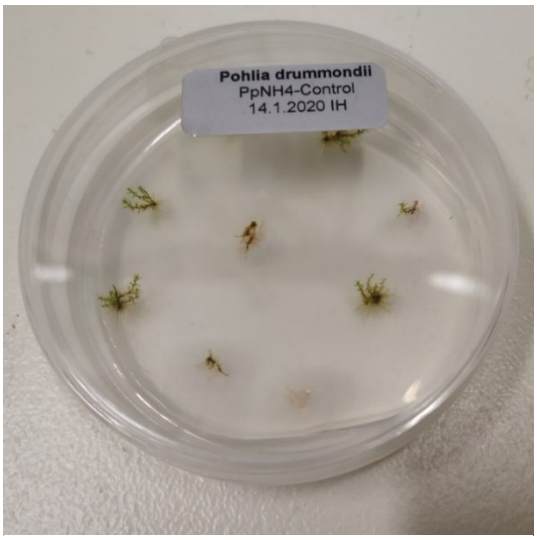


Fig.22 *Pohlia durmmondii*

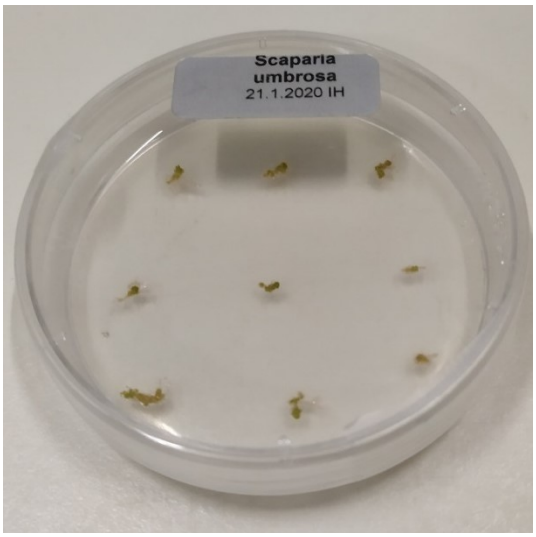


Fig.23 *Scapania umbrosa*

6.2 *P. PATENS* CULTIVATION BY TRANSPLANTS

The difference in the growth pattern between the plantlets of *P. patens* grown on $ZnSO_4$ and $CuSO_4$ media can be detected visually after 2 weeks (Fig.24, Fig.25, Graph 1) when the plantlets grown on $ZnSO_4$ media started producing more mass compared to the plantlets of the same age grown on $CuSO_4$ media and continued gaining mass gradually up until 5 months (Fig.30). The yield of the plantlets grown on $CuSO_4$ media was also gradual but only up until third week (Fig.24 - Fig.27, Graph 1). After that the plantlets did not change the yield distinctively.

The difference in discolouration could be detected after 3 weeks when the plantlets grown on $CuSO_4$ media started gaining brownish discolouration (Fig.27) and after 5 months they turned darker brown (Fig.31). The plantlets grown on $ZnSO_4$ media kept the same colour throughout the whole experiment same as the plantlets grown on the control media (Fig.32, Fig.34).

The control plantlets also gained the mass gradually. After 5 months some gametophores started losing chlorophyll and became “bleached” (Fig.33). This did not occur with the plantlets grown on heavy metal media. Some control gametophores became contaminated (Fig.34).

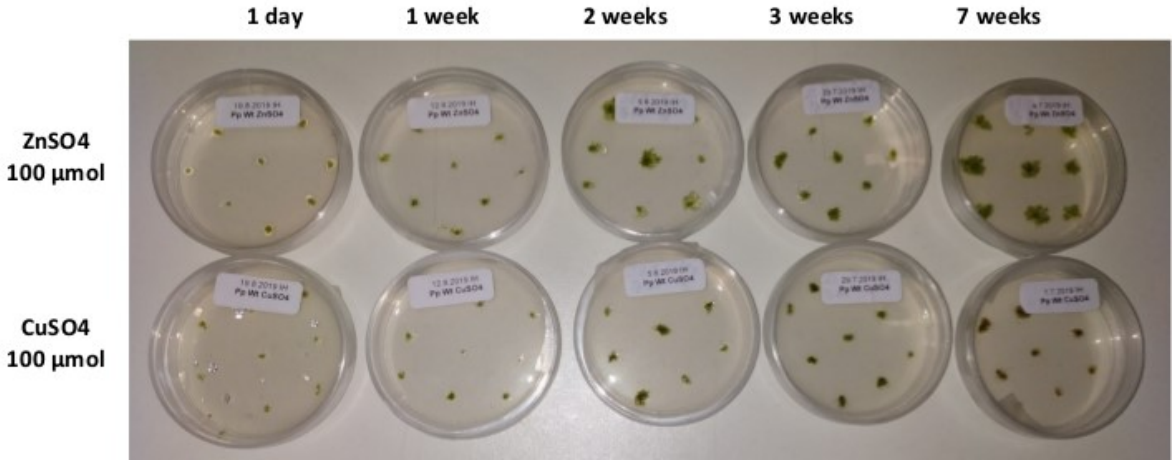


Fig.24 Series of *P.patens* plantlets grown on $ZnSO_4$ 100 µmol and $CuSO_4$ 100 µmol after 7 weeks, 3 weeks, 2 weeks, 1 week and 1 day (from right to left)

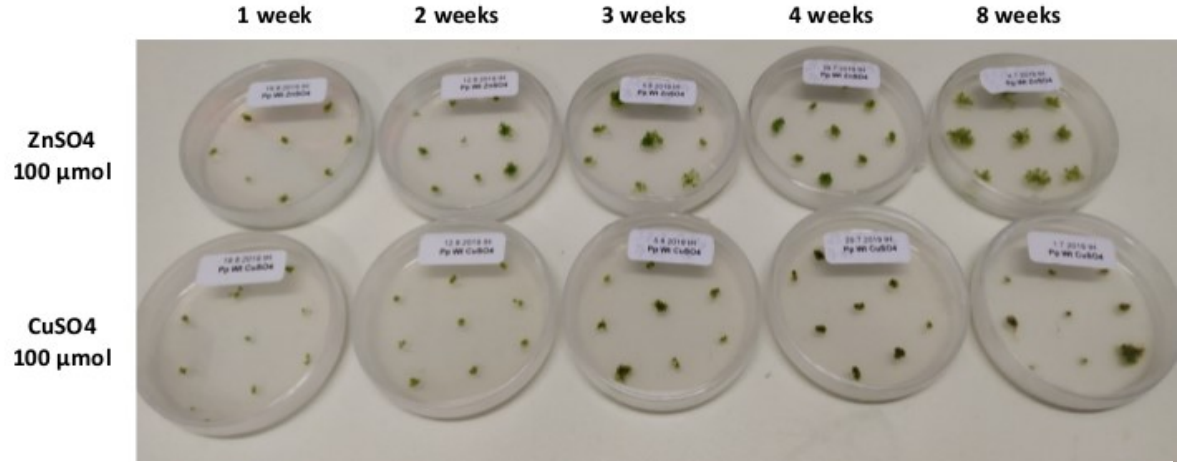


Fig.25 Series of *P.patens* plantlets grown on $ZnSO_4$ 100 µmol and $CuSO_4$ 100 µmol after 8 weeks, 4 weeks, 3 weeks, 2 weeks and 1 week (from right to left)

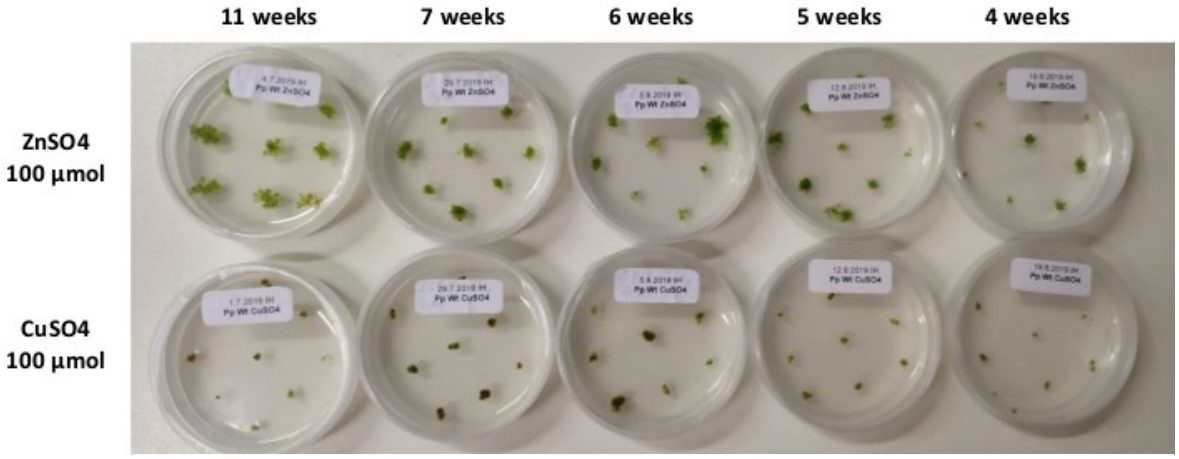


Fig.26 Series of *P.patens* plantlets grown on $ZnSO_4$ 100 µmol and $CuSO_4$ 100 µmol after 11 weeks, 7 weeks, 6 weeks, 5 weeks and 4 weeks (from left to right)

