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## „Drought stress and revival of mosses: cytological effects of de- and rehydration "

verfasst von / submitted by

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# Table of contents

# 1. Abstract

This work aims to elucidate the effects of drought stress on the morphology and physiology of a small selection of *bryophyte* species as droughts are becoming more common and mosses could serve as very easy to handle model organisms.

Four different *bryophyte* species were compared in their dehydration and rehydration behaviour. The mosses were compared in terms of shrinkage and regain of their leaf area, as well as, cell size (area), length and width and their photosynthetic activity.

This study investigated the effects of dehydration and the ability to recover from this severe dehydration on different moss species. Those species were, *Atrichum undulatum, Conocephalum conicum, Funaria hygrometrica* and *Physcomitrium patens.* Light stereo microscopy was used for the leaf area and the cell area/width/length measurement. The photosynthetic activity measurement was determined with a "chlorophyll fluorometer IMAGING-PAM". In all experiments the leafy gametophyte was examined.

The tested species showed significant differences in their reaction to dehydration and the following rehydration. The tested moss species differed significantly in their ability to withstand drought stress. Those results were proven by the photosynthesis activity measurement. Overall, there were unexpected differences between the almost randomly picked species in their reaction to dehydration and rehydration. All mosses were very resilient to dehydration but some more than others. The statement that *bryophytes* can withstand dehydration to a high degree by (Proctor and Pence, 2002) is confirmed by this study.

Different coping mechanisms of the moss species were found, some lost all their water within the cells very quickly while others contained their water relatively well within their cells. The same results were found in the photosynthesis activity measurement. Some species lower the photosynthesis activity very quickly and regain it quickly while others lower it much slower and do not regain it that fast. Significant correlations between the photosynthesis activity and the ability to survive severe drought were found. In the 3D recordings some species showed cytorrhysis, those species were also the most drought resilient species in the other experiments. No correlation was found between cell shape and their ability to withstand dehydration or recover from it. It was found that the only tested multilayered species (*C. conicum*) had the biggest problems in terms of coping with drought stress.

## 2. Zusammenfassung

Das Dehydrations- und Rehydrations- verhalten von vier verschiedenen Moosarten *Atrichum undulatum, Conocephalum conicum, Funaria hygrometrica* und *Physcomitrium patens* wurde untersucht und untereinander verglichen. Diese Arten wurden ausgewählt, da sie sehr verbreitet sind und auch teilweise (*P. patens*) als Modellorganismus Relevanz haben. Die Studie vergleicht welche Moosarten resilienter gegenüber Austrocknung sind bzw. wie sehr sie austrocknen und sich danach aber dennoch wieder erholen können.

Um diese Unterschiede festzustellen, wurde zuerst mittels Stereolichtmikroskop die Blattfläche eines einzelnen Gametophyten vermessen. Die Schrumpfung und die folgende Ausweitung der einzelnen Blätter konnten so über einen Zeitraum sehr detailreich bestimmt werden. Danach wurden auf zellulärer Ebene die Veränderungen der einzelnen Zellen betrachtet, auch mittels Stereolichtmikroskop. Es wurden Fläche, Länge und Breite der Zellen im hydrierten und im dehydrierten Zustand gemessen. Hier wurde dann festgestellt, dass manche Arten mehr schrumpfen als andere und in welcher Dimension diese schrumpfen. Es wurden hierbei signifikante Unterschiede zwischen den Arten festgestellt.

Die Photosynthese Aktivität der Arten während der Dehydration und Rehydration wurde mittels eines Chlorophyll Fluorometer gemessen. Auch hier gab es teils signifikante Unterschiede im Verhalten der Arten in Bezug auf deren Photosynthese Aktivität. Die Photosynthese Rate wurde bei manchen Arten sehr schnell verringert, während es bei anderen länger dauerte. Manche Arten stellten ihre Photosynthese Rate wieder sehr schnell um und erholten sich auch in den anderen Experimenten schneller. Um zu überprüfen ob die Moosarten auch nach solch extremen Dürre Verhältnissen auch weiter Lebens- und Wachstumsfähig waren, wurden sie ausgetrocknet und dann wieder in ihrem gewohnten Medium angesetzt. Erstaunlicherweise waren zwei der getesteten Arten (*A. undulatum, F. hygrometrica*) sehr robust und wuchsen selbst nach 20 min starker Austrocknung wieder.

Mit dem Stereomikroskop war es auch möglich 3D Aufnahmen der Zellen zu machen, bei diesen Aufnahmen war in manchen Arten Cytorrhyse zu sehen. Das waren auch die Arten die resilienter gegen Dehydration waren.

Diese Arbeit hat das Ziel die Auswirkungen von Austrocknung anhand einer kleinen Gruppe von unterschiedlichen Moosarten zu untersuchen. Durch den Klimawandel werden Dürreperioden immer intensiver und Pflanzen müssen sich daran anpassen. Meine Arbeit zeigt, Moose sind ideale und leicht zu händelnde Modellsysteme, wenn es um diese Thematik geht.

## 3. Introduction

Drought is an increasingly important limitation on plant productivity worldwide and climate change caused dry periods to become longer. Since the global surface temperature is predicted to increase by 2.6–4.8°C by the year 2100 (IPCC, 2014), water in the soil will be reduced. To exactly predict the impacts of climate change on natural vegetation and agriculture is difficult but the consequences for humankind could be devasting (Trivedi et al., 2022).

Plants must cope with these new conditions, as global environmental changes destabilize ecosystems (Hautier et al., 2015) and diminish biodiversity worldwide (Cardinale et al., 2012) with high extinction rates (Pimm et al., 2014). Climate change boosts regional mortality events but the prediction of those remain difficult because the physiological mechanisms of plants in terms of drought survival and mortality are poorly understood (McDowell et al., 2008). For the development of drought-tolerant crops and strategies for their survival, it is therefore important to understand the mechanisms of drought tolerance in plants (Hu et al., 2016). In comparison to *angiosperms*, *bryophytes* can withstand rapid dehydration to a much higher degree.

Vascular plants share fundamental genetic and physiological processes with non-vascular land plants (e.g., *bryophytes*), although the two lineages diverged very early in evolution. *Bryophytes* do not possess *tracheae* or *tracheids* which is found in vascular plants. They are especially interesting as they dry out very quickly and appear dead, but in some cases are easily revived again. Some of these *bryophyte* species are able to survive desiccation, which is a more severe state of dehydration than drought (Hu et al., 2016).

The exact drought coping mechanisms and differences between different moss species are often not known. Because *bryophytes* represent very basal land plants and are relatively easy to work with under laboratory conditions, they are interesting as a plant model system. Physiological changes associated with the drying of mosses are the same across a range of tissues, in both mosses (Pressel et al., 2009; Proctor et al., 2007) and liverworts (Pressel et al., 2009).

The physiological changes in *bryophytes* are also comparable with those described in vascular plants (Navari-Izzo et al., 2000; Quartacci et al., 1997; Vecchia et al., 1998), and include vacuolar fragmentation, chromatin condensation in the nuclei whilst membrane integrity is retained and changes in the internal structure of plastids and mitochondria (Pressel and Duckett, 2010). Nevertheless the exact relationship between vascular plants and *bryophytes* (hornworts, liverworts, and mosses) is still a matter of debate (Schaefer and Zrÿd, 2001). But it is known that *bryophytes* can withstand dehydration to higher degree than vascular plants (Proctor and Pence, 2002).

## 3.1 Bryophytes lifecycle

The *bryophytes* lifecycle, which is depicted in *figure 1* (Bruce Snyder, 2015), is dominated by a photoautotrophic haploid gametophytic generation that supports a simple and diploid sporophyte. The gametophyte is the haploid stage, this stage is characterized by two distinct developmental stages: the gametophore/leafy shoot, which differentiates by caulinary growth from an apical meristem; and the protonema, a filamentous network of caulonemal and chloronemal cells, which develops by cell division of the apical and subapical cells and apical growth (Schaefer and Zrÿd, 2001).

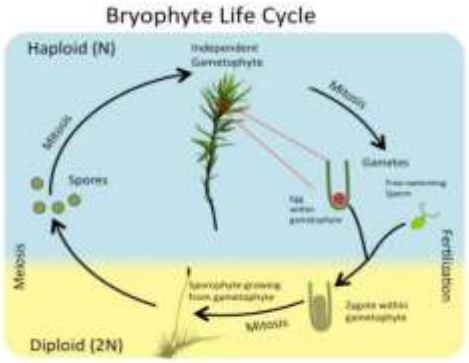


***Figure 1*** *Bryophyte life cycle is shown here, work by Eva Horne and Robert A. Bear (Bruce Snyder, 2015).*

As described above the gametophyte and the sporophyte are two distinct stages of the moss life cycle. This means that the green, leafy gametophytic tissue that is normally recognised as moss is haploid (has only one set of chromosomes). Therefore, gametophyte refers to all organs and tissues that are a part of the haploid generation (Brennan et al., 2022). In the following only the gametophyte, not the sporophyte is viewed at.

## 3.2 Bryophyte morphology compared to vascular plants

Since the first land plants have resembled extant *bryophytes* (Mishler et al., 1994), the understanding of the *bryophyte* morphology is essential to identify structural innovations that allowed early land colonization and understand more complicated body plans in vascular plants (Renzaglia et al., 2007). To reduce dehydration vascular plants are homeohydric and have a thick cuticle, vascular tissue to distribute water internally and roots to extract water from the soil.

Mosses, are depending on a surface film of water to maintain hydration, they are poikilohydric (Roberts et al., 2012). Flowering plants generate their lateral organs at the multicellular shoot apical meristem (Kuhlemeier, 2017), in *bryophytes* the leaves derive from a single shoot apical cell (Gifford, 1983; Harrison et al., 2009).

There are also major differences in the composition of the primary cell wall (PCW) between vascular and non-vascular plant taxa. The cell walls of basal mosses generally contain high concentrations of Glucuronacid and Galacturonacid, the cell walls of more advanced moss species contain a much lower concentration of Glucuronacid in its cell wall (Popper and Fry, 2003).

A very important difference between *bryophytes* and vascular plants is the missing vascular tissue in mosses. The vertical growth of *bryophytes* is limited because specialized conducting tissues are absence, therefore their height is determined by the time it takes for the water to diffuse between cells (Popper and Fry, 2003).

## 3.3 Anatomical/hydrostatical responses of all plants when dehydrated

One very prominent anatomical and hydrostatic responses of plant cells when dehydrated is for example wilt. Since many plants rely on turgor pressure within the cells, they are erect, but plants are constantly losing water through stomata this process is called transpiration. If transpiration causes more water to be lost through the stomata than it takes in, the water balance within the plant can get thrown off. The dehydrated cells no longer remain erect and the plant starts to wilt (Torkar et al., 2018). *Bryophytes* in contrast cannot maintain their turgor even when the water deficit is not severe (Proctor, 2000).

The hydrostatic skeleton in plant cells collapses when the cells are under water-deficit stress (Shafi et al., 2019). These events lead to cell collapse (= Cytorrhysis) the inner cell organs are diffuse distributed within the cell, also the general structure of the cell changes but the cell is not fully destroyed (Strugger, 1949). During the process of cytorrhysis the space for larger cell organelles, such as chloroplasts, becomes restricted to a small area inside the cells, the plasma membrane remains intact, and when the moss is hydrated again the cell organelles redistribute within the cell (Buchner and Neuner, 2010). The effect of cytorrhysis is later seen in the figures of *chapter 6.4 3D visualizations of selected moss species.* Cytorrhysis also occurs when mosses are frozen and keeps the cells from bursting and is therefore a helpful survival mechanism of mosses (Buchner and Neuner, 2010).

Another anatomical response of the plant to water loss is plasmolysis, here the protoplast (which is the inside of the cell) detaches from the cell wall. But plasmolysis is caused by a hypertonic environment of the cell (Lang et al., 2004).

## 3.4 Physiological responses of all plants when dehydrated

Photosynthesis happens inside the chloroplasts, it has two phases, the directly from light dependent light reaction and the dark reaction, which does not need light. The light reactions convert light energy into chemical energy in the form of NADPH and ATP. The dark reactions are linked to the light reactions in terms of utilizing NADPH and ATP, as well as pH and $Mg^{2+}$ gradients (TOLBERT et al., 1980). In these processes light energy is captured and used to convert carbon dioxide, water, and minerals into oxygen and organic compounds. The photosynthetic apparatus is a complex machinery, consisting of proteins and pigment complexes.

The photosynthetic apparatus is directly impacted by environmental stresses, through disrupting all major components of photosynthesis including the carbon reduction cycle, the stomatal control of the $CO_2$ supply and the thylakoid electron transport. In addition to that carbohydrates, peroxidative destruction of lipids are accumulated and water balance is disturbed (Allen and Ort, 2001). Stomata are controlling the entrance of water loss and $CO_2$ absorbability. Therefore stomatal closure is one of the first response to drought stress which results in a lower rate of photosynthesis (TOLBERT et al., 1980).

Because of that the photosynthesis activity is an important parameter on which the behaviour of a plant while dehydrating and rehydrating can be monitored.

As a main component of the chloroplast, the relative chlorophyll content has a positive relationship with the photosynthesis rate, when chlorophyll contents are lost the inactivation of photosynthesis follows, a main cause of this is water/drought stress (Allen and Ort, 2001).

Chlorophyll content reduction which has been induced by drought stress, has the following characteristics: the loss of chloroplast membranes, excessive swelling, distortion of the lamellae vesiculation, and the appearance of lipid droplets (Kaiser et al., 1981). This disorder and partly destruction of the chloroplasts is visible when the cells are examined under the microscope.

The photosynthesis activity in my experiments was measured with a chlorophyll fluorometer (IMAGING-PAM). Thereby three parameters were measured.

In each moss species, minimal fluorescence $F_0$ (After dark adaptation normally all PS II reaction centres are open and maximal photochemical quenching is observed) and maximal fluorescence Fm (The Fm-value is assessed at the plateau level reached during application of a Saturation Pulse) were measured. The Effective PS II quantum yield, Y(II) was measured too, this value shows how much absorbed quanta are converted into chemically fixed energy and how much is converted to heat and fluorescence, it represents a benchmark of the plant's wellbeing. These parameters show the physiological condition of the photosystem of each moss species.

The chlorophyll fluorometer (IMAGING-PAM) works as described in *chapter 5.6 Photosynthetic activity of the mosses*.

## 3.5 Investigated bryophyte species

Bryophytes are divided into the following phyla, the liverworts or *Hepaticophyta*, the hornworts or *Anthocerotophyta*, and the mosses or true *Bryophyta*. The phyla which were examined here were *Hepaticophyta* and true *Bryophyta.* The tested species are *Atrichum undulatum (Figure. 2 A), Conocephalum conicum (Figure 2 B), Funaria hygrometrica (Figure 2 C)* and *Physcomitrium patens (Figure 2 D)*. All species but one *(C. conicum)* were mosses (*true Bryophyta*).

The species which are used, are picked because of their physiological differences, ecological niches they live in, their availability and their relevance in the research. All the used species are widespread across the northern hemisphere ("Atlas-of-British-and-Irish-Bryophytes-V1-69.pdf," n.d.).

*Atrichum undulatum* is a basal representative of the moss phylogenetic tree and as a generalist a desiccation-tolerant plant (Hu et al., 2016). It was also very easy to propagate under laboratory conditions. *A. undulatum* lives on shady, humus-rich, more or less moist, loamy soils, such as on embankments in deciduous forests ("Atlas-of-British-and-Irish-Bryophytes-V1-410.pdf," n.d.).

*Conocephalum conicum* is a common liverwort (*Hepaticophyta*) in the northern hemisphere and therefore it has ecological relevance ("Atlas-of-British-and-Irish-Bryophytes-V1-69.pdf," n.d.). It was picked for these experiments as a representative of a multi cell layered species,

although there is little literature on this species, comparable species (e.g., *Marchantia polymorpha*) have been a subject of various studies (Godinez-Vidal et al., 2020). *C. conicum* is associated with calcareous substrates and is found on sandy banks, wet rocks and open woodlands, it even occurs in high attitudes ("Atlas-of-British-and-Irish-Bryophytes-V1-69.pdf," n.d.).