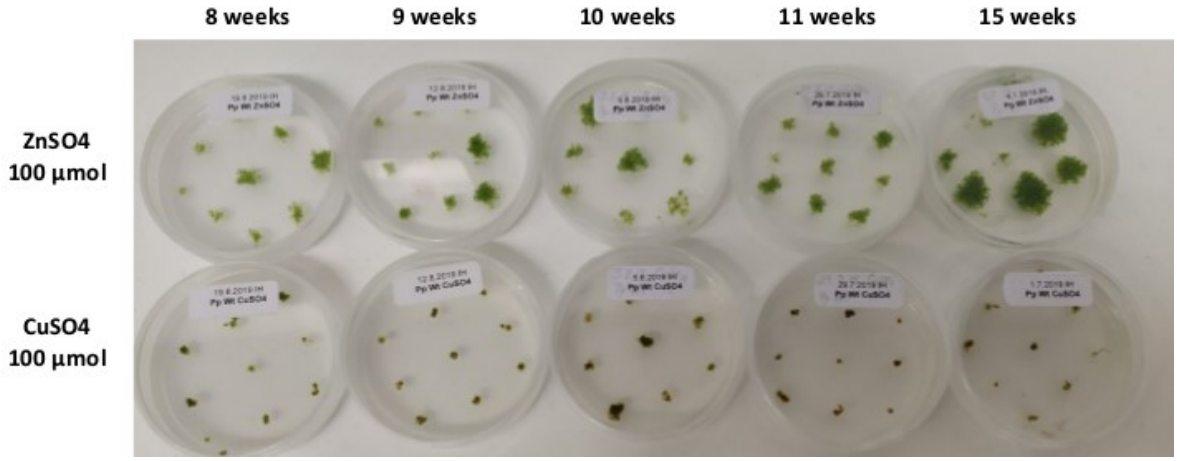
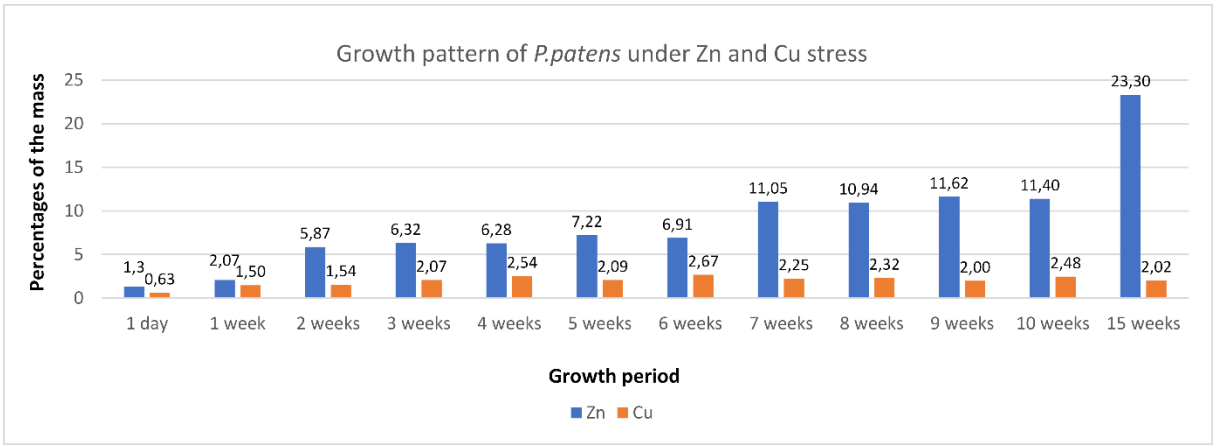


Fig.27 Series of *P. patens* plantlets grown on $ZnSO_4$ 100 µmol and $CuSO_4$ 100 µmol after 15 weeks, 11 weeks, 10 weeks, 9 weeks and 8 weeks (from left to right)



Graph 1 Growth pattern of *P.patens* grown on agar medium spiked with $CuSO_4$ and $ZnSO_4$ (100 µmol) over period of 15 weeks

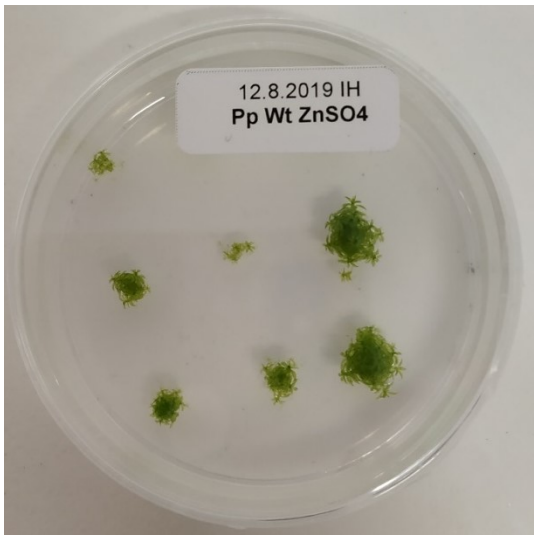


Fig.28 *P. patens* grown on media spiked with $ZnSO_4$ 100 µmol after 2 months

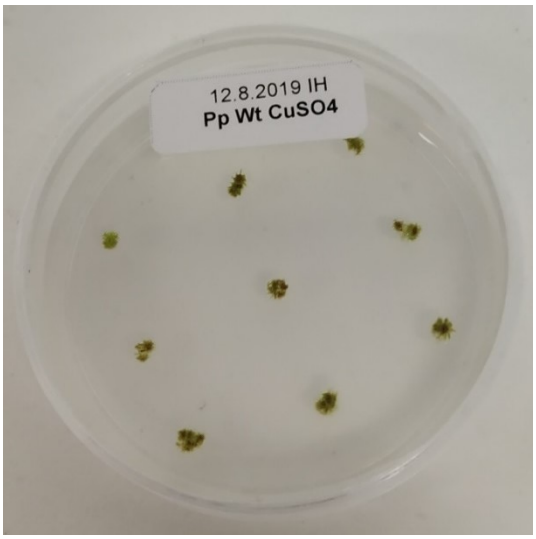


Fig.29 *P. patens* grown on media spiked with $CuSO_4$ 100 µmol after 2 months

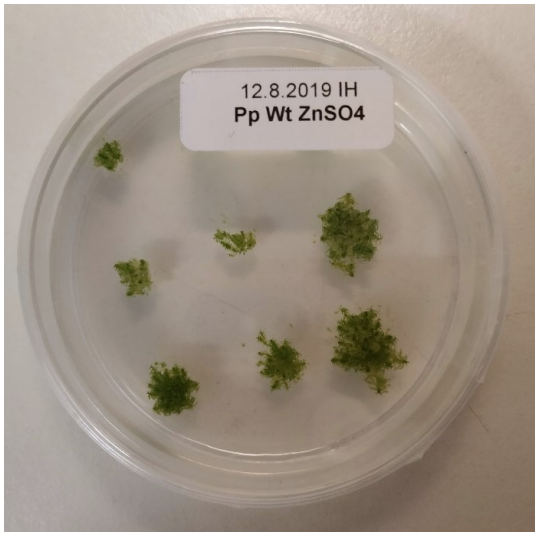


Fig.30 *P. patens* grown on media spiked with $ZnSO_4$ 100 µmol after 5 months

Fig.31 *P. patens* grown on media spiked with $CuSO_4$ 100 µmol after 5 months

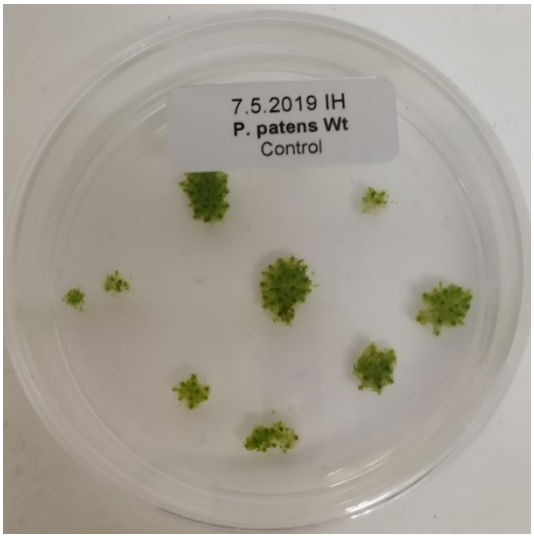


Fig.32 *P. patens* plantlets grown on control media after 5 months

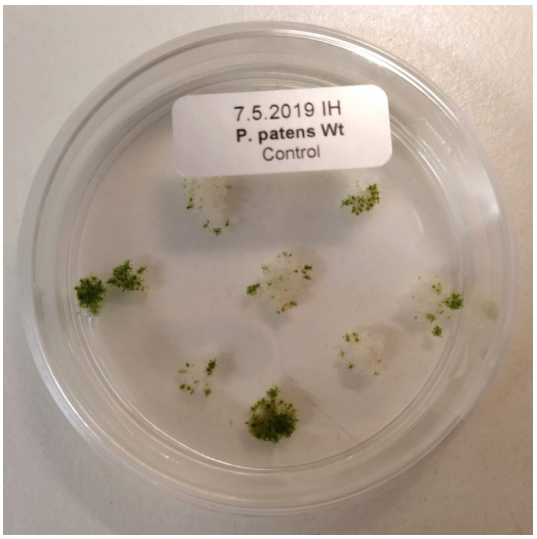


Fig.33 *P. patens* plantlets grown on control media after 7 months

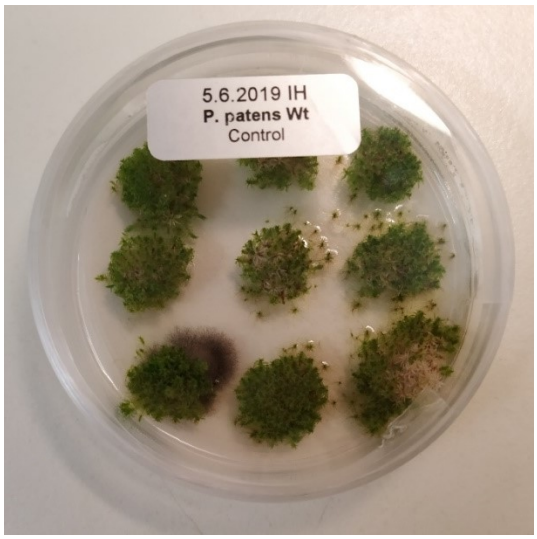


Fig.34 *P.patens* plantlets grown on control media after 8 months

6.3 *P. PATENS* CULTIVATION USING HOMOGENISED PLANTLETS

This cultivation technique was part of the experiment in order to grow and harvest more protonemata for microscope investigation, however under our laboratory conditions and choice of media only very little protonemata were formed and development shifted towards shoot growth.

Homogenised moss fragments could be detected on the media after one week, especially on $ZnSO_4$ media and control media. There was hardly any growth visible on $CuSO_4$ media (Fig. 35). At six weeks the growth trend changed in favour of the moss grown on $ZnSO_4$ media where the moss started forming green dense shoots compared to the moss grown on the control media. There was not much additional growth of the moss on $CuSO_4$ media. Interestingly, mainly the moss shoots differentiated from the moss fragments and almost no protonemata were visible at this stage (Fig.36). The same observation was recorded after 10 weeks (Fig.37). After 28 weeks the shoots are most developed on control media with only a little protonemata formed. The shoots are also visible on $ZnSO_4$ media where they cover a whole surface in the Petri dish. Again, the protonemata grew only a little. The moss fragments on $CuSO_4$ manifested a poor growth and brownish discoloration (Fig.38). The same was recorded at 30 weeks (Fig.39).

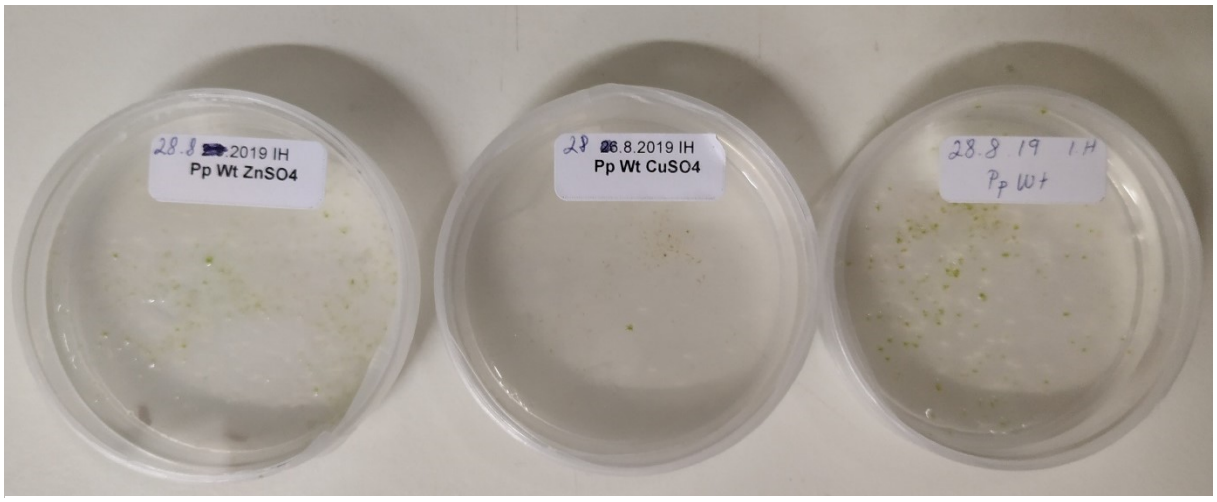


Fig.35 Homogenised tissue of *P. patens* grown on $ZnSO_4$ 100 µmol (left), $CuSO_4$ 100 µmol (middle) and control (right) plates after 1 week

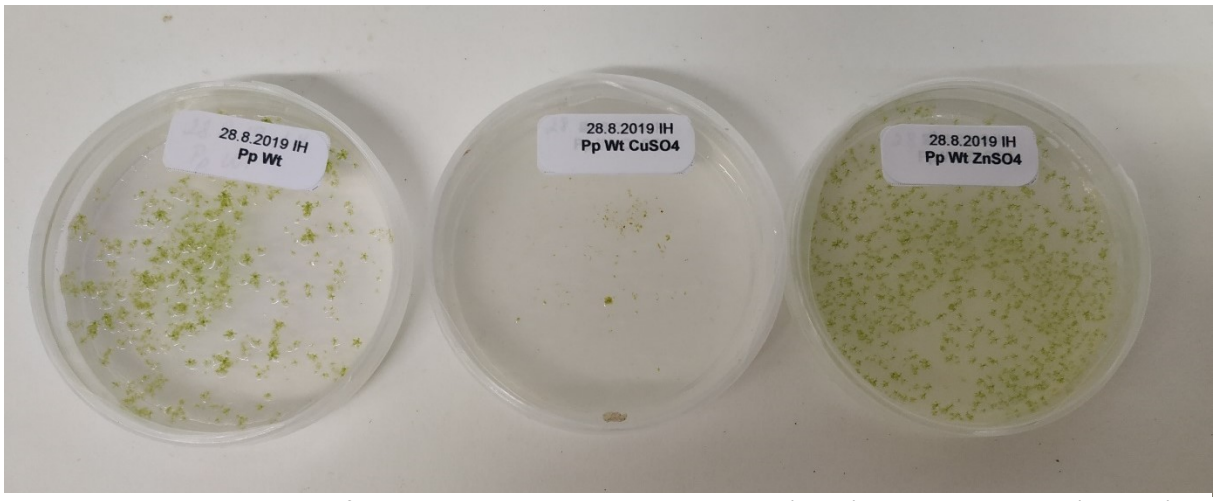


Fig.36 Homogenised tissue of *P.patens* grown on $ZnSO_4$ 100 µmol (right), $CuSO_4$ 100 µmol (middle) and control (left) plates after 6 weeks

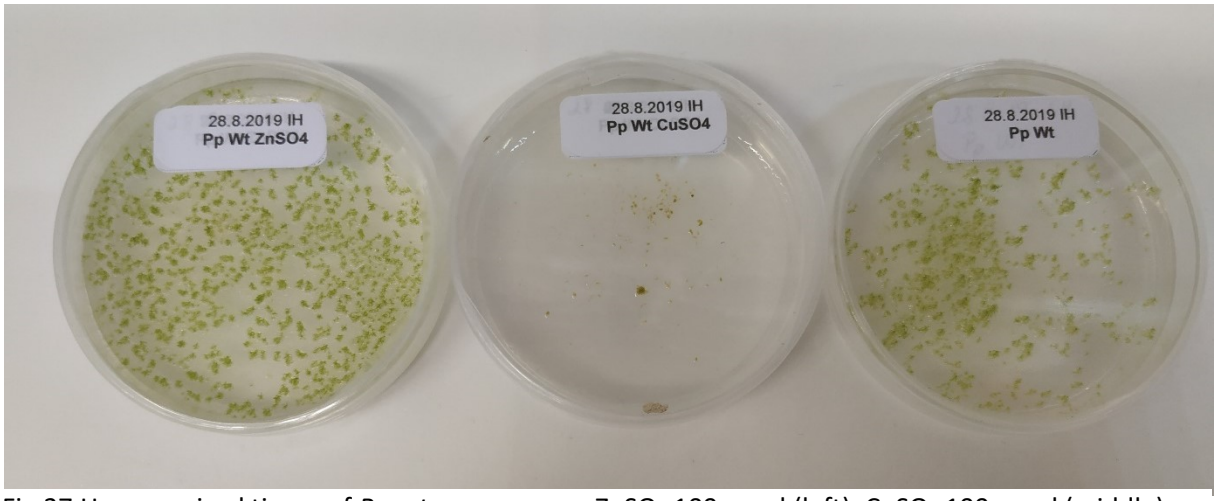


Fig.37 Homogenised tissue of *P. patens* grown on $ZnSO_4$ 100 µmol (left), $CuSO_4$ 100 µmol (middle) and control (right) plates after 10 weeks