Werner et al. (1991) has shown that the protonemata of *Funaria hygrometrica* can recover very well, but only if the rate of drying is slow (Pressel and Duckett, 2010). Many studies of plant physiologists have focused on species like *Funaria hygrometrica*, *Atrichum undulatum*, and *Physcomitrium patens* (Schaefer and Zrÿd, 2001). So, these species were a relatively well-known model system and therefore interesting to compare to each other. *F. hygrometrica* inhabits open, nutrient-rich, mostly light, fresh to moist locations and prefers sandy, loamy, clayey soil, humus, or gravel. Natural locations are often burn sites and muddy waterfronts ("Atlas-of-British-and-Irish-Bryophytes-V1-441.pdf," n.d.).

*Physcomitrium patens (or Physcomitrella patens)* was picked in this study because there are many papers on it including some, which are about dehydration. *P. patens*, which is often used as a model system for basal land plants, is able to tolerate several abiotic stresses, including dehydration (Xiao et al., 2018). "*P. patens* is the first moss to be successfully transformed" (Schaefer et al., 1991) and has been used as the first land-plant and eukaryote, in which gene targeting occurs with a high efficiency which is comparable to that of yeast (*Saccharomyces cerevisiae*) (Schaefer and Zrÿd, 2001).

*P. patens* is widespread across Eurasia and North America. It grows on clayey or muddy soils, especially in drained ponds and on dried-up riverbanks and its natural occurrence ends at higher altitudes (Gesellschaft, 2004).

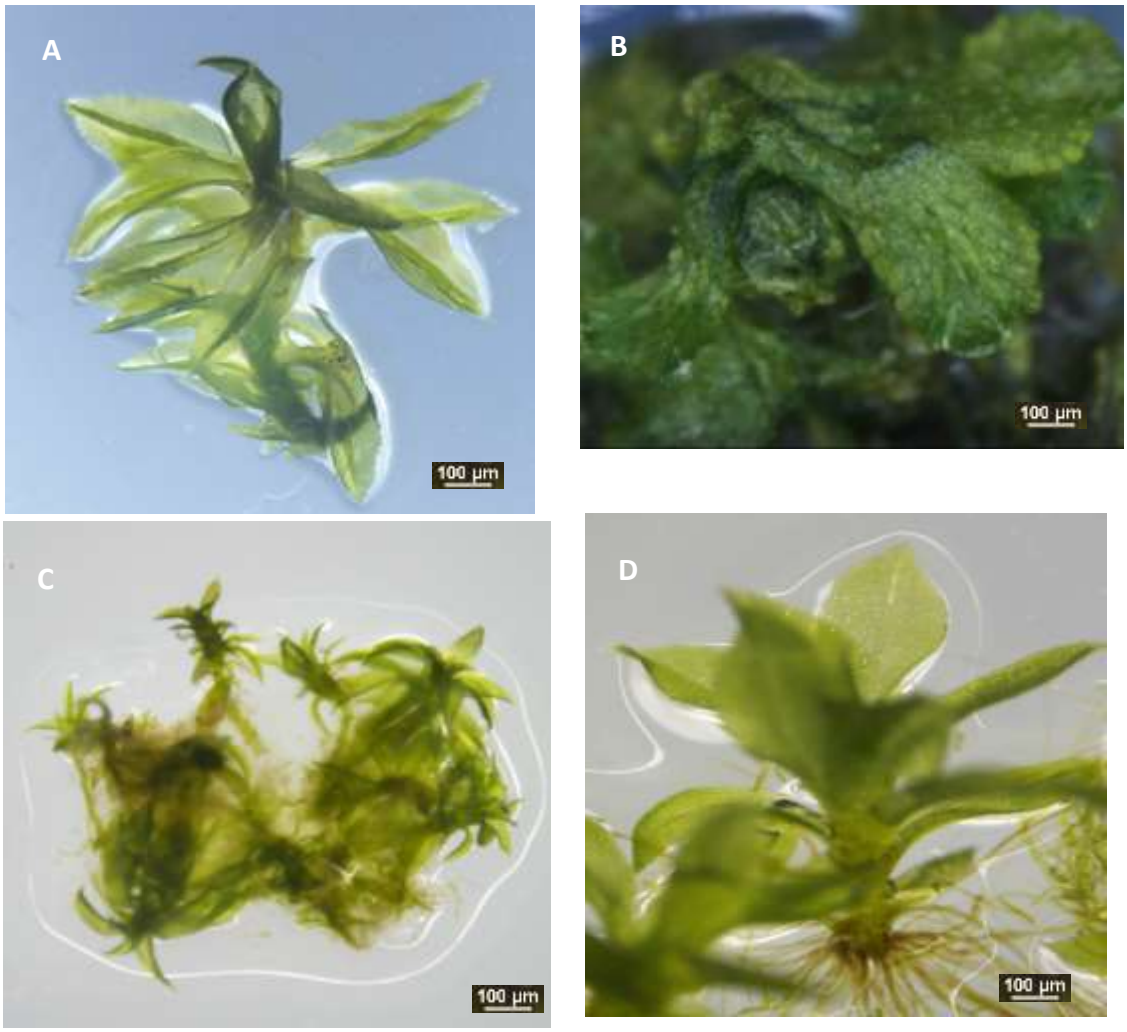


***Figure 2.*** *These species were selected for the experiments. A) Atrichum undulatum, B) Conocephalum conicum, C) Funaria hygrometrica, D) Physcomitrium patens*

## 4. Goal of the experiments

This study aimed to test a hypothetical relation between drought stress tolerance and the cell's physiological condition in four bryophyte species (*Atrichum undulatum, Conocephalum conicum, Funaria hygrometrica* and *Physcomitrium patens*).

The experiments were conducted and monitored on the leafy gametophyte as a whole and on single leaves of the gametophyte.

The consequences of drying and rewetting in bryophyte vegetative tissues are the shutting down and resumption of metabolic activities associated with de- and rehydration which are accompanied by major cytological changes that are equally profound (Pressel and Duckett, 2010).

In the modern biotechnology high-throughput approaches are widely used to research such desiccation tolerance mechanisms. Although high-throughput approaches were not feasible as the amount of moss samples was limited, the focus of my work was to characterize physiological and cytological aspects of dehydration stress response in the plants.

# 5. Material and Methods

## 5.1 Planting moss cultures

The first step in these experiments was to culture the species (*Atrichum undulatum, Conocephalum conicum, Funaria hygrometrica* and *Physcomitrium patens*) on sterile growth media, the used media contained of:

| | |
|---|---|
| Plant Agar (Duchefa Biochemie); Exp date: 31-01-2022 | 5g |
| Murashige & Skoog Medium (Duchefa Biochemie); 4414.09 mg/l; Exp date: 12-06-2022 | 1.5g |
| dest. $H_2O$ | 0.5l |

After autoclaving, petri-dishes with 6cm radius were then half-filled with the medium, which hardened after 10 min.

Gametophytes and protonemata were then picked from an original moss culture. Each moss part was then placed in nine culture spots on the medium (*Figure 3 A*). This happened in the laminar flow cabinet (under sterile conditions). The petri dishes were sealed with parafilm® or sterile tape (3M) and placed in the growth cabinet (Conviron, *Figure 3 B*). The day and night regime were set to 14h/day and 10h/night, the humidity was set to 60%, although most petri dishes of the mosses were sealed with parafilm®, so no humidity could get out or into the moss cultures.

Each moss species grew for at least 2 weeks in the growth chamber before use for the experiments or working under the microscope. Continuous subculturing was done every 3 weeks.

Because some species did not grow very well, the ingredients of the used growth media was adjusted to minimal nutrient content:

| | |
|---|---|
| Plant Agar (Duchefa Biochemie) | 5g |
| Murashige & Skoog Medium (Duchefa Biochemie); 4414.09 mg/l; Exp: 12-06-2022 | 0.75g |
| dest. $H_2O$ | 0.5l |

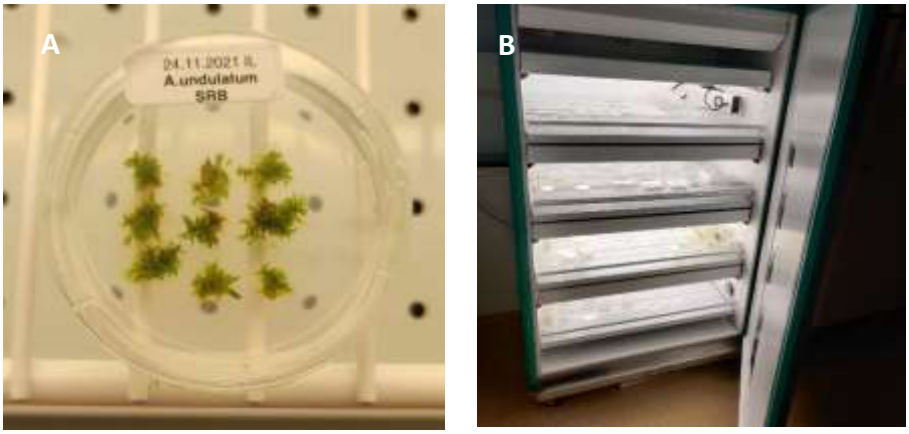


***Figure 3*** *A) The 6cm plates with the mosses inside are depicted here. B) The growth chamber (Conviron), where the mosses are grown.*

Contamination with fungi was a problem in some species more than others, although working under the same sterile conditions. *Funaria hygrometrica* and *Conocephalum conicum* were especially prone to fungal infection. In addition, *Funaria hygrometrica* grew very slow.

## 5.2 Experimental setup for de- and re-hydration

The mosses were always treated with a fast-drying protocol, which means the gametophyte was put out of its growing petri dish and onto a glass slide, without any medium. The following rehydration of the gametophyte was also done on that glass slide without medium.

In the dehydration experiment, each gametophyte was placed on the glass slide and instantly 5 µl of distilled $H_2O$ were added so the moss does not dry out while focusing with the microscope. The excessive water was afterwards removed by absorption with a paper towel. Then, every minute a photo of the drying moss was made for 60 minutes. These photos were put together into short videos which are available via the permalinks from *chapter 10.1*.

As it turned out later, dead cells were not easily distinguishable from living cells. After around 20 minutes the cells of all species did not look good, they lacked organized contents, were devoid of plastid pigmentation and sometimes even collapsed. Nevertheless, two of the tested species (*Atrichum undulatum, Funaria hygrometrica*) survived this procedure. As they were able to grow again after more than 20 minutes of dehydration, despite of looking dead (Chapter: *2.5. Survival of the mosses after dehydration*).

The rehydration experiment proceeded immediately with the dehydrated moss on the glass slide. This gametophyte was drizzled with 30 µl of distilled $H_2O$. After around 20 minutes another 10 µl of distilled $H_2O$ were added to prevent the drying of the moss.

A photograph was taken of the rehydrating moss every minute, for 50 minutes. The cells already seemed fully turgid after 50 minutes and there was no difference visible in the structure of the mosses compared to the mosses which rehydrated for 60 minutes. The rehydration photos were made into short videos too, which can be seen via the permalinks from *chapter 10.1.*

## 5.3 Examination of the gametophytes on two levels

To determine the grade of dehydration/rehydration and differentiate between the species it was necessary to examine the behaviour of the gametophytes on two levels. On the macroscopic level, the whole leaves and plantlets were examined. In a more detailed approach, the focus was on the individual leaflet cells of all species.

### 5.3.1 Dehydration of mosses (Gametophyte tip)

In this experiment single leaves of the gametophytes of each species were monitored for their loss in size. For this a gametophyte was selected from a plate, then taken with a tweezer (under unsterile conditions) and put on a glass slide. From this selected gametophyte a single leaf was examined.

Then macroscopic high-resolution images were taken with the "*Nikon SMZ1500 Stereo Microscope Bino Fluo*" and the used camera was a "*Nikon DS-Ri2*". For those pictures, the "*HR Plan Apo 1x WD 54 Nikon*" objective (nosepiece) and a ring light was used, for even light distribution. The Zoom of the microscope was set to 8.0x. The recordings were then analysed manually using the program "*NIS-Elements BR (Nikon)*".

The area of a single selected leaf was then measured as described in *chapter 5.7*.

To give the moss maximum hydration at the beginning, 10 µl distilled $H_2O$ were being drizzled on the moss after being placed on the slide. The water was then partially absorbed by a filter paper, to give the moss no water reservoir.

The microscope was set to take High-resolution images every minute, which was possible because of the used "*NIS Elements BR 5.11 software*". Since the moss changed its structure while dehydrating, focusing was necessary so this process required human attendance and wasn't fully automated. After around 40 min in every moss species, there was no significant drying visible anymore, but the experiment ran 1h for every moss species.

Photographs of the dehydrating gametophytes were taken every minute, for 1h. Then the dehydration process was done.

The output of this area measurement was provided by the program in µm². The data for each run were then used for statistical analysis (Chapter: 5.7 Statistics and data processing).

### 5.3.2 Rehydration of mosses (Gametophyte tip)

The same gametophyte from the dehydration experiment on the glass slide was rehydrated by adding around 30 µl distilled $H_2O$ to it. The outcome in this scenario was different between the species but in almost all species after 20 min the gametophytes appeared rehydrated. After 30 min, an additional 10 µl distilled $H_2O$ were added to the gametophyte. The rehydration of each moss ran for 50 min, and pictures were taken every minute.

The settings under which the rehydration pictures were taken, were the same as *in chapter 5.3.1*.

The output of the rehydration area measurement was also provided by the program in µm² and then used for statistical analysis (*Chapter: 5.7*).

### 5.3.4 Light microscopy of selected moss species on a cellular level (single leaf)

We looked for more detail on the cellular level by analysing the size of the individual cells of each species in its hydrated and its dehydrated form. For these closeup pictures, the light microscope (Nikon Eclipse Y-IM), with a 40x/NA 0.75 objective (Nikon Plan Fluor 40x/0.75) was used.
The fresh leaves of each hydrated moss species were put on a glass slide and covered with distilled $H_2O$, this was then covered by a coverslip and sealed off with hot/liquid Vaseline. Since only single leaves of the gametophytes were examined the dehydration process was much faster, so after 20 minutes the leaves were fully dried. The fully dehydrated leaves were covered with an UV resin ("*UV Resin hard*" by "*DecorRom*"), which hardened after irradiation with UV light. The resin was used, so no air bubbles were in between the leaf, to conserve its structure to that timepoint and to keep the leaf in place.

The "*Nikon NIS-Elements BR software*" was then used to make multilayered images and measure the area within the cells as (previously) described in *chapter 5.3.1*.

The stereo Microscope Nikon Eclipse Y-IM microscope was used to examine the cells of the moss leaves. The recordings were transferred to the computer, where they were analysed for cell size, length, and other parameters in NIS-Elements BR. These parameters were measured manually by framing or measuring each cell by hand on the computer. *C. conicum,* because it is a multi-layered species was especially difficult to bring into focus.

Around 40-50 cells in each species were measured and then the results were tested for their power. All species except *C. conicum* showed a power value of far over 80% high power in the power test which is significant. *C. conicum* had a power value of 77%, this can be led back to the fact that *C. conicum* has more than one cell layer in its leaves. The cells of *C. conicum* do not dry out in such a uniform matter as in the other, one cell layered species. The exact number of cells (=n) which were measured per species is depicted in *appendix 1.*

Based on the gained data, the area, the length, and width of the cells of each moss species was measured (*Chapter: 6.2*).