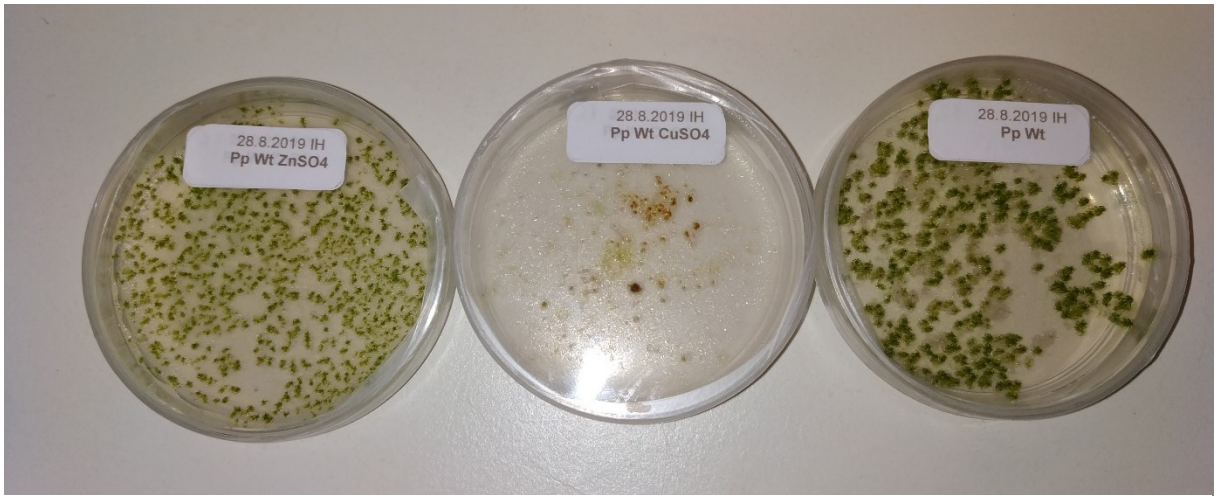


Fig.38 Homogenised tissue of *P. patens* grown on $ZnSO_4$ 100 µmol (left), $CuSO_4$ 100 µmol (middle) and control (right) plates after 28 weeks

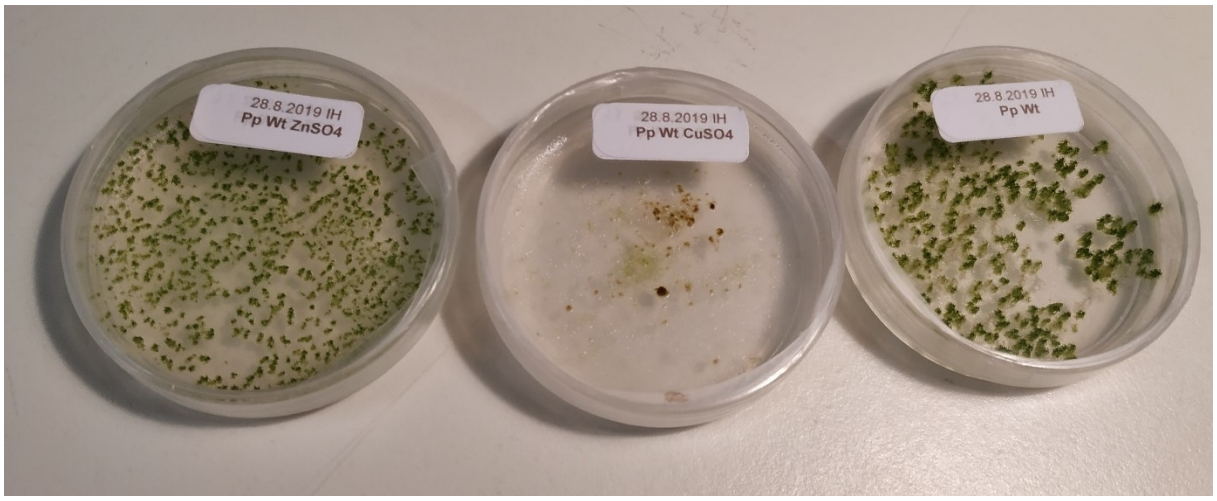


Fig.39 Homogenised tissue of *P. patens* grown on $ZnSO_4$ 100 µmol (left), $CuSO_4$ 100 µmol (middle) and control (right) plates after 30 weeks

6.4 LIGHT MICROSCOPY

Cell wall gives mosses ability to withstand some heavy metal stress. Homogalacturonan, a component of pectin in the cell wall can form complexes with heavy metals and thus prevent them to enter inner cells. Our hypothesis is that copper and zinc grown mosses develop a thicker pectinous cell wall for metal protection.

To address this question, we had to get high resolution images of the cell wall. Therefore, semi thin sections of the moss samples were performed and analysed by light microscopy.

We used protonemata tissue for its direct contact with the medium and even penetration by agar low viscosity resin during embedding. For embedding, we used protonemata grown on copper and zinc metals, both 3 weeks and 6 weeks old and protonemata grown on control medium that were 6 months old. Though semithin sections were planned for protonemata grown on metal and control media only the control samples were sectioned.

The process of embedding consists of several steps. Firstly, protonemata needed to be fixed with 5% glutaraldehyde fixative in 0,2 Mol buffer solution of sodium cacodylate to retain its ultrastructure. Then, we immersed the samples in solution of osmium tetroxide to preserve lipids in membranes. This step was followed by dehydration in graded concentration series of ethanol and acetone. Dehydrated samples were embedded in agar low viscosity resin poured in plastic moulds and placed in the oven for polymerisation. After three days the hard polymerised blocks were ready for sectioning. As mentioned above, only the control samples were sectioned by microtome using glass knives. We cut sections 0,5 µm thin and stained the

cells walls with toluidine blue on a glass slide. Later we put a drop of agar low viscosity resin on the sections covered with a glass and put it in the oven to polymerise. The prepared sections provided data about the thickness of the cell wall of the *P. patens* protonemata. The cell wall highlighted purple by toluidine blue can be easily distinguished under a light microscope (Fig.40 – Fig.43). Even the highest possible magnification of 100x produced a slightly blurred image with no sharp boundaries of the cell wall and other organelles, like chloroplasts (Fig.41, Fig.42, Fig.43). There are also visible creases possibly caused by resin slicing and sample preparation.

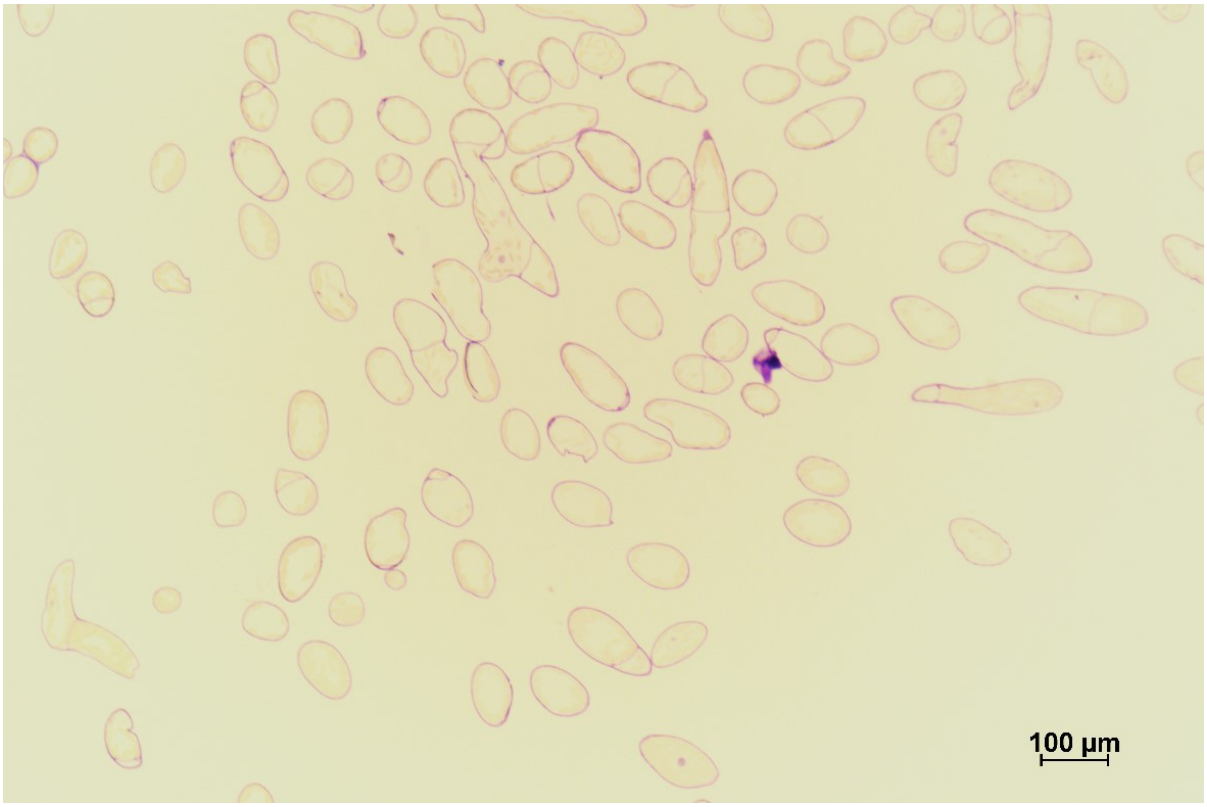


Fig.40 Microtome resin cross-section of *P. patens* protonemata grown on control agar media