### 5.3.5 3D visualization of selected moss species

To show the structure and height differences in the hydrated and dehydrated moss leaflet cells, 3D videos of the mosses were made. These 3D recordings were possible because the light microscope (Nikon Eclipse Y-IM) in combination with the "Nikon NIS-Elements BR". The software took high resolution images of multiple image layers, it automatically focused on individual depth within a given thickness of the leaf ("extended focus" setting). The single images were saved.

The focused pictures of all depth grades were then stacked on each other to create a 3D image of the moss. With another function in the program, this 3D element was then turned in all directions to make 3D screenshots as well as 3D videos, the permalinks for these videos are listed in *chapter 10.2*.

## 5.4 Survival of the mosses after dehydration

To answer the question if these four tested moss species will survive the dehydration and then still live, another experiment was done.

Under sterile conditions in the laminar flow, each moss species was dried on a glass slip for timepoints of 0, 5, 10, 20 minutes. Those timepoints were chosen according to previous dehydration experiments (see also *figure 10)* and indicated as sectors on the bottom of the agar plate.

The petri dishes were then sealed with parafilm® and placed in the growth cabinet (Conviron, *Figure 3 B*). Day and night regime were set to 14h/day and 10h/night**.**

Right after the mosses were implanted onto the petri dish all mosses from all timepoints looked healthy. To further analyse their survival on the petri dish, later pictures were taken after 0, 2, 10 days to analyse revival of the plants.

## 5.6 Photosynthetic activity of the mosses

The photosynthetic activity correlates with water stress in plants (Allen and Ort, 2001). Therefore, photosynthesis is an important parameter to test the stress level in de-hydration and re-hydration of mosses.

The photosynthetic activity was measured with a fluorometer which indicates the chlorophyll fluorescence of the mosses (not the direct photosynthesis rate). Fluorescence is emitted by the chloroplasts due to the fact, that the energy absorbed by a photon raised the chlorophyl molecule to an excited state. In our experiments, the moss received a short red-light pulse, which brought the chlorophyl molecules into the excitation stage. Then the light was turned off again this brought the molecule into the relaxation stage. In this stage, the emitted fluorescence is measurable.

Before the experiment started, the mosses were put under light deprivation for at least 10 minutes to completely turn off the plant's photosynthesis activity. If this hadn't been done, the photosynthesis activity would interfere with the measured fluorescence. The mosses were measured macroscopically, on a stereo microscope stand. The used hardware was the "*Chlorophyll Fluorometer IMAGING-PAM (Walz Mess- und Regeltechnik*)", including the headpiece, "*model: IMAG-MIN/R (Walz Mess- und Regeltechnik*)", where the light flashes were emitted. The analysis program which was used is called "Imaging Win".

The moss gametophytes of two different colonies from the same species were put on a glass slide which was then placed under the camera (AVT Manta 145B (Mini)). Every 120 seconds a photo was taken (the light which was emitted by the camera for a few milliseconds had no influence on the photoactivity of the moss). The light intensity in the program was set to level 3, this was a very low level which was needed to not overexpose the relatively small mosses.

The tested mosses were dehydrated for 1 hour. Then, the mosses were rehydrated for 50 minutes, again every 120 seconds a measurement of all parameters was taken. Two runs of this experiment were done, on two days.

## 5.7 Statistics and data processing

<u>Macroscopic leaf area measurement:</u>

To measure the area of a single leaf through time in every timepoint, the RGB value was used. The RGB value determines in the picture on the computer screen what colour each pixel has. Each pixel corresponds to a certain area in $\mu m^2$. Therefore, to measure the leaf area all green pixels are measured, as the leaf gets smaller, less pixels on the screen are green. So, the area of all green pixels corresponds to the resizing of the gametophyte. Since only a single leaf needed to be measured, this single leaf was framed and only the (green) area within this frame was measured. The measurement was taken as depicted in *figure 4 A)*.

Many tries had to be done to get the leaf alignment right, this factor was determined by chance as, single leaves were often overlayed by other leaves those photographs would have been useless later in the area measurement. In addition to that the recording had to be started instantly as the leaves of the gametophytes dried out very quick.

The results of the area measurements were then provided by the program (Nikon NIS-Elements BR) via excel sheet in µm².

Cell area measurement:

Cell length and width were measured manually. The output of the area measurement was provided in µm.

In the cellular area measurement experiment, the cell areas were measured manually with the Nikon-microscope intern program "*NIS-Elements BR*". The cells were framed and within this frame the area was measured. With the obtained data from the area of the cells (in µm²) in all 4 moss species, a power test was done. The power test determines how many cells one must measure to say with at least 80% confidence, that there is a significant difference between the two groups. The two tested groups are in each moss species: hydrated moss leaves and dehydrated moss leaves. The cellular area was measured as depicted in *figure 4 B)*.

The power test was done using R. The quantity of the counted cells is n of each species were 50 for *A. undulatum*, 42 for *C. conicum*, 40 *F. hygrometrica* and 40 for *P. patens*. The values of the power test were 98 % for *A. undulatum*, 77 % for *C. conicum*, 99 % *F. hygrometrica* and 100 % for *P. patens*. So, there was a significant difference in the dehydrated size of the cells and the hydrated size of the cells. The respective codes can be found in the *appendix 1*, *chapter 10.*

Cell length and width were measured manually, as depicted in *figure 4 C), D)*. The output of the area measurement was provided in µm. These measurements were also taken manually.

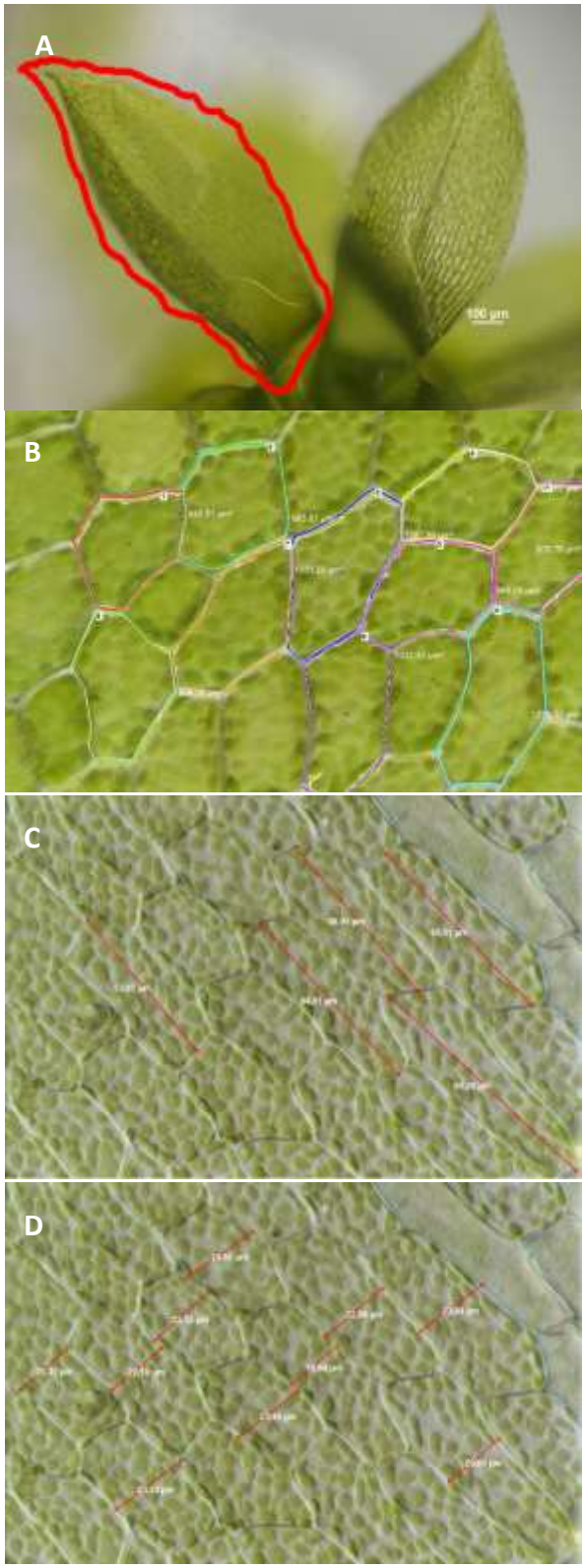


***Figure 4*** *macroscopic leaf area measurement in A), cell area measurement in B), cell length measurement in C) and cell width measurement in D)*

Treatment of the data:

Since the software programs delivered the data as Microsoft excel tables. Some statistics were done in these excel tables. Also, the excel tables were used through R to create statistical figures.

The data in the different experiments were always normed to percent, so that it was possible to compare the different species and different experiments to each other. The initial measurement was set to 100 % from this value the loss or gain of area, length, width, and photosynthetic activity was shown.

The examination of the leaves and in a more detailed approach the examination of the individual cells as well as the photosynthesis activity measurement were all done this way.

With the obtained data from the cell length and width measurements boxplots were made with R. In other cases, bar graphs fitted the representation of the data better, those were done directly in Microsoft Excel.

# 6. Results

## 6.1 Dehydration & rehydration of leaf area in mosses

The four bryophyte species, *A. undulatum, C. conicum, F. hygrometrica* and *P. patens* were screened for sensitivity to water stress, to determine if there was variation in the response to dehydration and the following rehydration. Single leaves were examined for their dehydration and rehydration behaviour. The measurement was done as described in *chapter 5.7 Statistics and data processing*.

In the following picture series (*figure 5*) the representative dehydration and rehydration process of a moss are shown in *F. hygrometrica*.

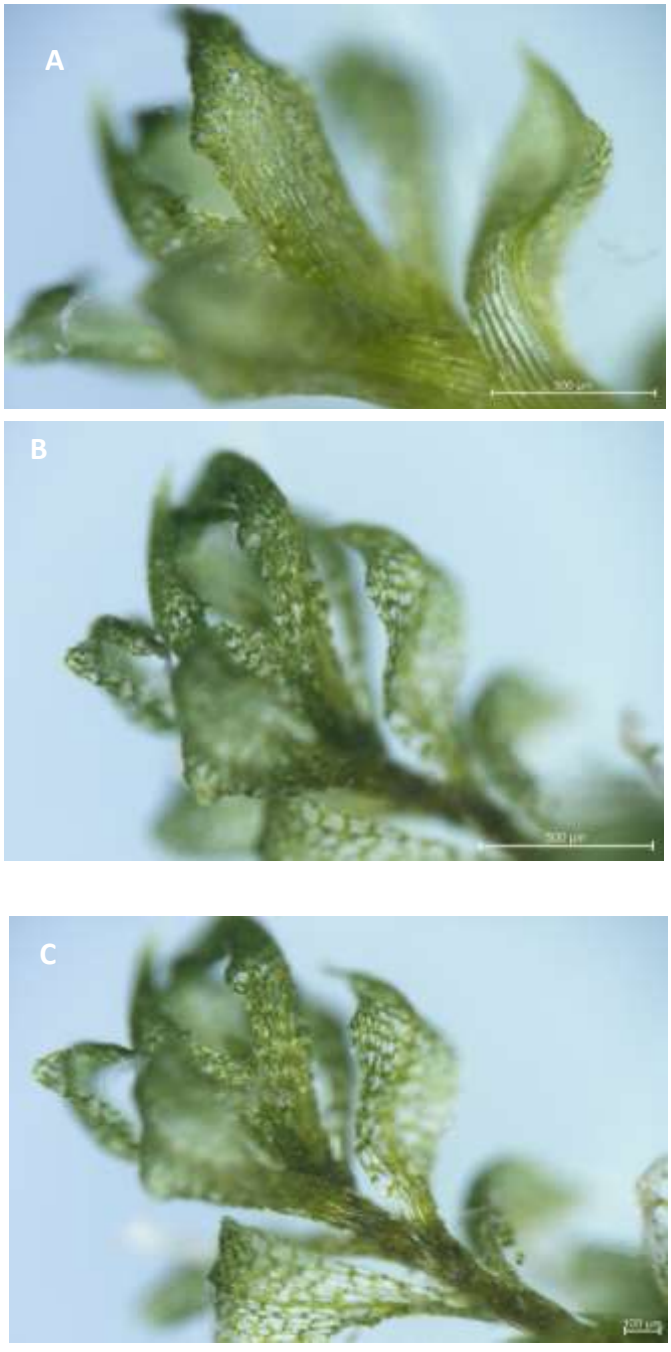


***Figure 5*** *Representative snapshots of Funaria hygrometrica fully hydrated in A), dehydrated in B) and rehydrated in C).*

*Funaria hygrometrica*, is depicted in *figure 5*. In its hydrated form *A*) it had a bright green colour with chloroplasts evenly distributed in the leaves and a turgid healthy appearance. *Figure 5 B)* shows, after 60 min of dehydration the green was much darker, the chloroplasts were only on the cell edges which made the leaves look full of holes, also the leaves were very crumbled together in the direction of the stem.

After 50 min of rehydration in figure 5 *C)* the moss was still dark green, the chloroplasts were still on the edges of the cells, but the leaves were more unfolded.

Photos of the leaves were taken, in the dehydration scenario for 60 minutes (*figure 6*) and in the rehydration scenario for 50 minutes (*figure 7*). The area of those leaves in each time step was measured in µm². The initial values which were gained from the area measurement are set as 100 %, from this value the other timepoints are shown in percent relative to the initial value.

The dehydration of *A. undulatum, C. conicum, F. hygrometrica* and *P. patens* is shown in *figure 6*. *A. undulatum* lost 40 % of its former hydrated area when dehydrated for 20 min after that the area was constant. *C.conicum* lost 65 % of its former hydrated area, after 30 min most of the dehydration has happened. *F. hygrometrica* lost 50 % of its former area, while all the shrinkage happened after 20 min of dehydration. *P. patens* was very stable in terms of its behaviour when dehydrated, it lost only 37 % of its former hydrated leaf area.

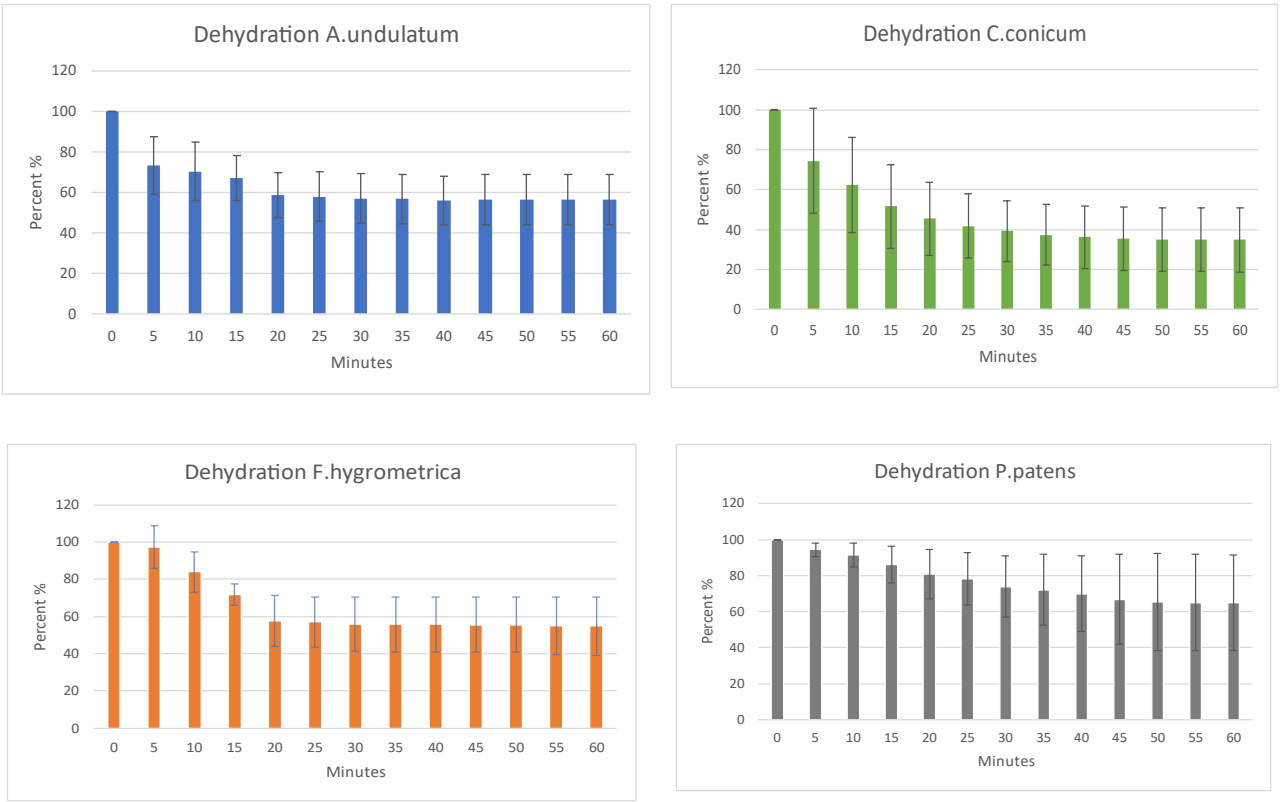


***Figure 6*** *Dehydration of the tested moss species; Data obtained from the Nikon SMZ 1500 microscope*

The rehydration process of each tested moss species is depicted in *figure 7*. *A. undulatum* gained 50 % compared to its dehydrated state after 10-15 min. *C. conicum* gained 30 % compared to its dehydrated state after 20 min. *F. hygrometrica* gained almost 200 % compared to its dehydrated state after 20 min. *P. patens* gained 130 % compared to its dehydrated state after 10 min.


***Figure 7*** *Rehydration of the tested moss species; Data obtained from the Nikon SMZ 1500 microscope*

In a rough overview, the moss species seem to behave similar to each other but there are huge differences in the reaction of these species to water stress. This fact is made clear when the data of all moss species are applied on one graph (*Figure 11*).

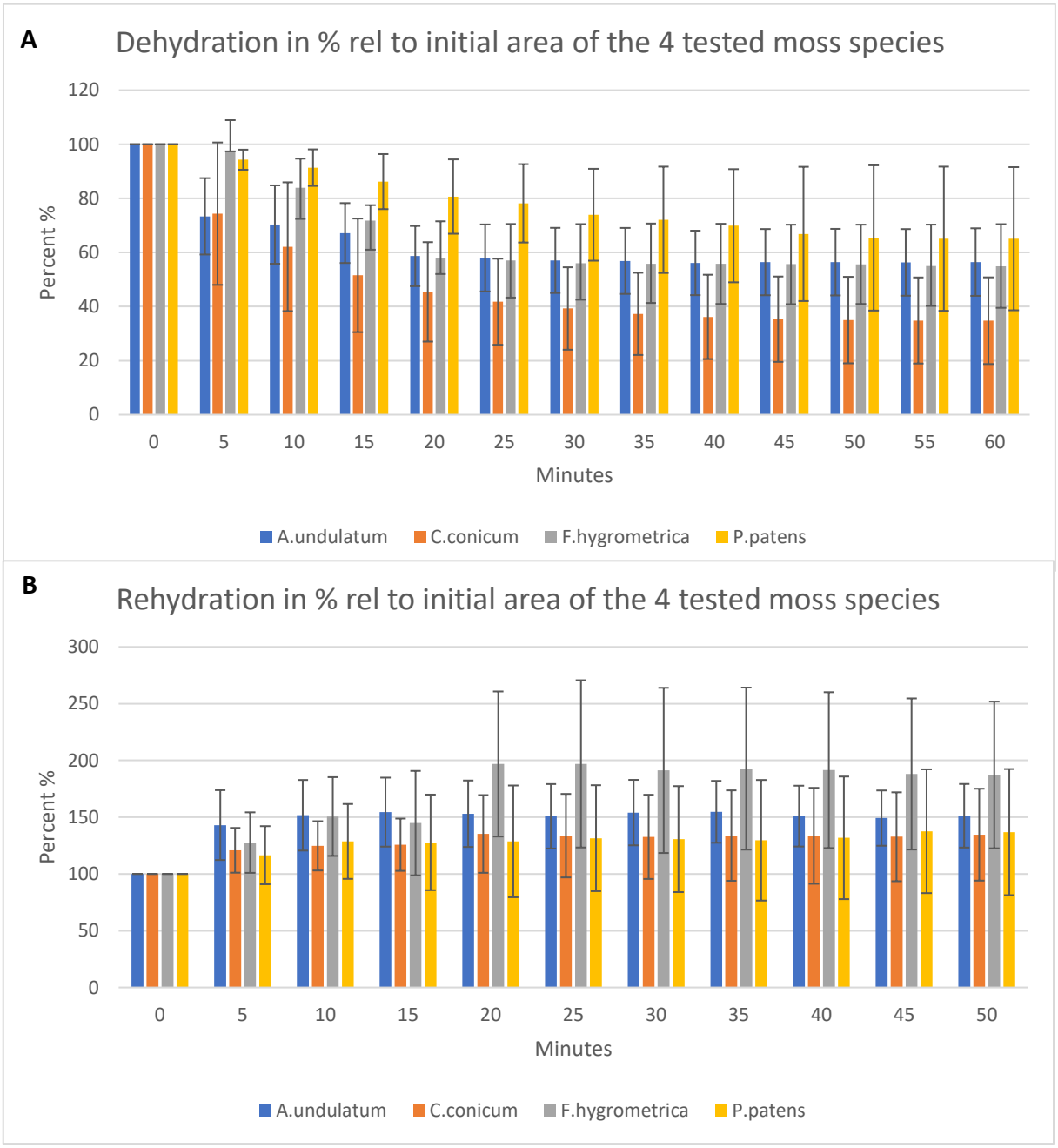


***Figure 8*** *Experimental setup. Data obtained from the Nikon SMZ 1500 microscope. A) The dehydration progress through time, of the leaf area of the 4 tested moss species are shown. B) The rehydration progress through time, of the leaf area of the 4 tested moss species are shown.*

This experiment generally showed that there are significant differences in the de- and rehydration behaviour of the tested moss species. *Figure 8 A*) shows that *Physcomitrium patens* lost the least of its area, while in comparison *C. conicum* shrank the most out of all these species.

In terms of dry stress tolerance, the most resilient species from these experiments were *Funaria hygrometrica* and *Atrichum undulatum*, as they did not lose much of their leaf area and regained a lot of their former leaf area *(Figure 8 B*).

In the following experimental setup, a photo was taken every minute and the area of a single selected leaf was measured. In the representative photos of *figure 9* and *figure 10* only 0, 10, 30 min are shown because at that timepoints the largest differences in dehydration and rehydration conditions could be seen.

In addition to the pictures shown in *figure 9* and *figure 10* videos were made, these videos are available via the permalinks from *chapter 10.1.* The pictures which were made, were lined up to create a short video of the dehydration & rehydration of the mosses.

Representative photos of all tested species dehydrating are shown in *figure 9,* the species rehydrating are shown in *figure 10*. *Conocephalum conicum* lost a lot of its former area (*figure 9 B*). In contrast to that, *Physcomitrium patens* lost less area while under dehydration *figure 9 D).* This representative picture series shows how the data from *figure 8* looked under the microscope, from a single leaf as shown in *figure 9* and *figure 10* the area in all the tested mosses was measured.

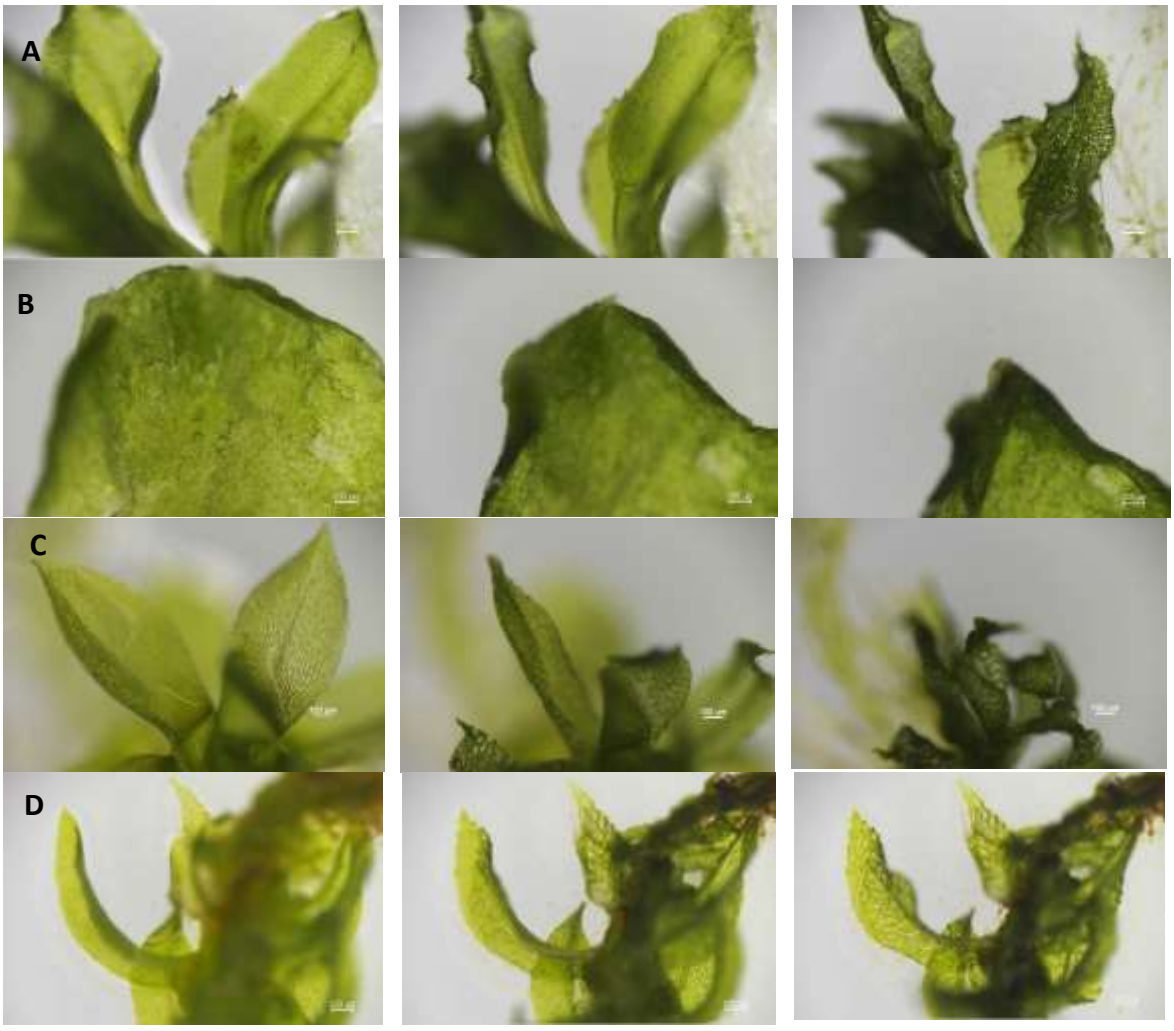


***Figure 9**, Representative "Dehydration" photos. Data obtained with the Nikon SMZ 1500 microscope. In both pictures the timesteps 0-30 min, when the most happens are depicted. A) Atrichum undulatum, B) C. conicum shows the most shrinkage of the leaf area, C) Funaria hygrometrica, D) P. patens shows less shrinkage of the leaf area.*

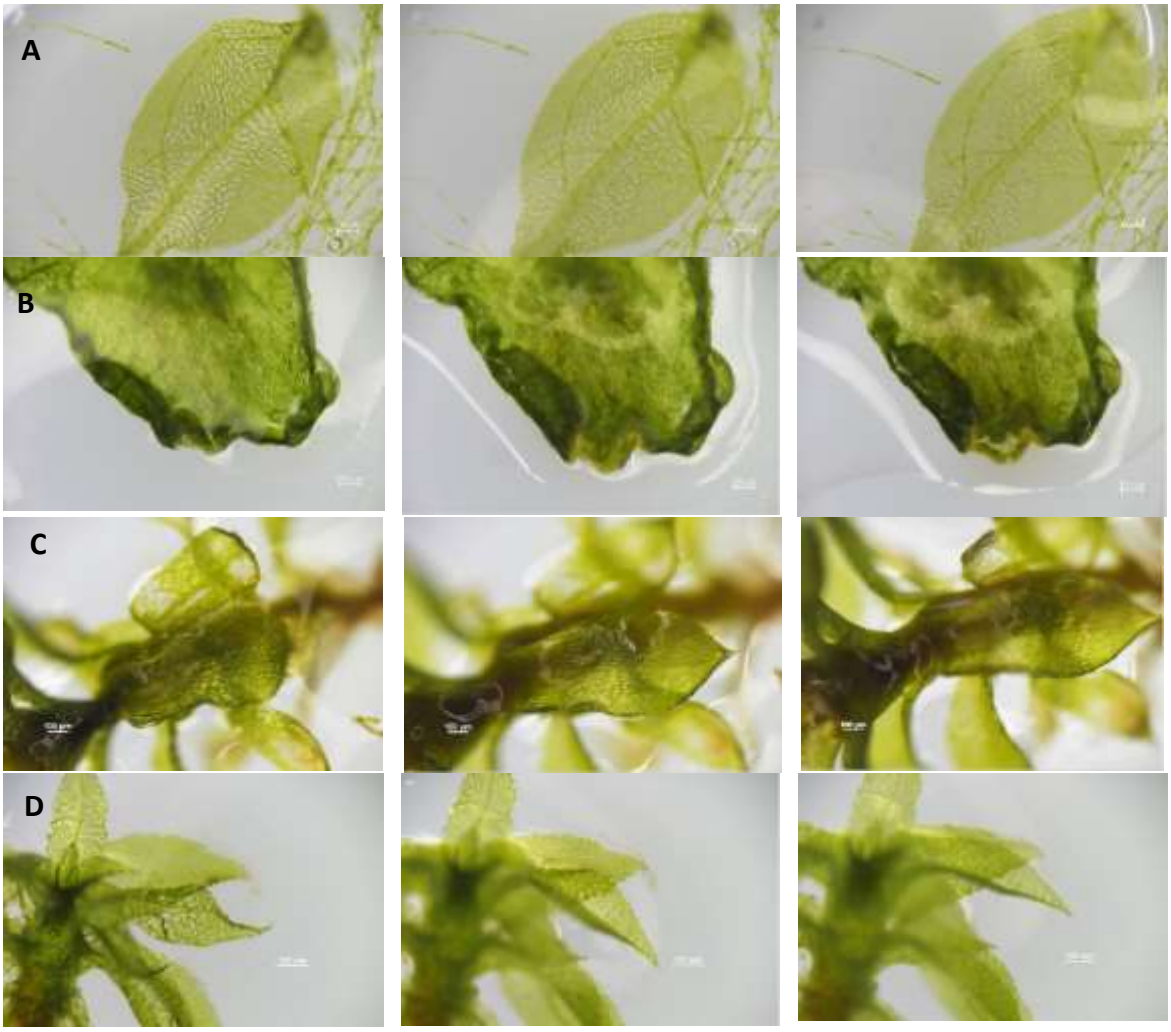


***Figure 10**, Representative "Rehydration" photos. The data is again obtained with the Nikon SMZ 1500 microscope. In both pictures the timesteps 0-30 min, when the most happens are depicted. A) Atrichum undulatum, B) C. conicum regains the least amount of leaf area. C) F. hygrometrica regains the most of its former leaf area, D) P. patens.*

In *figure 10* representative photos of rehydration are shown. *C. conicum (figure 10 B)* which does not recover very well in terms of its leaf area and *F. hygrometrica (figure 10 C)* which recovers well. In the "Dehydration" as well as in the "Rehydration" event, again only the areas of single leaves were measured in each species.