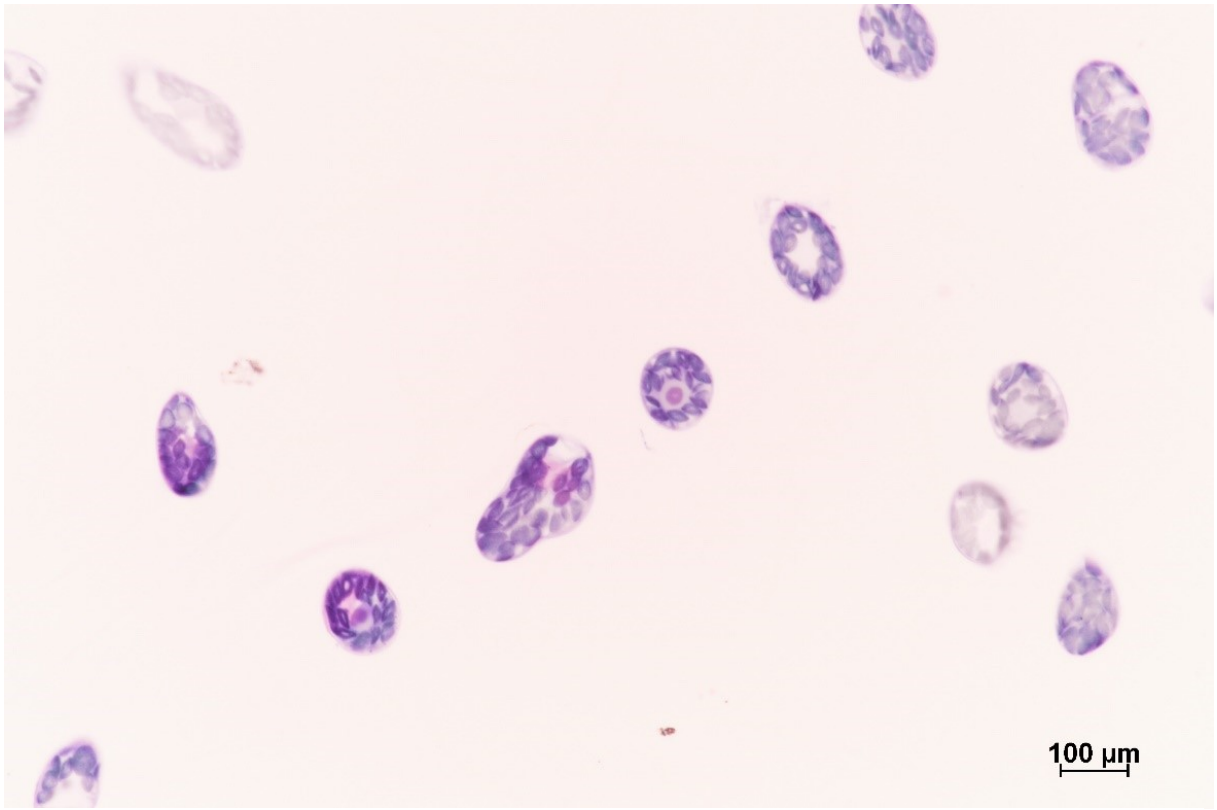


Fig.41 Microtome resin cross-section of *P. patens* protonemata grown on control agar media

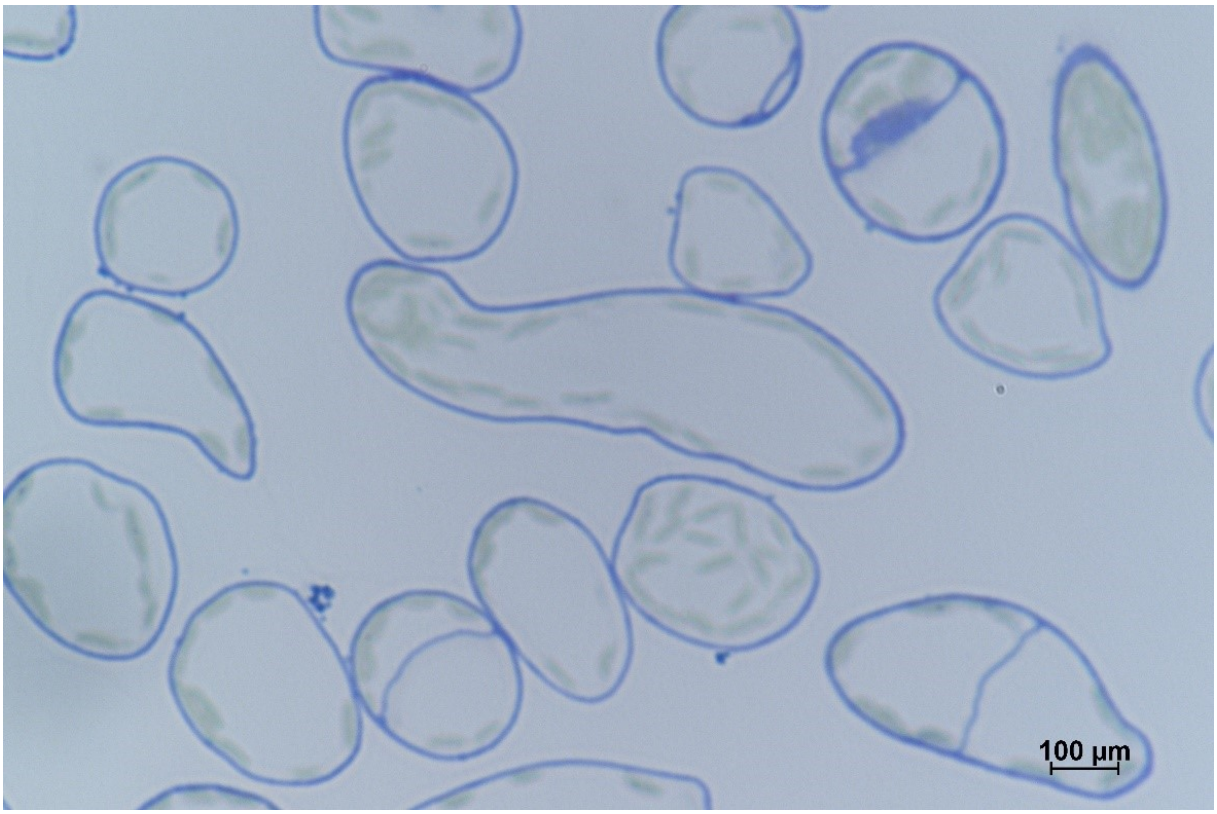


Fig.42 Microtome resin cross-section of *P. patens* protonemata grown on control agar media

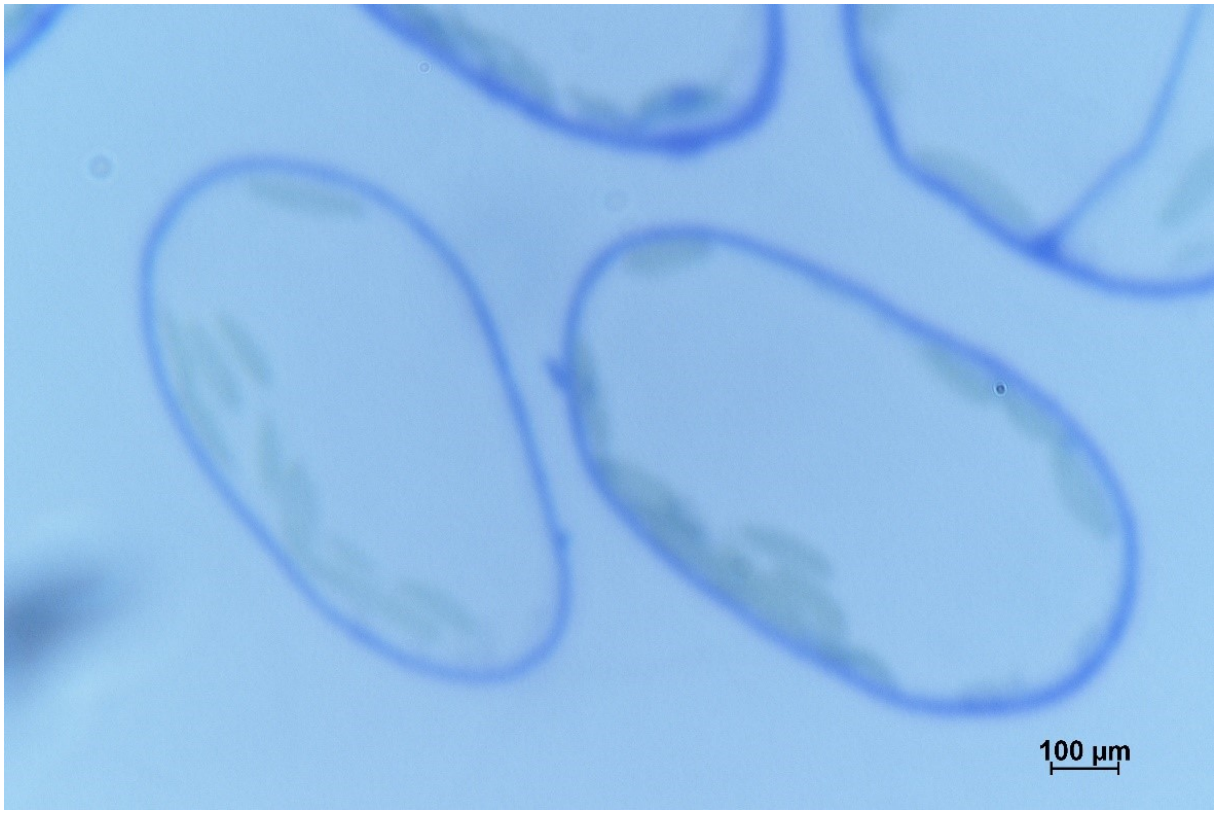


Fig.43 Microtome resin cross-section of *P. patens* protonemata grown on control agar media

6.5 IMMUNOLABELLING

We suspect that homogalacturonan, a component of pectin, changes cell wall architecture in response to heavy metal stress. Two types of homogalacturonan can be detected by anti-pectic polysacharide antibodies, un-esterified and methyl esterified. The main goal of our study was to detect homogalacturonan its localisation and amount in the tissue of *P.patens* under heavy metal stress.

Immunolabelling process consisted of several steps. First, the cells were fixed with paraformaldehyde fixative in PM5 and washed afterwards. To get larger molecules of antibodies into the cell it was necessary to remove lipids from cell membranes which was achieved by a permeabilisation detergent Triton X. Again, washing samples after this treatment was required. Primary antibodies were applied on the drained samples positioned on cover slips. They were let incubated over night followed by application of secondary antibodies that were covered with tin foil due to high light sensitivity.