## 6.2 Area measurements at the cellular level

With the goal to go into more detail single cells of the 4 moss species were observed especially their appearance in its hydrated and dehydrated state. With the gained data from the area measurement, a boxplot was made (*Figure 11)*. This Plot shows visually that there is a difference between the size of the dehydrated cells and the hydrated ones. This boxplot also shows that cells of some species dry out more than others. *Funaria hygrometrica* has when compared the mean cell sizes from 1006 µm$^2$ in its hydrated and 402 µm$^2$ in its dehydrated state the biggest difference in cell area which is a 60% decrease in cell size.

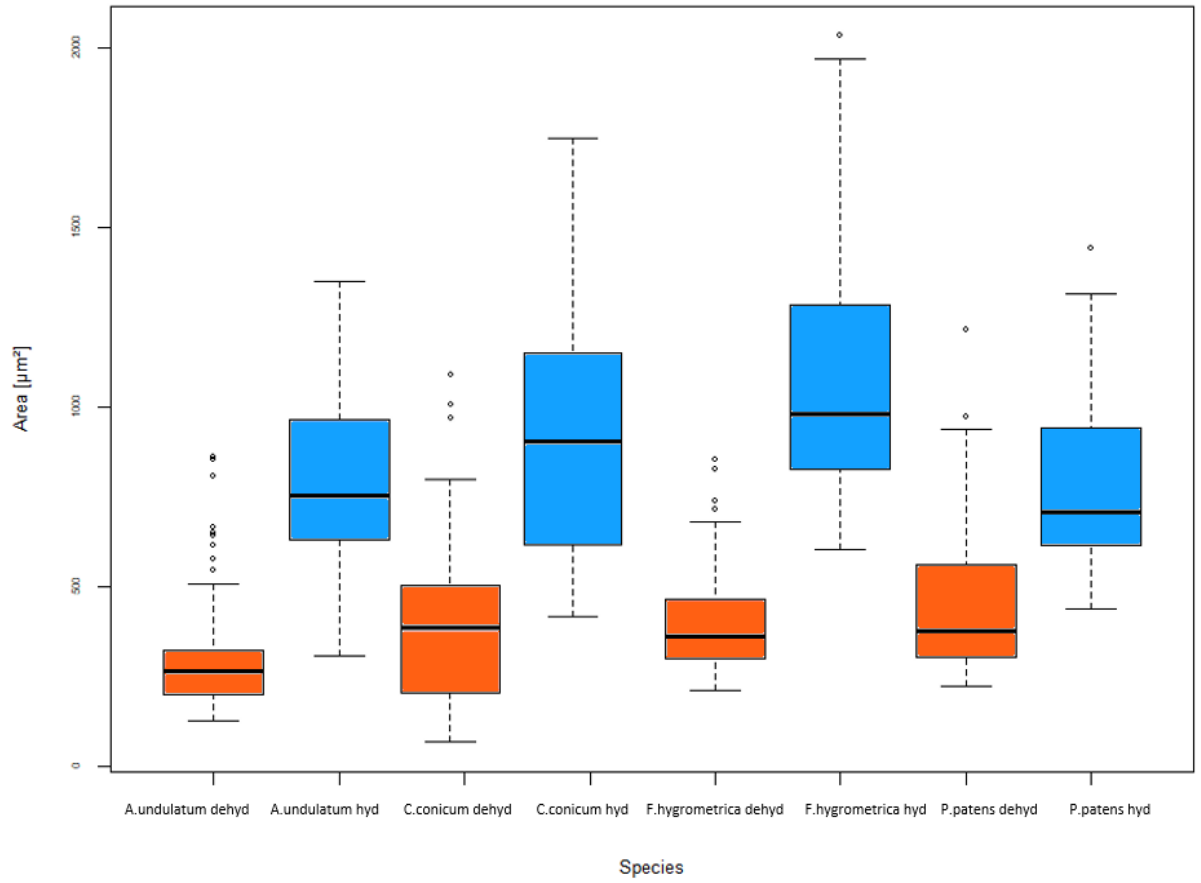


***Figure 11,*** *Cell area measurement boxplot. Data obtained from the Nikon SMZ 1500 microscope. The measured cell areas of the moss species in its dehydrated (orange) vs. its hydrated (blue) state are shown.*

*Atrichum undulatum* went from its mean area of 777 µm$^2$ in its hydrated state to 325 µm$^2$ in its dehydrated state, which is a 58 % decrease in cell size. *Physcomitrium patens* went from 840 µm2 in its hydrated state to 464 µm2 in its dehydrated state, which is a 44.8% decrease in cell size. This species had the least difference in cell area when dehydrated and hydrated cells are compared. *C.conicum* went from its mean area of 939 µm$^2$ in its hydrated state to 408 µm$^2$ in its dehydrated state, which is a 56 % decrease in cell size.

Hydrated Dehydrated

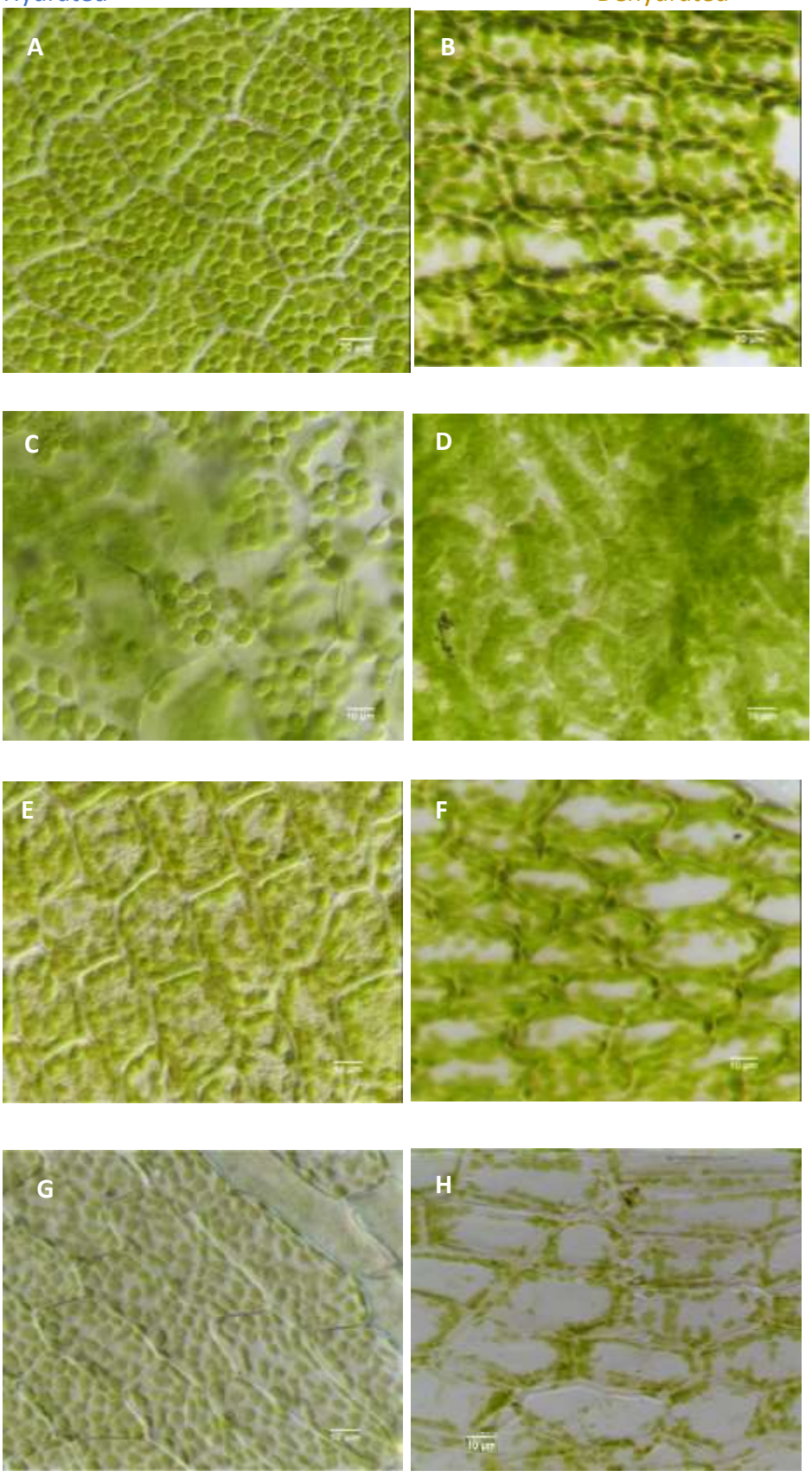

***Figure 12*** *Closeup photographs of selected moss species in hydrated and dehydrated state. A) A. undulatum dehydrated; B) A. undulatum hydrated; C) C. conicum hydrated; D) C. conicum dehydrated; E) F. hygrometrica hydrated; F) F. hygrometrica dehydrated; G) P. patens hydrated; H) P. patens dehydrated.*

The cells depicted in *figure 12* were used for the area measurements at the cellular level. As it can be seen in *figure 12*, all the moss species do have very different cell forms. Of those species *P. patens* had the most rectangular and longish cell appearance (*Figure 12 G, H*). The other single cell layered species had a more hexagonal cell shape (*Fig. 12 A, B, E, F*). The cells of *C. conicum* did not have a clear distinct form (*Figure 12 C, D*).

In the dehydrated cells, the chloroplasts tend leave the centre of the cell and the cells seem to shrink in width (*Figure 12 B, F, H*). In dehydrated *C. conicum* the chloroplasts more or less stay in their former hydrated position (*Figure 12 D*).

## 6.3 Cell length and width when dehydrated

The shape of the moss cells differed between the tested species in the hydrated condition. To analyse the effect of cell form in the process of dehydration, cell length and width were measured on leaflets of all four bryophyte species. Around 60 measurements were taken before and after drying the mosses for 60 minutes on a glass plate. The results are shown as a box plot in *figure 13*, *table 1* and *figure 14*, *table 2*, respectively.

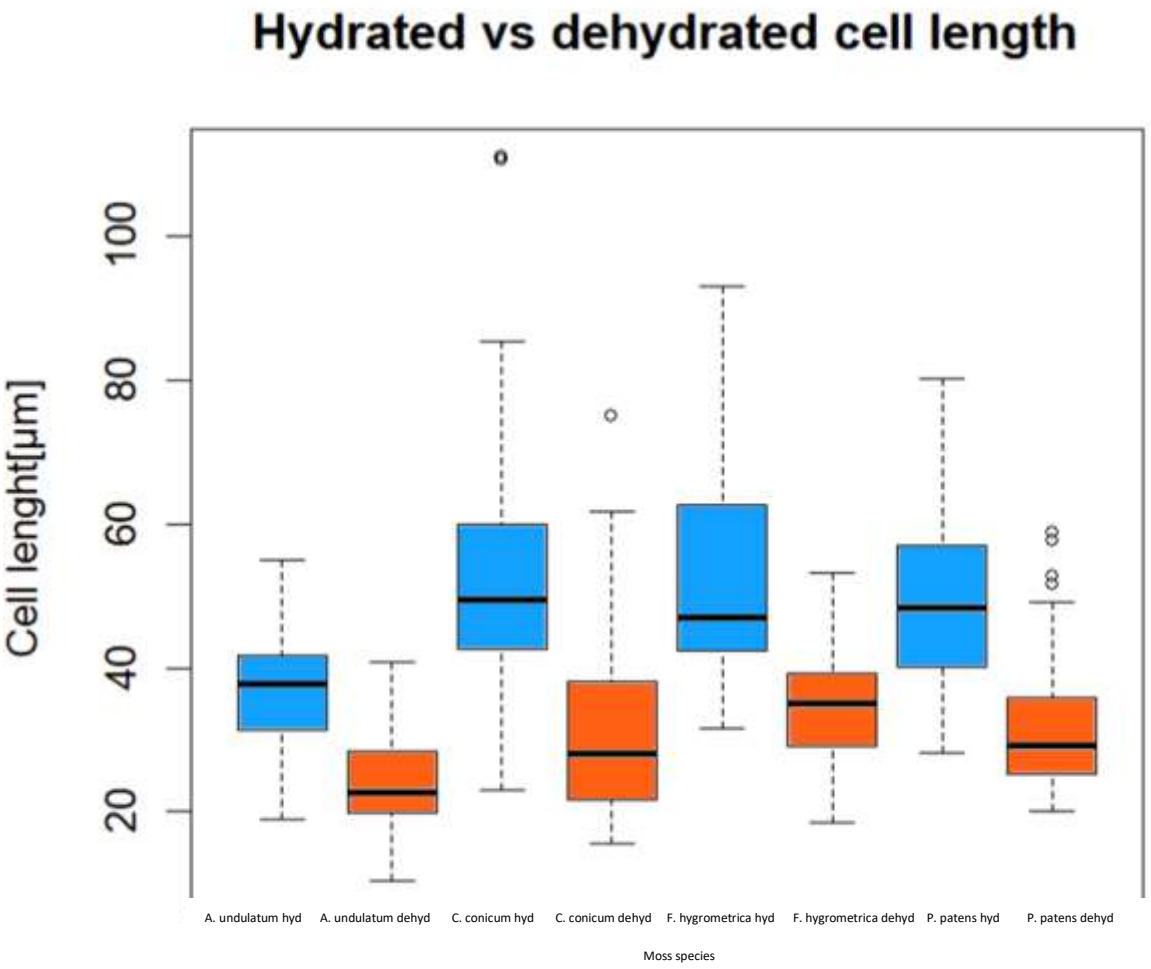


***Figure 13*** *Here the length difference from the cells of each moss species from its dehydrated (orange) vs. its hydrated (blue) state are shown. Also, it can be seen here clearly that some species shrink in their length more than other species.*

The mean lengths of the hydrated and dehydrated cells are shown in *figure 13* and *table 1*, all species shrink in length very similar to each other. The relative shrinkage of the cell length is shown in percent in *table 1*. *A. undulatum* has the shortest cell length. Interestingly the lengths of the cells are all very similar to each other, apart from *A. undulatum*.

| Cell length | Hydrated | Dehydrated | Shrinkage in % |
|---|---|---|---|
| *A. undulatum* length | 36.30 µm | 23.57 µm | 35% |
| *C. conicum* length | 52.57 µm | 31.39 µm | 40% |
| *F. hygrometrica* length | 53.02 µm | 34.76 µm | 34% |
| *P. patens* length | 49.50 µm | 31.49 µm | 36% |

*Table 1 comparison of cell length in the tested species*

The mean widths of the cells are shown in *table 2* and *figure 14*, the species in this measurement also display a similar reaction when dehydrated compared to the cell length measurement. The differences of the hydrated and the dehydrated form within the same species are also between 30-40 % as in the length measurement. In its hydrated form the species when compared are very similar to each other and the cell width ranges from 20.79 µm for P. patens, to 24.97 µm for A undulatum, 24,6 µm for C conicum and 25,44 µm for *F. hygrometrica*, respectively.

| Cell width | Hydrated | Dehydrated | Shrinkage in % |
|---|---|---|---|
| *A. undulatum* width | 24.97 µm | 16.74 µm | 33% |
| *C. conicum* width | 24.60 µm | 13.98 µm | 43% |
| *F. hygrometrica* width | 25.44 µm | 14.89 µm | 41% |
| *P. patens* width | 20.79 µm | 14.48 µm | 30% |

*Table 2 comparison of cell width in the tested species*

The difference in the shrinkage of the cell width and length are very similar. *F. hygrometrica* shows the biggest shrinkage in width, this shrinkage is also seen in the 3D image of this species, where *F. hygrometrica* crumbles together more than the other species (*Chapter: 6.4 3D visualizations of selected moss species*).

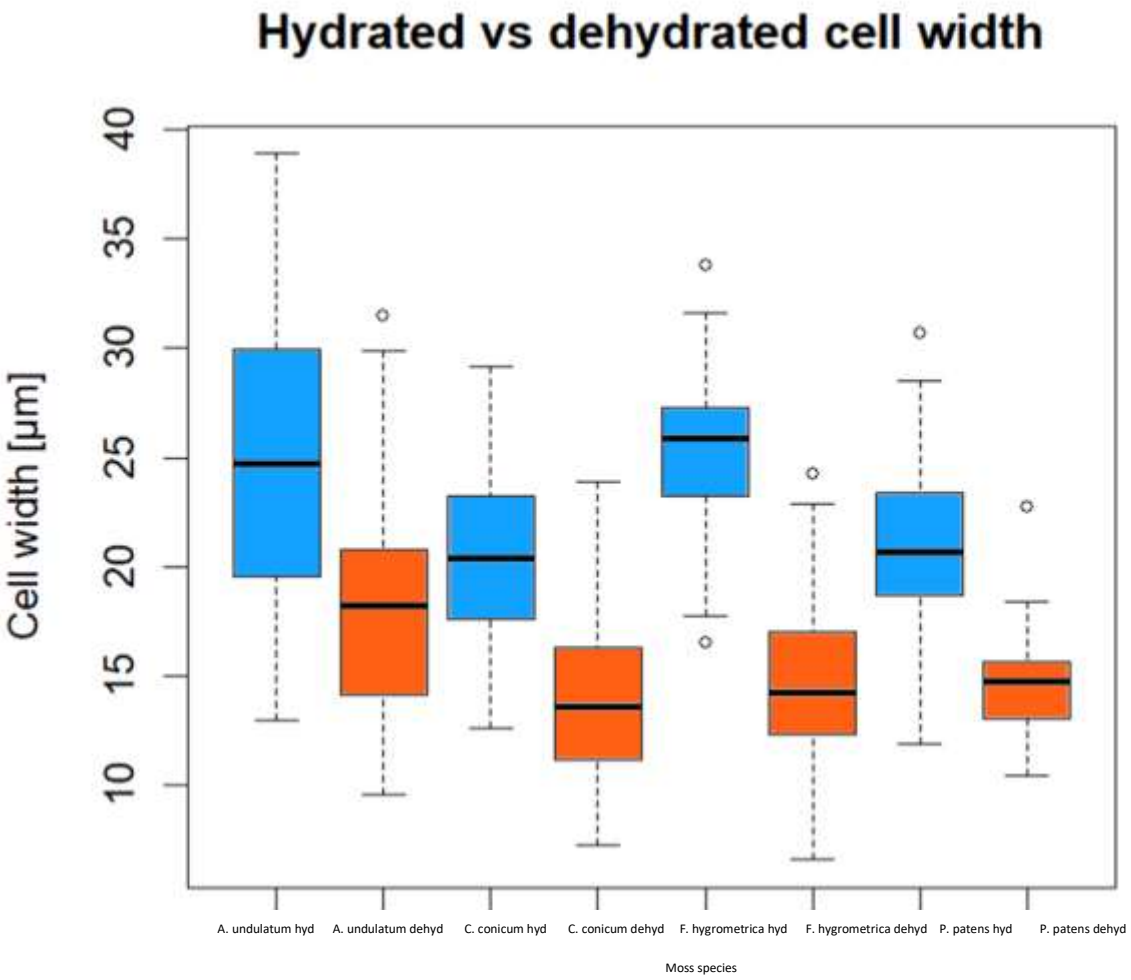


***Figure 14**, Here the width difference from the cells of each moss species from its dehydrated (orange) vs. its hydrated (blue) state are shown.*

## 6.4 3D visualizations of selected moss species

The stacking of the "Extended Focus" function allows for optical sectioning of the samples and the reconstruction of 3D images from each moss species. Such 3D-reconstructions were made in the hydrated and dehydrated state (after 20 minutes of dehydration). In the single cell layered species (*Atrichum undulatum, Funaria hygrometrica and Physcomitrium patens*), it can clearly be seen, when dehydrated, the chloroplasts move to the cell walls (cytorrhysis). From these 3D images, short videos were made, where the mosses were viewed in every angle, those videos are available via the permalinks from *chapter 10.2*.

*A. undulatum (Figure 15)* has a very uniform bubbly cell arrangement and structure in its hydrated state. The cells are fully turgescent and about 5-8 µm thick. The chloroplasts are evenly distributed in the cortex of the cells. When dehydrated still some structure is visible and the chloroplasts wander on the edges of the cell (cytorrhysis). The thickness of the cells is reduced to 2-5 µm.

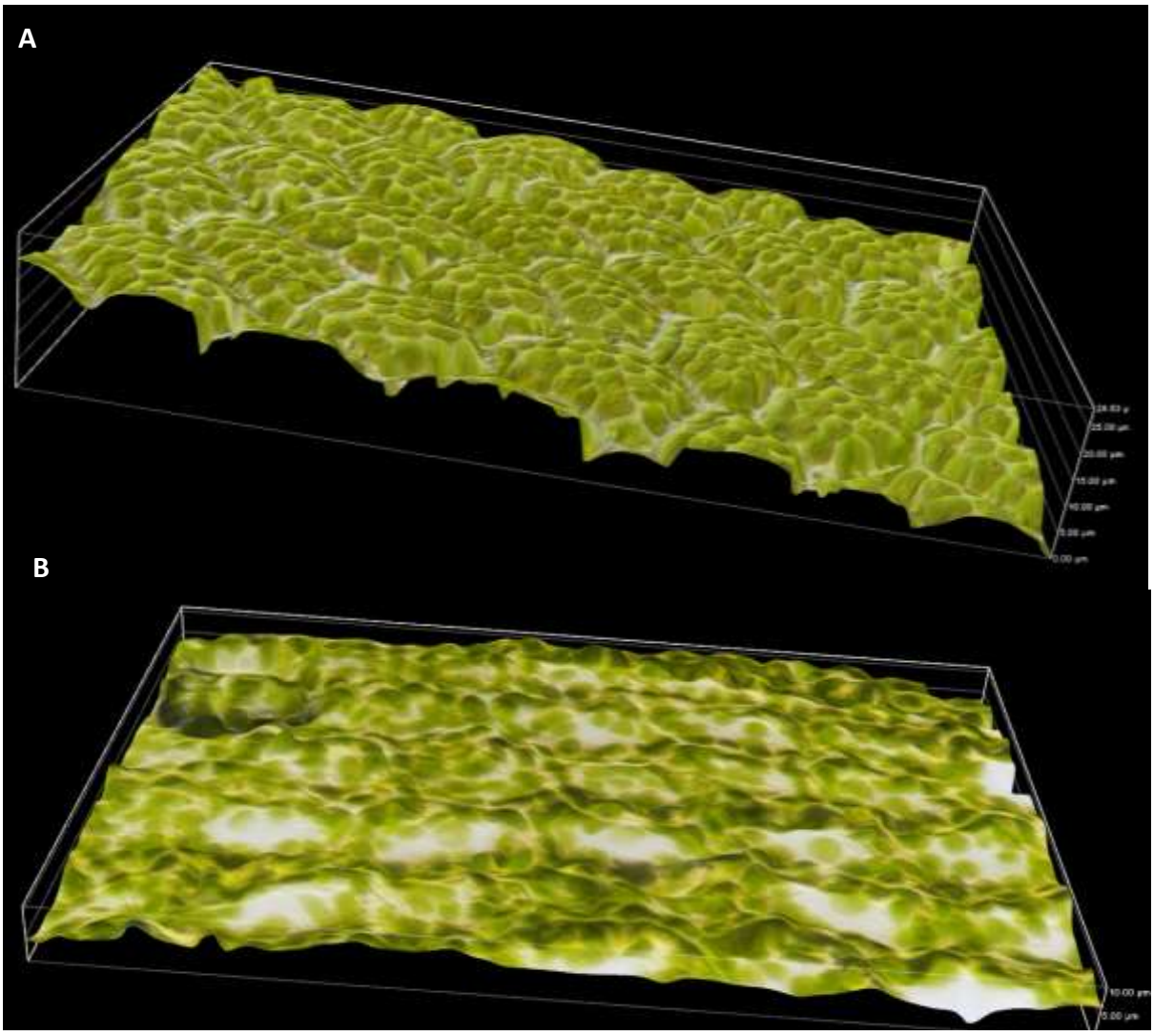


***Figure 15**, 3D photographs of selected moss species. A) A. undulatum hydrated; B) A. undulatum dehydrated.*

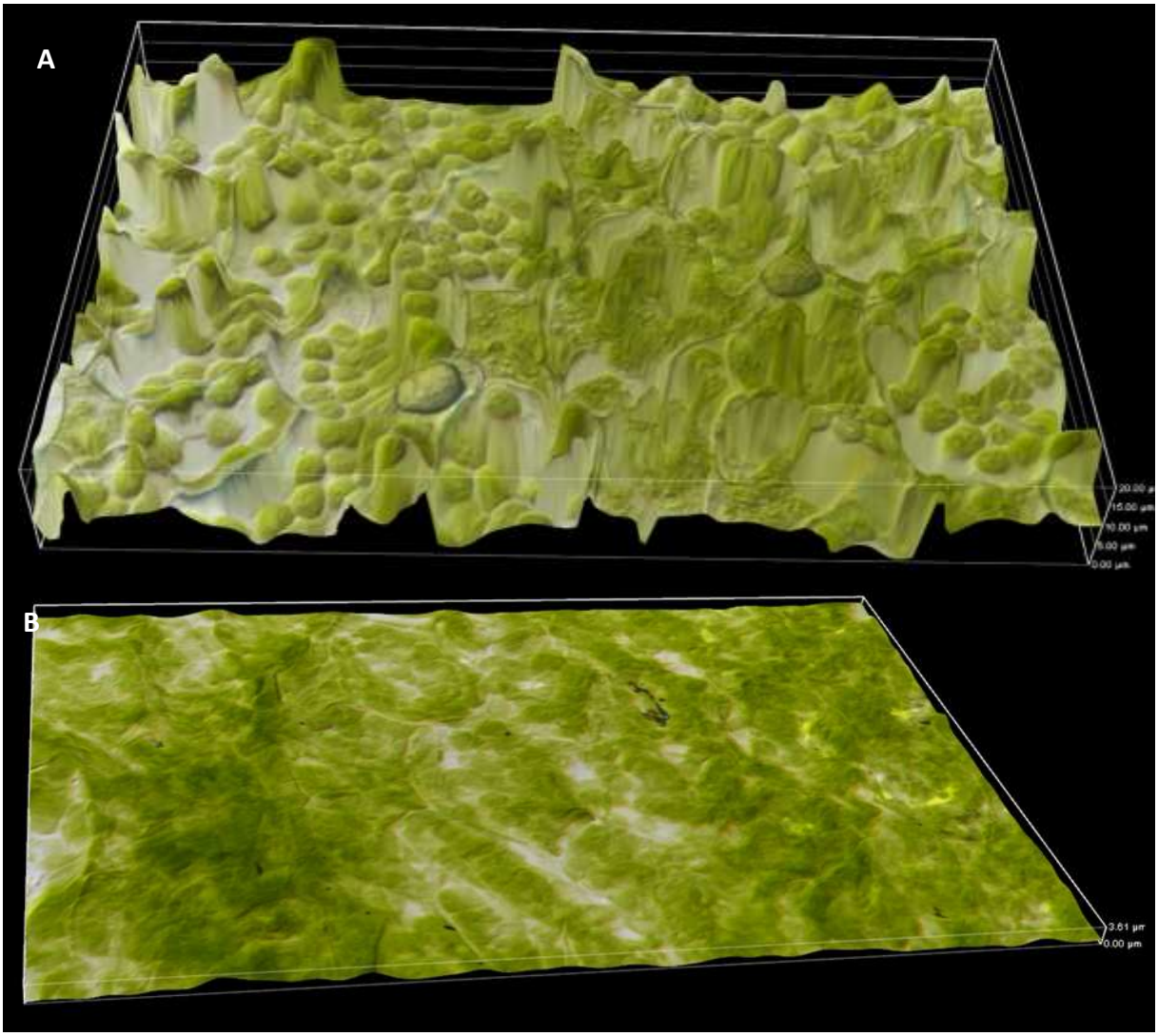


***Figure 16**, 3D photographs of selected moss species. A) C. conicum hydrated; B) C. conicum dehydrated.*

*C. conicum (Figure 16)* is a multiple cell layered moss species and a liverwort species, the differences in thickness compared to the other species are clearly visible when hydrated. The chloroplasts are evenly spread within the cells when hydrated and when dehydrated. When fully hydrated, the leaflet measures up to 20 µm in thickness. When dried out, this species becomes very flat, with no structure visible anymore and a height of only about 3 µm.

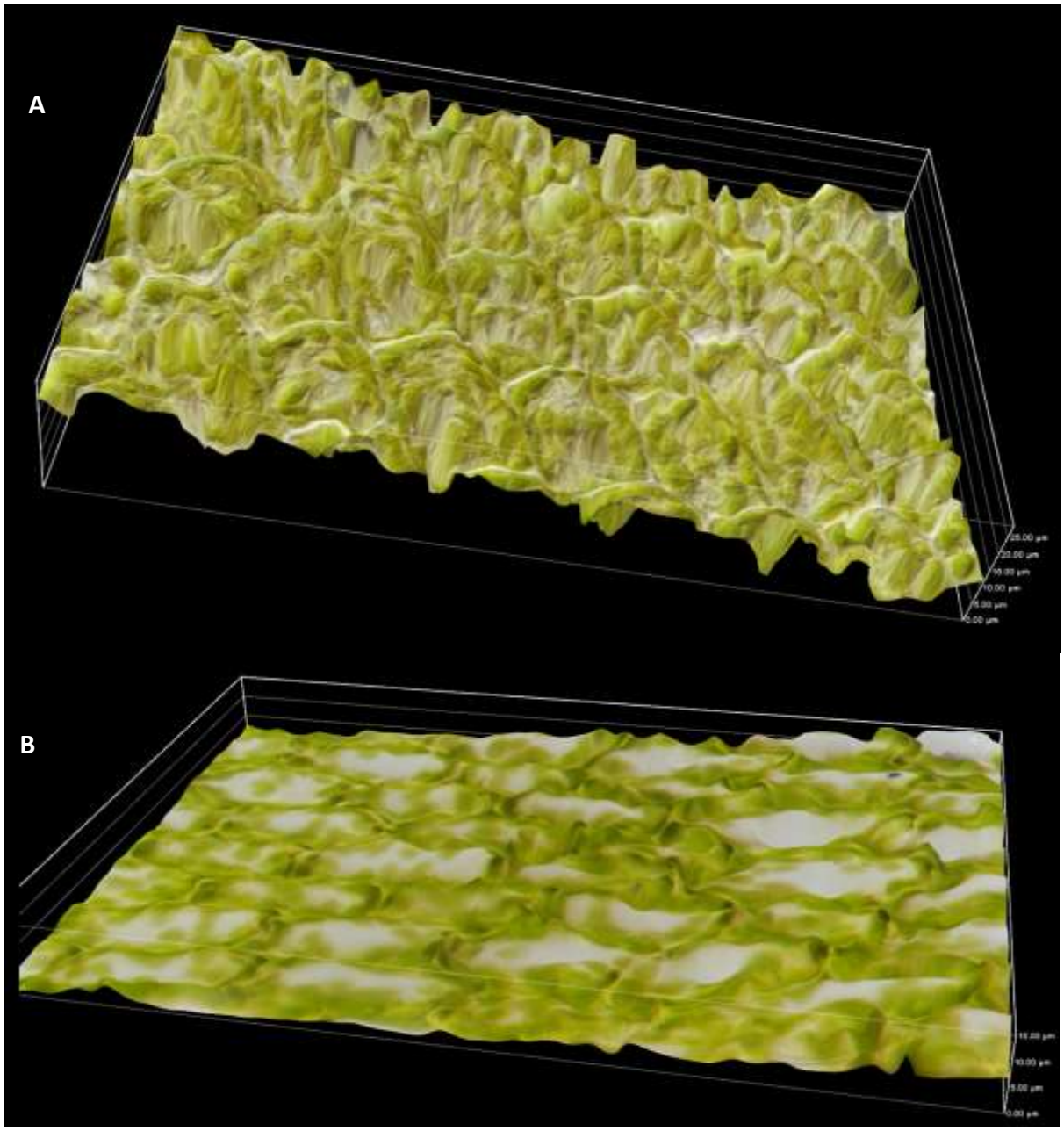


***Figure 17**, 3D photographs of selected moss species. A) F. hygrometrica hydrated; B) F. hygrometrica dehydrated.*

Although *F. hygrometrica (Figure 17)* has a very uneven structure in its hydrated form, height differences up to 15 µm in thickness. The species shows a similar behaviour as *A. undulatum* when dehydrated, the height differences are then only about 5 µm. When hydrated, the chloroplasts are spread evenly within the cells, while dehydrated they are gathered on the edges of the cells. The dehydrated cells are wrinkled in the same way as they are in *A. undulatum,* cytorrhysis is visible in *figure 17 B).*