The primary antibodies JIM7 and LM20 used in the study detected methyl esterified homogalacturonan in the cell walls of protonemata and leaves of *P.patens*. Secondary antibodies used in the study were Rabbit Anti-Mouse and Goat Anti-Rat labelled with FITC tag.

6.5.1 IMMUNOLABELLING WITH PRIMARY ANTIBODY LM 20

A methyl esterified homoglacturonan in the leaf of the control sample detected by antibody LM20 formed higher intensity spots with fairly even distribution in the cell walls or at places where two cells connected (Fig.44, Fig.45) compared to the homogalacturonan in the leaf of $ZnSO_4$ sample labelled with the same primary antibody where it was located mainly at the tip of the leaf (Fig.47). More experiments are needed to find out if heavy metal environment affects only tips of leaves or other tissues as well. The pectin was also detected in the cell walls of protonemata where it is distributed over the entire cell (Fig.46).

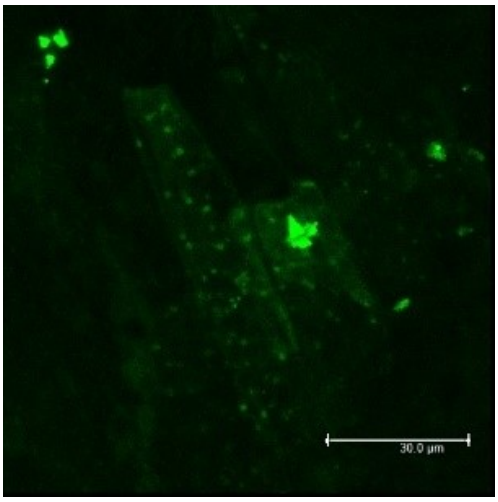


Fig.44 Leaf of *P. patens*, control sample. Antibodies LM20/FITC

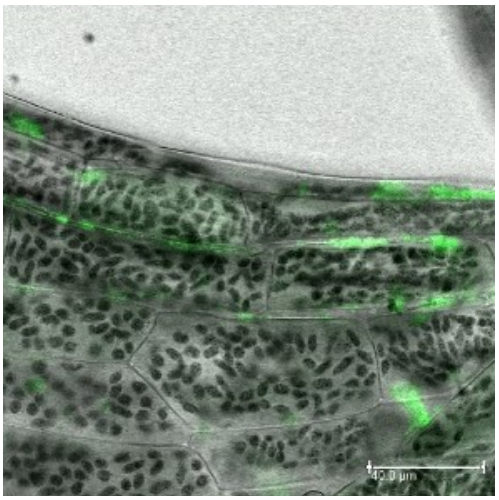


Fig.45 Leaf of *P. patens*, control sample. Antibodies LM20/FITC

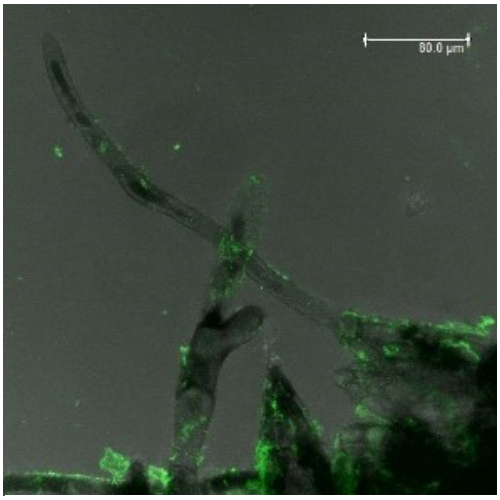


Fig.46 Protonemata of *P. patens*, control sample. Antibodies LM20/FITC

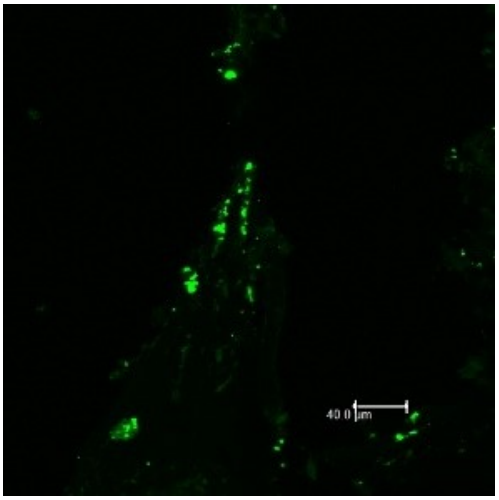


Fig.47 Leaf tip of *P. patens*, $ZnSO_4$ sample. Antibodies LM20/FITC

6.5.2 IMMUNOLABELLING WITH PRIMARY ANTIBODY JIM 7

An even distribution of pectin in the cell walls of protonemata was also detected by using a primary antibody JIM 7 (Fig.46). This antibody gave strong signal in the leaf tissue but with signal patches remaining even after the washing process.

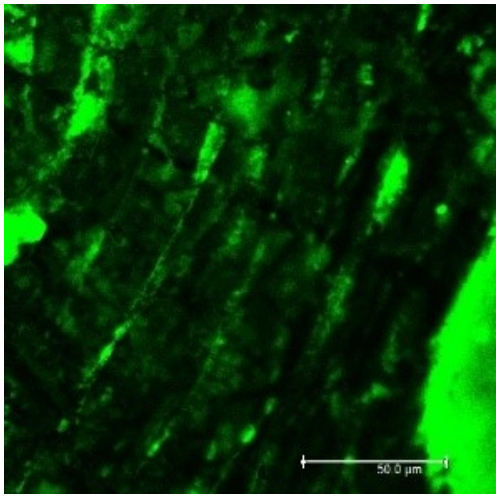


Fig.48 Leaf of *P .patens*, control sample. Antibodies JIM7/FITC

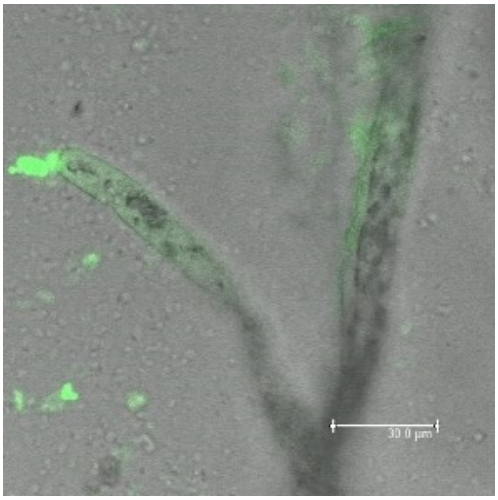


Fig.49 Protonemata of *P. patens*, control sample. Antibodies JIM7/FITC

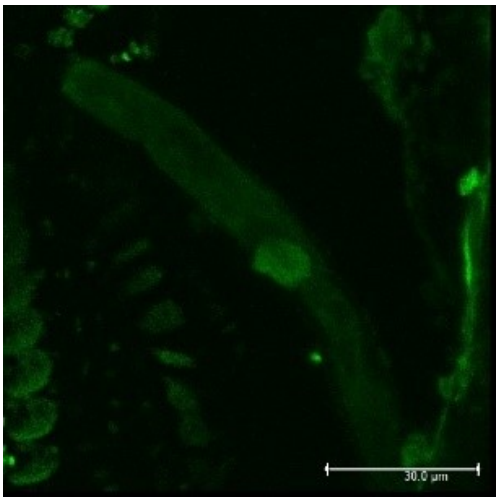


Fig.50 Protonemata of *P. patens*, control sample. Antibodies JIM7/FITC

7.DISCUSSION

Our study presented methods used in detection of ultrastructural changes in the cell wall of *P.patens* under heavy metal stress. The methods consist of steps beginning with propagating and harvesting the moss, preparing the specimen for visualisation under both light and confocal microscope and collecting the data. Our experiment was carried out in controlled laboratory conditions on the moss grown on agar medium. Most mosses used as biomonitors are ectohydric, that means they gain nutrition with the whole body therefore our study would present only partial information on how mosses deal with the stress because it imitates heavy metal sorption only from soil.

7.1 CELL WALL AS A BARRIER AGAINST HEAVY METAL STRESS

It is already known that moss cell wall acts as a selective barrier against heavy metal stress in bryophytes and higher plants as it was observed in some research, e.g. in Basile et al. (2012) where different heavy metals were immobilised extracellularly probably in the cell wall of the moss *Scorpiurum circinatum* and its ultrastructural changes were investigated under an electron microscope following treatment in heavy metal solutions. Similarly, Zn was sequestered at the cell wall of the moss *Pohlia drummondii* which aided tolerance against the metal (Lang and Wernitznig 2011). The study by Konno et.al (Konno, Nakashima, and Katoh 2010) deals with copper uptake by metal-tolerant moss *Scopelophila cataractae* and its tight binding to homogalacturonan in the protonemata cell wall. The authors of the study however hint that the cell wall thickening might have been cause by rearrangement of the cell wall architecture rather than increased volume of homogalacturonan. Cell wall thickenings caused by increased amount of pectin were also observed in *Funaria hygrometrica*, a lead tolerant moss (Krzesłowska et al. 2009).