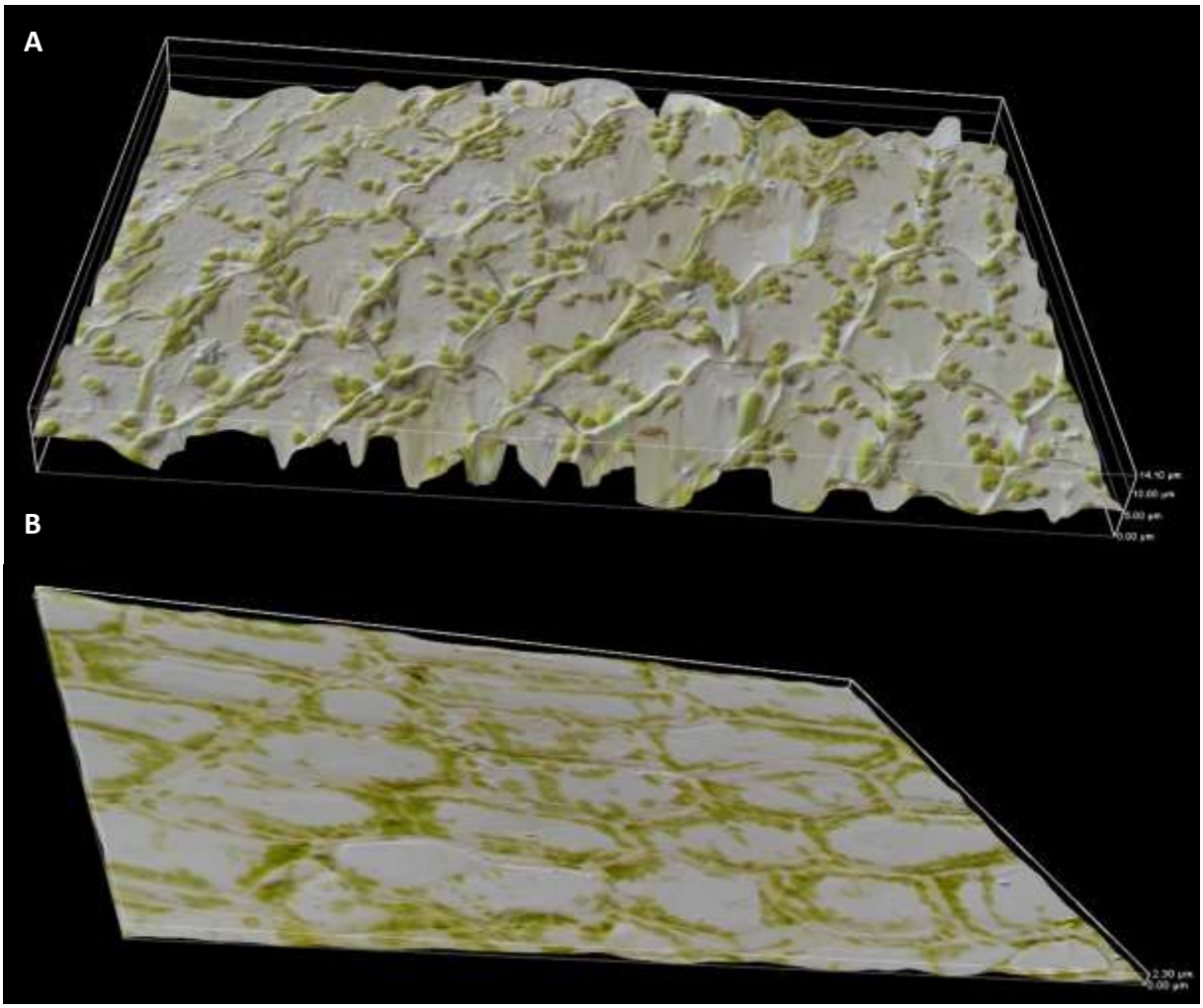


***Figure 18**, 3D photographs of selected moss species. A) P. patens hydrated; B) P. patens dehydrated.*

The microscope photos of *P. patens (Figure 18)* show that it has a lot of space (water) in the cells and fewer chloroplasts than the other species within the cells, compared to the other moss species. The thickness of these cells when hydrated is about 10 µm. When dehydrated the chloroplasts wander to the edges of the cells (cytorrhysis), like the previously described single cell layered mosses. But after dehydration there is almost no structure visible anymore and the leaf appears completely flat which is very different to *A. undulatum* and *F. hygrometrica*. The height of this moss when dehydrated shrank to about 1-2 µm.

## 6.5 Survival of the mosses after dehydration

To see if the four tested moss species will survive the dehydration and then still live afterwards, we performed a time-dependent dehydration experiment for 0, 5, 10, 20 minutes. The agar plate on which the mosses grew was split in four parts which represented the time of how long the mosses were dried out before planted back onto the plate. Pictures were taken after 2 days and 10 days to check for survival. The test for survival was just a visual test, the judgement of survival was based on my experience.

After 2 days, the photos showed that *P. patens* and *C. conicum* looked unhealthy when dried out for 20 minutes which is shown in *figure 19*. After the next measurement (10 days) it became obvious that *P. patens* and *C. conicum* died when dried out for 20 minutes. Unfortunately, the mosses were almost all infected with fungi after a while as depicted in *figure 20*.

The reason for that was probably that the glass slide on which the mosses were dried out wasn't sterilised properly. Nevertheless, this experiment showed which species survive dehydration in the long term.

At the timepoints 0, 5 and 10 minutes all mosses seemed to have survived. The 0-minute test was the control group which has proven the meaningfulness of this test, as every moss species survived. 5 and 10 minutes of dehydration was not enough to kill any moss species.

*Table 3* gives an overview of the results of this experiment after the last timepoint, 20 minutes. All the mosses were alive at day one. At day 2 still all mosses were alive, although *P. patens* and *C. conicum* looked bad (brownish appearance). On day 10 it was clear that *P. patens* and *C. conicum* did not survive (*Figure 20*), as they were totally brown and sometimes bleached and there was a lot of fungi within their petri dish.

| Survival of the mosses after 20 minutes of dehydration | | | |
|---|---|---|---|
| | Day 0 | Day 2 | Day 10 |
| A. undulatum | + | + | + |
| C.conicum | + | + | - |
| F.hygrometrica | + | + | + |
| P.patens | + | + | - |

*Table 3 survival of the mosses after dehydrated for 20 minutes*

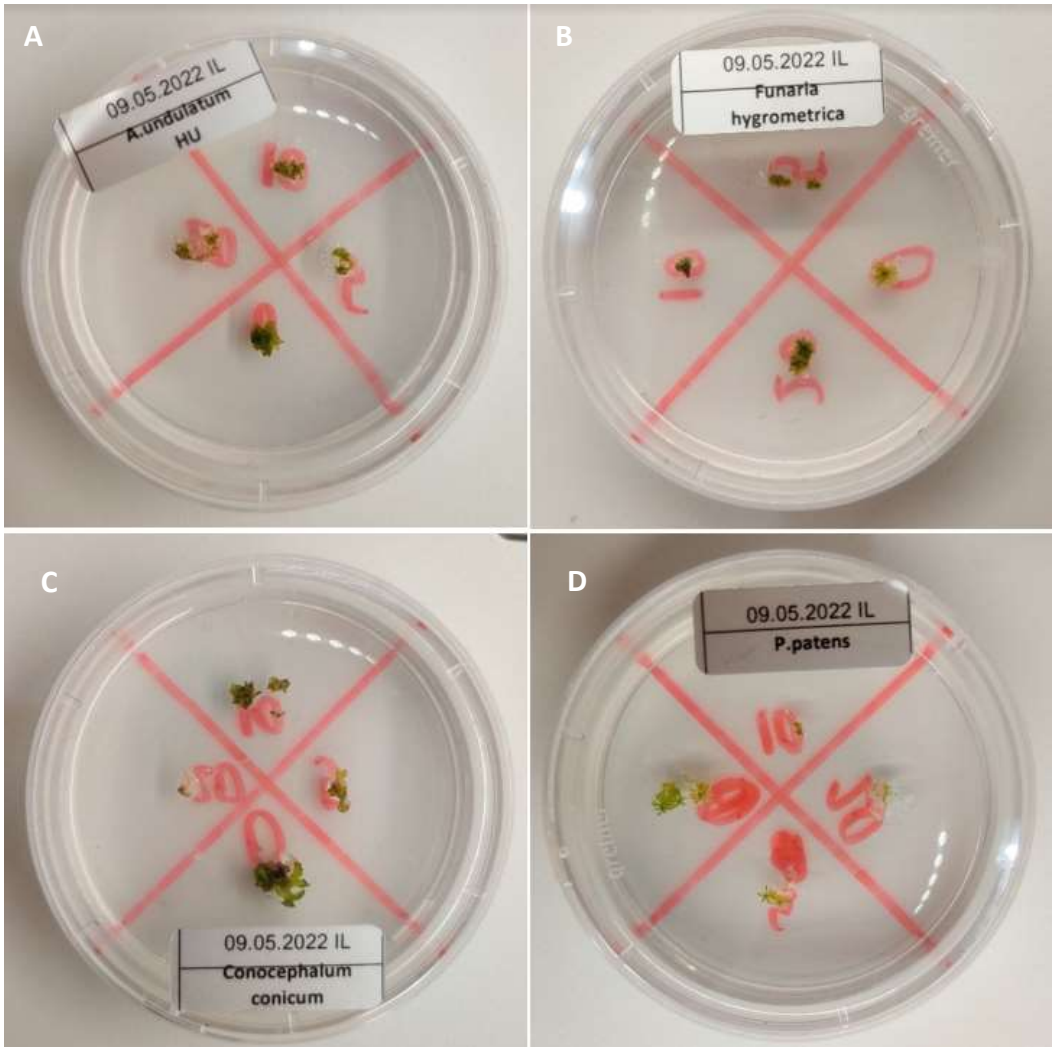


***Figure 19**, The dehydrated mosses after 2 days in the growth chamber. A. undulatum A) and F. hygrometrica B) survived all timepoints, while C. conicum C) and P. patens D) look unhealthy when dried out for 20 minutes.*

After 10 days *C. conicum* and *P. patens* died when dehydrated for 20 minutes before replanting into the medium. This result correlates with the data from *Figure 8 B)*, as those species also did not regain their former area when rehydrated.

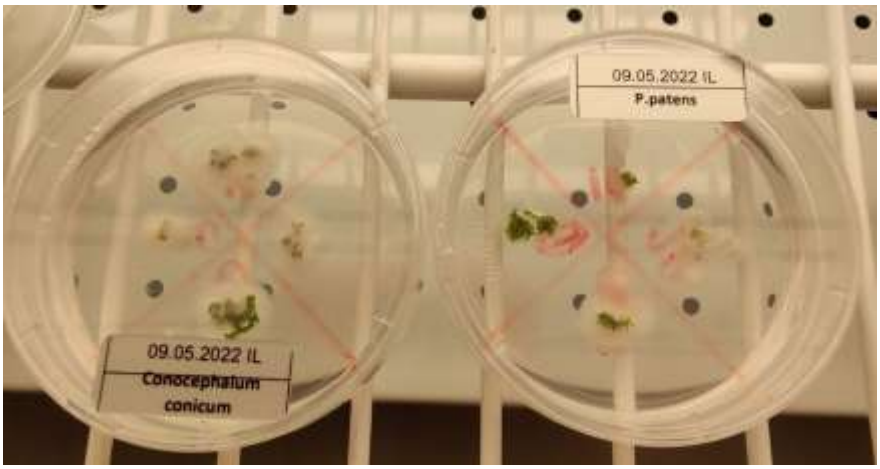


***Figure 20**, The dehydrated mosses after 10 days in the growth chamber. P. patens and C. conicum still did not recover and died when dried out for 20 minutes before planting.*

## 6.6 Photosynthetic activity and physiological effects

The four moss species, *A. undulatum, C. conicum, F. hygrometrica* and *P. patens* were screened for their photosynthetic activity while dehydrating and rehydrating, to determine a potential physiological variation.

Photos of the leaves were taken every two minutes, in the dehydration scenario for 60 minutes and in the rehydration scenario for 50 minutes. The fluorescence of those leaves at each time point was measured, normed to percent, and compared in the following charts (*Figure 22 & 23 & 24*).

The measurement of the data of the parameters $F_0$, $F_m$ and Y(II) looked as depicted in *figure 21*. Random places (within the white frames) on the moss were measured for their activity. The colour code ranges from black via red, orange, yellow, green, blue and violet to purple. The colours code for numbers between 0 and 1, a value of 0 means no fluorescence at all while a value of 1 is the maximum measurable fluorescence (Walz, 2009). When the moss was dried out there was much less area to choose from on the moss, this meant the frames had to be put on other places at the start of the rehydration experiment.

Because of the shift of the frames which measure the fluorescence, different end of dehydration and start of rehydration values are given in the following, as only in very few cases the same frame was used from the beginning of the experiment to the end of the experiment. From each parameter the mean value of all frames of each timepoint was then calculated.

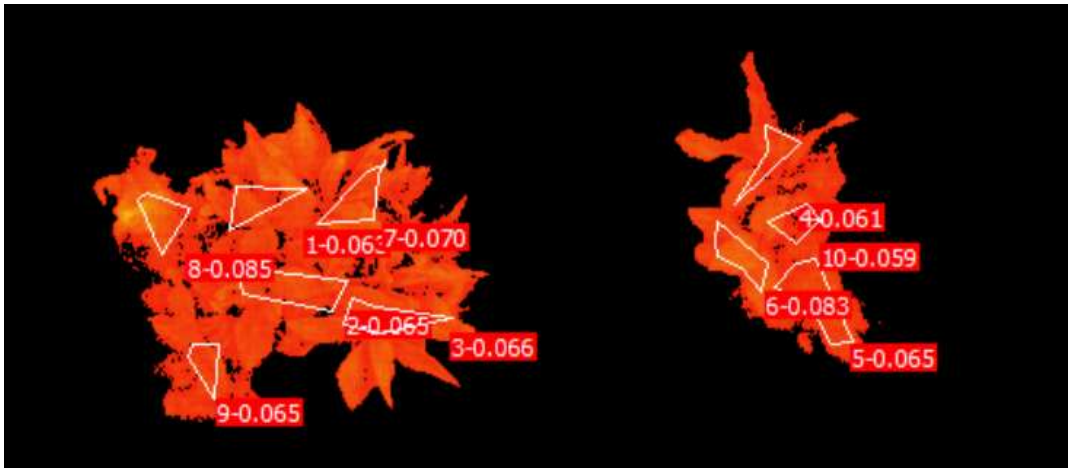


***Figure 21*** *Representative "Photosynthesis activity" photo. This is A. undulatum at the beginning of the dehydration. The redder the area is the more fluorescence is measured. After time it sometimes fades completely, or it goes yellow which means less fluorescence.*

### 6.6.1 Dark fluorescence yield ($F_0$)

One of the measured parameters, dark fluorescence yield ($F_0$) is measured after dark adaptation and shows the minimal fluorescence yield of each moss species (Walz, 2009). In a plant which would survive the dehydration and rehydration, it is expected that this value declines when dehydrated over time and rises when rehydrated again. The data is presented in *figure 22*.