Apart from sequestration in cell wall, bryophytes may deal with toxic concentrations of heavy metals by immobilization in vacuoles or cytoplasm vesicles with help of phytochelatins or glutathione (Basile et al. 2012). In the cell wall of higher plants, responses to heavy metal stress can be different than in bryophytes e.g., upon abiotic stress higher plants produce reactive oxygen species accompanied by secondary cell wall lignification (Berni et al. 2019). The composition of the cell wall of bryophytes is also slightly different to vascular plants. It contains uronic acids, mannose-containing hemicellulose as well as 3-O-methyl rhamnose and

instead of lignin it contains lignin-like polymers. Numerous ion exchange sites (formed from uronic acid for example) give cell wall ability to absorb heavy metals (Parrotta et al. 2015). Another valuable observation on how higher plants may deal with applied metal stress is increased amount of xyloglucan contrary to previous hypothesis on Cd immobilisation by homogalacturonan in the cell wall of *Medicago sativa.* Xyloglucan is not as affected by pectin methyl esterase (PME) activity as homogalacturonan is, therefore stays in its methyl esterified form and does not expose any binding sites for the metal and thus prevents its entry to the cell wall or apoplast (Gutsch et al. 2019). Similar strategy was observed in metal tolerant population of *Silene paradoxa*. Under copper stress pectin concentration lowered in the cell wall of the root however its methylation degree was higher which protected the protoplast against copper toxicity (Colzi et al. 2012).

7.2 COPPER AND ZINC TOXICITY

Cu and Zn are transition metals that are structurally very similar, however it is generally assumed that divalent copper is more toxic than zinc. The toxicity of heavy metals is attributed to binding of heavy metals to thiol groups -SH of different proteins, changing their steric conformation and affecting their functionality. The difference in toxicity is due to different affinity of metals to proteins where they form complexes. Transition metal cations have high electronegativity and can form covalent bonds in these complexes. Toxicity of copper is rather complex, and it is dependent on both outside and intrinsic mechanisms of the cell. Both metal cations have similar chemical features and size to charge ratios however copper can efficiently remove other transition metals from the complexes due to its higher affinity to the thiol groups. This agrees with The William Irving order of stability of bivalent transition metal complexes (Mn2+<Fe2+<Ni2+<Zn2+). Cu is also part of Fenton reactions when free radicals are produced. This is not so much case for zinc which cannot change oxidation state as a free ion. (D H Nies 1999; Dietrich H Nies 2016; Walker, Enache, and Dearden 2003).

In our study, naked eye observations proved higher toxicity of copper in *P.patens* compared to toxicity of zinc. After 3 weeks of steady growing, the process reached plateau, copper grown *P.patens* changed shoot colour to brown and stopped gaining any yield. After this point all the copper shoots stayed the same size. This was not so much case for zinc grown *P. patens* where

the moss was not affected phenotypically, and it prospered throughout the whole experiment. Our results are comparable with the study by Sassmann et al. (2015a).

7.3 MOSS PROPAGATION

In general, we could not observe any growth pattern in bryophytes on group level however 3 species in moss group were thriving best after the propagation. We did not include enough species in liverworts or hornworts groups and the time period for recording the growth pattern after propagation was not sufficiently long. Duckett et al. (2004) states that liverworts are the most difficult for vegetative propagation as they do not regenerate so well as mosses or hornworts.

The propagation medium contained SO_4^{2-} anions because they provide high number of free cations (Cu^{2+}, Zn^{2+}) which can interact with protons in the cell wall. The availability of free cations is discussed in the study by Sassmann et al. (2015b). The growth pattern of all moss species was recorded by taking images regularly. In the future experiments we should standardised the images to get more exact data. According to manuals for propagation of *P.patens* by Cove and Bezanilla, Petri dishes are recommended to be sealed with micropore tape to allow exchange of gases. Parafilm sealing may cause poorer growth and development (Bezanilla 2008; Frank, Decker, and Reski 2005) however we opted for this sealing to avoid the samples drying out too early and allowed us to track some samples for a longer period of time.

7.4 INDUCED GROWTH OF PROTONEMATA

The measurements of the cell wall thickness of protonemata in *P.patens* was preferred to cell wall thickness of shoots. Since the protonemata are only one cell layer thick and are in a direct contact with the medium they tend to be more sensitive to changes in the environment. To harvest enough protonemata for the experiment we used a special blender and fragments of the moss were cultivated on solid agar medium. Surprisingly, mainly shoots with very little protonemata were present after weeks of cultivation.

The differentiation of the moss organs is regulated by intrinsic and external factors, temperature, light intensity, phytohormones, trophic state or pH of the media according to many studies. *P.patens* can be cultured on both solid and liquid media. On solid media the moss undergoes full development starting with the growth of chloronema differentiating into

caulonema filaments with bud initials which later grow into leafy shoots. The subculturing of the moss on the solid media is recommended at monthly intervals. The moss culture in liquid media can be initiated from mechanically disrupted gametophore or protonemata. *P.patens* has a great regeneration ability and its disrupted tissue in liquid medium leads mainly to the growth of protonemata (Frank, Decker, and Reski 2005). In closely related species *P.turbinatum* the development of leafy shoots were also formed less in the liquid media than on solid one (Meyer 1940). *P.patens* can grow in a wide range of pH between 4,5 and 7,0. Adjusting the pH of the media can tweak the process of differentiation. According to study of semi-continuous bioreactors of *P.patens,* at lower pH 4,5 only chloronema filaments were developed whereas at pH 5,8 to 7,0 caulonema filaments were formed (Hohe et al. 2002). Another way how to influence the growth of protonemata culture and differentiation process is by adding ammonium tartare. This substance halts the transition from chloronema filaments to caulonema and inhibits formation of buds (Hohe et al. 2002). In a study of peat moss regeneration it was recorded that weekly mechanical wounding keeps the moss culture in a protonemata stage and inhibits further differentiation (Zhao et al. 2019). Also, blending the moss tissue should be carried out approximately for 2 minutes because over blending may cause poor regeneration outcome (Cove et al. 2009). In our study the blending processes was not carried out at exact same times which may have interfered with the protonemata development. In some moss species high density of protonemata in liquid culture may affect gametophore development. This might be caused by two phytohormones auxin and cytokinin with different concentration in the medium (Zhao et al. 2019). Auxin and cytokinin play a crucial role in cell differentiation. Under standard conditions most caulonemal side branch initials grow into new protonemata filaments and around 5 percent of them change into buds which grow into leafy gametophore. Adding an exogenous auxin can affect shoots development in different ways which depends on its concentration, but generally low 2,4-D auxin promotes shoots growth and its higher concentration stunts it or even completely suppresses it (Thelander, Landberg, and Sundberg 2018). The effect of 2,4-D at various concentrations was tested on the moss *Rhodobryoum giganteum* where the results showed that induction of protonemata is dependent on auxin concentration e.g., low concentration of the phytohormone induces protonemata growth whereas above certain conversation the growth ceases. This is also dependent on the type of tissue used for the propagation (Y. Y.

Chen et al. 2009). Protonemal suspension culture of *Funaria hygrometrica* was also successfully cultured in liquid medium in the study by Itouga et al. (2017).

7.5 SHOOT DISCOLORATION UPON COPPER STRESS

Accumulation of copper in *P.patens* led to impaired growth and shoot discoloration. Plants under Cu stress, in general, manifest discolouration symptoms due to impaired respiration and photosynthetic processes. The chlorophyll biosynthesis especially chlorophyll a and b are decreased, chlorophyll structure is altered as well as the composition of the thylakoid membrane. The changes of thylakoid membrane mainly the fatty acid composition negatively affect fluidity of the membranes and thus the function of photosystems. More sensitive to damage caused by Cu is PSII photosystem of which donor and acceptor sites are exposed to the toxic metal which leads to electron flow blockage and low oxygen evolution in PSII (Shakya, Chettri, and Sawidis 2008; Yruela 2005).

As mentioned in previous paragraph, the chlorophyll was affected by copper stress however its degradation by zinc solution at concentration 100 µmol was not observed, even after 9 months phenotype was comparable to the control sample. Lower toxicity of zinc compared to other metals was also observed in study by Shakya, Chettri, and Sawidis (2008) where impact of copper, zinc and lead on chlorophyll content was recorded in mosses.

7.6 MOSS CULTURE CONTAMINATION

Very challenging task in the study was to keep the moss cultures axenic. To avoid infecting the plantlets in Petri dishes, all the propagation was carried out in laminar flow cabinets with instruments either disinfected with ethanol or autoclaved. At any moment before the fume hood was entered our gloves were disinfected with ethanol and changed regularly. The propagation took place on average every second week only from the cultures which were sterile without a sign of contamination. Only tips of plantlets were cut off and moved to a new sterile agar plate in order to lower the risk of cross-contamination. The younger shoots further away from the agar medium probably harbour less bacteria or fungi than the shoots in a direct contact with the medium. Some moss species seemed to be more resilient to the bacteria than others but in general most cultures had signs of infection. To improve sterility of moss cultures in future experiments, we recommend using a set of autoclaved instruments for each moss species. A study by Carey recommends embedding protonemata in antibiotic-containing agar

medium for initiation of moss culture. Even if antibiotics significantly reduced bacterial replication and moss outgrown from the media, the use of antibiotics prophylactically may lead to the growth of resistant bacterial strains which would be very difficult to remove (Carey, Payton, and McDaniel 2015). Widely used chemical for plantlets disinfection is sodium hypochlorite (NaOCl). A study dealing with axenic bryophyte cultivation used 70% ethanol for 30 seconds prior to application of NaOCl with different concentrations on moss and liverworts gametophyte followed by a rinse in autoclaved tap water. Suitable substrate for propagation is also recommended here (Beike, Horst, and Rensing 2010). The drawback of using NaOCl is that its higher concentrations can kill plant material when sterilising the surface of the moss as it happened in the study of establishment and propagation of the moss *Hypnum cupressiforme* (Vujicic M, Sabovljevic A, and Sabovljevic M. 2011). The concentration needed for killing bacteria harmed the moss as well and even if the moss survived the bleaching process, after transferring it on the medium it was outgrown with bacteria, algae and fungi again. The study had more success with setting up axenic cultures *in vitro* using spores rather than gametophyte.

7.7 PREPARATION OF RESIN SECTIONS

Preparation of samples embedded in epoxy resin is a multistep procedure beginning with diluting solutions for sample fixation. First, samples are fixed in glutaraldehyde which helps to preserve an ultrastructure of cells followed by fixation in osmium tetroxide to retain the lipids in the cell membranes. The resin used in the process is hydrophilic therefore dehydration in ascending ethanol or acetone grades is needed. To achieve polymerisation process and thus hardening of the resin, the moulds are heated up at 60 °C in the oven. Hard blocks are later sectioned by microtomes and the sections observed under a light or electron microscope (Pacheco 2019). The solutions of sodium cacodylate and glutaraldehyde have a short shelf life of one month and needed to be stored in the fridge with appropriate signs of expiry date and hazardous chemicals. After their use, they were disposed into a special toxic container. The chemical, osmium tetroxide, used as a fixative must always be stored in glass veils and never in plastics because it can penetrate plastic material. Its solid form is volatile therefore working in a fume hood with protective clothing and laboratory glasses is required to avoid its inhalation. Osmium tetroxide is always disposed in a special container because it is highly poisonous.

The epoxy resin has exceptional preservation properties therefore it is widely used in transition electron microscopy (TEM). It can maintain ultrastructure of sample by uniform hardening, and it does not change its volume a lot during a process of curing. After hardening the epoxy resin is easily sectioned and very stable under an electron microscope. There are some disadvantages to working with resins as they are highly irritable and toxic. For this reason working in fume hood is mandatory (Glauert and Lewis 1998). An alternative to toxic epoxy resin, there is less harmful option, London Resin White which penetrates samples easier and due to its aromatic cross linkage it has improved stability under electron beam (Skepper and Powell 2008).

Our prepared resin slides had creases visible under the light microscope which may have been caused by poor spreading of the resin sections on the glass slides. We were recommended to use GloQube Glow discharge system® (Quorum Technologies) to change surface energy of the glass slides and thus improve their hydrophilicity and change the contact angle. Water droplets then spread out evenly on the glass surface however the effect is not permanent therefore it is needed to add the sections in the drop of water straight after the treatment of the glass slides in the discharge system. When a water droplet is spread out evenly on the hydrophilic glass slides then the ultrathin sections get into an even position as well. Later the slides are heated, water droplets evaporate, and the sections have improved spreading.

The glow discharge plasma is a common method used for modifying surface energy of various materials. It is based on low-pressure gas ionisation. In the gas chamber there are energetic electrons which collide with gas molecules leading to ionisation or excitation of these molecules causing formation of free radicals and ions. Glowing can be seen when the radicals and ions relax into lower energetic state releasing photons (Walkiewicz A 2020) The ionisation affects the surface of the material.

For future experiments we recommend using thinner resin slices and clean new microscopy glass for improved visualisation under a light microscope.

The cell wall thickness of *P.patens* was not measured in our experiment, but we would advise using protonemata slices cut perpendicularly to the wall plane to get the most accurate information about the width of the wall. The measurements of the cell wall thickness can be

affected by resin section preparation and type of resin used for embedding. This is discussed in the study measuring cell wall thickness of algae *Vaucheria frigida* (Mine et al. 2016)

7.8 MICROSCOPY

The cell wall has very complex architecture and to follow all the changes and interactions upon heavy metal stress it would be necessary to use array of monoclonal antibodies to detect whole composition of different epitopes in the cell wall and get "better picture" of its ultrastructural alterations throughout the whole experiment.

Homogalacturonan in the cell wall can be detected with the use of various imaging technology and staining. The best resolution can be obtained by TEM in combination with immunostaining. An alternative to detection of pectin in the cell wall *in situ* with fluorescent tags, is localisation of pectin via immunogold-labelling method with better resolution and relative quantification. In order to view specimen in TEM, it needs to be fixed, sectioned and stained. Conventional way of fixing the specimen is chemically with aldehydes and osmium tetroxide followed by dehydration in organic solvents. Fixed specimen is then embedded in different plastics. Another possibility is cryo-fixation where the specimen is quickly frozen in a liquid propane or ethane at –180 °C or under high pressure at 2100 atmospheres within 100 ms.

Both these methods are used mainly for transverse sections of specimen however they both have some downsides, immunofluorescence has poorer resolution in comparison to immunogold TEM which on the other side has limited sample size detection (Domozych 2012; Sun, Sun, and Juzenas 2017).

Immunogold-labelling is a method used since 1980s. This technique gives valuable information about interaction of proteins or polysaccharides in cells at ultrastructure level with help of colloidal gold particles of the same size conjugated to secondary antibodies. The protocol is not too demanding, and the staining can be done either prior to embedding, sometimes with substantial loss of spatial resolution or post-embedding (on-grid labelling) with better preserved ultrastructure detail and antigenicity (Jones 2016). Immunogold staining is not visible using bright field microscopy therefore silver particles can be deposited on colloidal gold to increase the size and improve the detection of stained specimen by colouring it black. A silver enhancement method uses a physical developer as in photography which contains

silver ions and reducing solution in optimal pH environment (Oliver 1994). This method can be used to avoid overlap of different sizes of gold particles, one ultrasmall and other larger, both silver-enhanced as presented in study by Yi et al. (2001). A protocol for fixation in immunogold-labelling has been improved over time. The latest suggestions are that a fixative, osmium tetroxide used in immunogold TEM degrades cell components in high concentrations and reduces antigenicity of specimen when applying immunogold staining. Lower concentrations of this fixative can help protect ultrastructure of biological membranes and the epitopes. Another factors influencing post-embedding immunolabelling may include incubation time for both primary and secondary antibodies ("speed immunolabelling") or size of gold particles where "ultrasmall" gold particles work best for signal intensity (Flechsler et al. 2020).

In our study, homogalacturonan in the cell wall of *P.patens* was detected with primary antibodies JIM7 and LM20 and secondary antibodies conjugated to fluorescent tag FITC in combination with confocal microscopy. FITC is a very common green fluorochrome rather sensitive to changes in pH and photobleaching expressed between 494 – 520 nm (A. K. Chen et al. 2009). Unfortunately, we managed to use only antibodies for detecting methyl-esterified homogalacturonan which has not ability to bound heavy metals sufficiently enough and gave us only limited information on its localisation. In the protocol for fixation and application of antibodies we did not include the step with application of driselase, enzyme for cell wall digestion. Same as with preparation of resin sections, the samples were first fixed to retain the ultrastructure. Some images had very low signal due to application of unsuitable secondary antibodies. Even if we had not the best image quality, we managed to detect methyl-esterified homogalacturonan in our control samples, shoots and protonemata of *P.patens.* Although probes JIM7 and LM20 detected homogalacturonan in both cell types, they showed slightly different results. JIM7 signal on protonemal cell was more consistent and stronger compared to LM20 signal. On the other hand, homogalacturonan in the leaf recognised by JIM7 and LM20 showed different ultrastructure. Some images of leaf tissue with JIM7 probe had patches of signal which was not consistent throughout the sample. This may have been caused by antibodies application after their expiry date or that the storage tube had not been shaken well before the usage. Leaf tissue with LM20 epitope had "dotted like"

structure and rather strong labelling. Two probes for methyl-esterified homogalacturonan were tested in *P.patens* in another study and gave different results as well (Berry et al. 2016).

We also managed to detect distribution of homogalacturonan in the leaf tissue under zinc stress. Methyl-esterified homogalacturonan was present mainly in the leaf tip. One explanation for this may be that metal ions were incorporated in homogalacturonan and immobilised but did not reach the leaf tip from agar medium. The internal translocation plays a key role in our experiment despite the ability of ectohydric mosses conducting water by external rapid translocation. Translocation is aided by tension forces and in ectohydric mosses water can move in two directions. (Glime 2017).

We did not detect homogalacturonan under copper stress but the study by Antreich, Sassmann, and Lang (2016) measured copper enrichment in the stem of *P. patens* grown on 0,1 mM of $CuSO_4$ using semiquantitative SEM-EDX analyses. Copper was distributed evenly throughout the whole stem unlike in other two moss species *Mielichhoferia elongata* and *Pohlia drummondii* used in the experiment.

Antibodies used for research are usually produced in laboratory or farm animals like mouse, rat, goat, sheep, chicken or rabbit. The process starts with injecting immunogen (target antigen) into an animal. An immunogen consists of specific purified antigen and carrier protein. Antibodies are then formed in the serum of the animal, collected and later purified. Sometimes polyclonal antibodies can answer all the research questions without using monoclonal antibodies which need a special epitope. It takes about a month to produce polyclonal antibodies and about 3 to 6 months to produce monoclonal antibodies. The welfare of the animals should always be considered in selection of monoclonal or polyclonal antibodies. Monoclonal antibodies are produced by induction of special B cell needed for hybridoma establishment. Very often primary antibodies are monoclonal because of their high epitope specificity. Secondary antibodies should be produced in an animal species that is not closely related to the animal species used for production of primary antibodies (Leenaars and Hendriksen 2005; Lipman et al. 2005).

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