The photosynthetic activity of *A. undulatum* was very constant while dehydrating and while rehydrating, which leads to the conclusion that this species can withstand water stress the best. In dehydration the $F_0$ value fell from 0.075 to 0.060, in the rehydration the value rose from 0.0621 to 0.0629.

*C. conicum* had a constantly decreasing photosynthetic activity during dehydration as well as during rehydration. This moss was probably in the process of dying. In dehydration the $F_0$ value fell from around 0.096 to 0.048, in the rehydration the value further decreased from 0.064 to 0.0534. This suggested that the moss wasn't dead but did not recover even with the help of water, at least not in the experimental time frame.

*F. hygrometrica* had the expected course of how a moss expects to behave when dehydrated. It lost the most of its photosynthesis activity when dehydrated and regained the most of all tested species when rehydrated. In dehydration the $F_0$ value fell from around 0.15 to 0.030, in the rehydration the value rose from 0.04 to 0.07.

In *P. patens* there was a big drop in its photosynthetic activity during dehydration. Later it gained its photosynthetic activity in the rehydration experiment. In dehydration the $F_0$ value fell from around 0.13 to 0.021, in the rehydration the value rose from 0.05 to 0.08.

The given values were always mean values of the measurements.

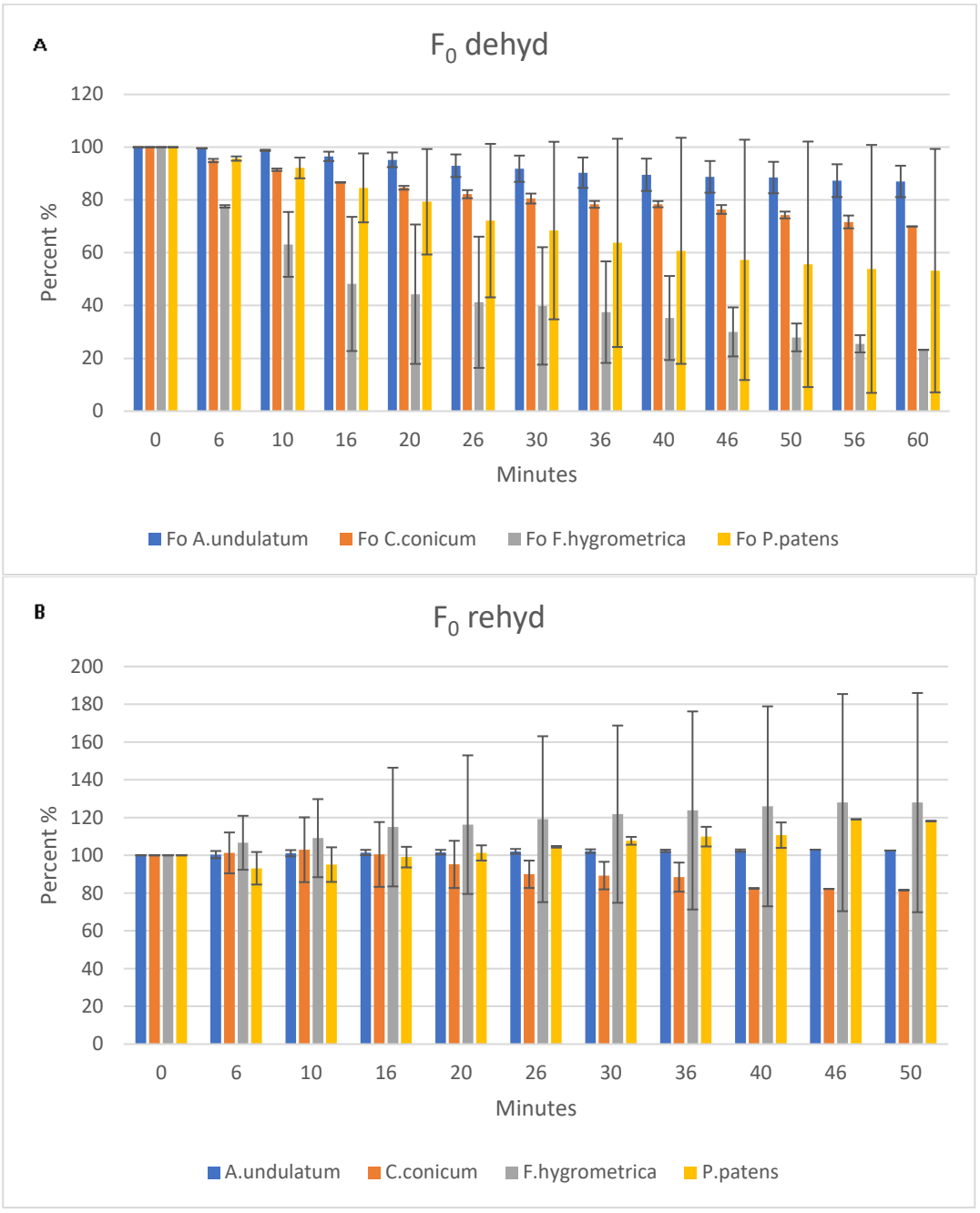


***Figure 22**, $F_0$ values of the macroscopic data obtained from Imaging Win. A) The photosynthetic activity through time when the mosses are dehydrating, of the leaf area of the 4 tested moss species is shown. B) The photosynthetic activity through time when the mosses are rehydrating, of the leaf area of the 4 tested moss species is shown.*

### 6.6.2 Maximal fluorescence yield ($F_m$)

Maximal fluorescence yield, $F_m$, is reached during application of a Saturation Pulse (*figure 23*). The outcome of the measurement of the parameter $F_m$ was very similar to that of $F_0$, when looked at the different moss species relative to each other.

The $F_m$ value of *A. undulatum* was relatively constant while dehydrating and while rehydrating, which leads to the conclusion that this species can withstand water stress to the highest degree in the tested species. While dehydrating the $F_m$ value fell from 0.071 to 0.059, in the rehydration the value rose from 0.06 to 0.065. Although the values in these experiments were lower they correspond with the photosynthetic measurement data in (Hu et al., 2016)

*C. conicum* had a constantly decreasing photosynthesis activity during dehydration as well as during rehydration. In dehydration the $F_m$ value fell from 0.092 to 0.051, in the rehydration the value further decreased from 0.057 to 0.049. This suggested that the moss wasn't dead but did not recover even with the help of water.

*F. hygrometrica* had the expected course of how a moss expects to behave when dehydrated. It lost the most of its photosynthesis activity when dehydrated and regained the most of all tested species when rehydrated. In dehydration the $F_m$ value fell from around 0.16 to 0.031, in the rehydration the value rose from 0.043 to 0.081.

In *P. patens* there was a big drop in its photosynthetic activity during dehydration. Later it gained its photosynthetic activity in the rehydration experiment. In dehydration the $F_m$ value fell from around 0.16 to 0.026, in the rehydration the value rose from 0.041 to 0.073.

In general, during the dehydration a linear downward trend was visible, and, in the rehydration, a linear uptrend was visible in all species, some reacted more than others to the water stress.

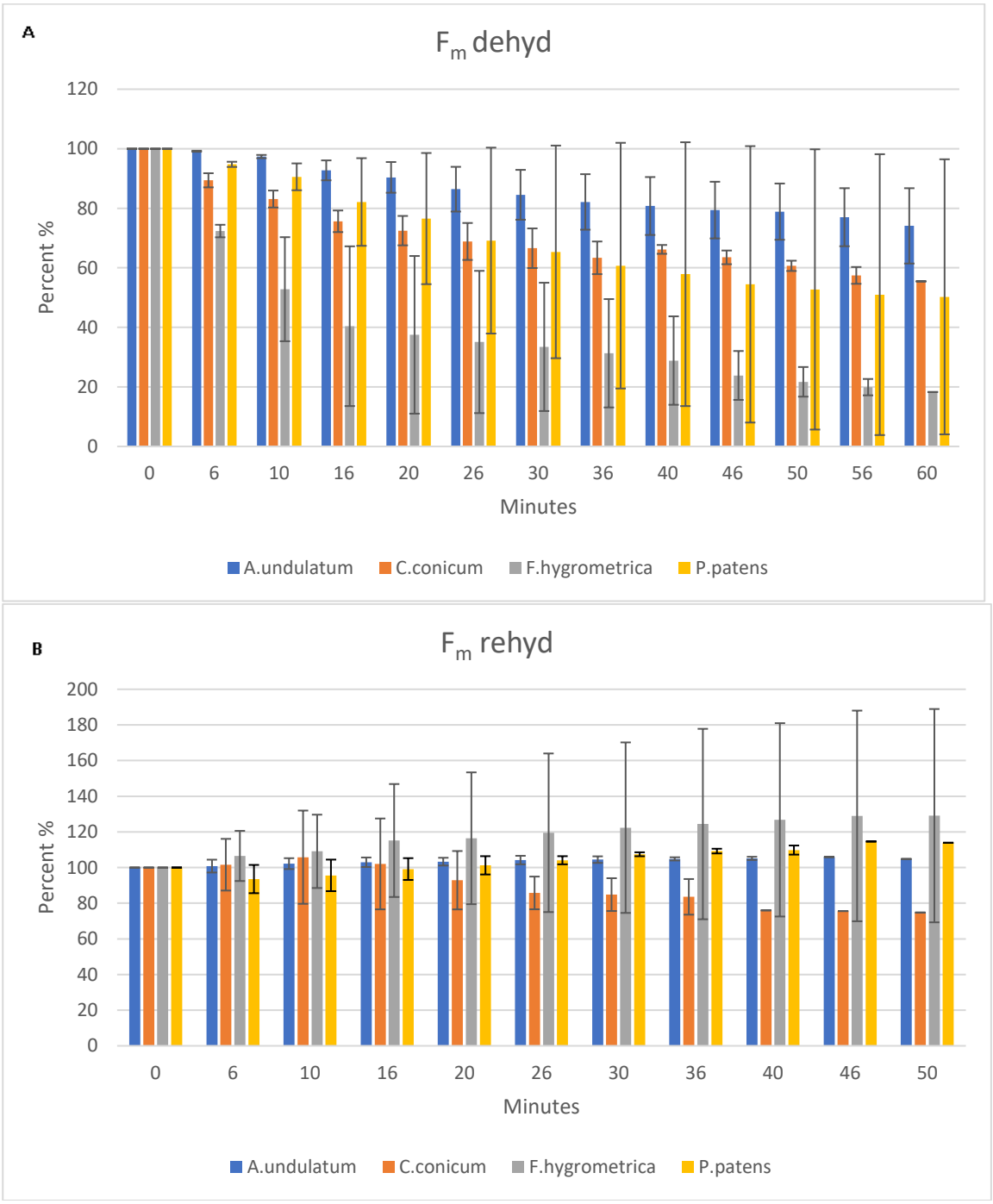


***Figure 23,*** *$F_m$ values of the macroscopic data obtained from Imaging Win. A) The photosynthetic activity through time when the mosses are dehydrating, of the leaf area of the 4 tested moss species is shown. B) The photosynthetic activity through time when the mosses are rehydrating, of the leaf area of the 4 tested moss species is shown.*

### 6.6.3 Effective PS II quantum yield, Y(II)

The effective PS II quantum yield, Y(II), which shows how much absorbed quanta are converted into chemically fixed energy and how much is converted to heat and fluorescence (Walz, 2009), is depicted in *figure 23*. It is a parameter which shows the general wellbeing of the plant, the higher the value, the better is the condition of the plant.

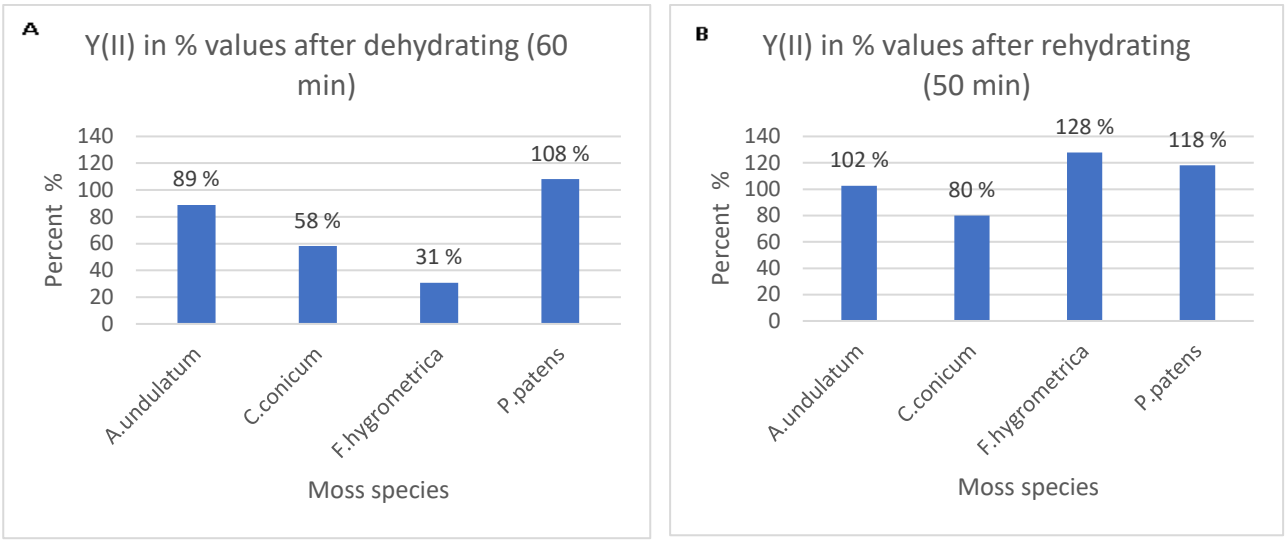


***Figure 23**, The effective PS II quantum yield, Y(II) values of the macroscopic data obtained from Imaging Win, only the last timepoint is shown. A) Y(II) values when moss is dehydrated, after 60 minutes B) Y(II) values when moss is rehydrated, after 50 minutes*

*Figure 23* shows the Effective PS II quantum yield of the last timepoints in the dehydration (*figure 23 A*) and rehydration (*figure 23 B*) scenarios. The mean values of Y(II) at the end of the experiments are depicted in percent relative to the Y(II) starting values of each experiment, which were set to 100 %. The last timepoints of the experiments are the most relevant, in the dehydration and rehydration scenario.

This means for *A. undulatum*, it can fix 89 % of the absorbed quanta into chemically fixed energy after 60 minutes of dehydration. When rehydrated this species chemically fixed energy stays constant from the beginning on from 100 % to 102 %.

C.conicum can fix 58 % of the absorbed quanta into chemically fixed energy after 60 minutes of dehydration. When rehydrated this species still loses its ability to chemically fix energy, from 100% in the beginning to 80 % after 50 minutes, so it has lost 20 % of its Y(II) value while rehydrating for 50 minutes.

*F. hygrometrica* can only fix 31 % of the absorbed quanta into chemically fixed energy than at the start of the experiment. When this species is rehydrated, 28 % more of its (dehydrated) starting Effective PS II quantum yield is achieved.

*P. patens* fixes over 100 % of the absorbed quanta into chemically fixed energy, as it could do at the start of the experiment. After 50 minutes of rehydration this species has also gained almost 18 % more of its starting Effective PS II quantum yield.

After rehydration *F. hygrometrica* regained its former PS II quantum yield, Y(II) value. *A. undulatum and P. patens* were very constant through the experiment.

*C. conicum* did not recover when rehydrated but rather lost photosynthesis activity through the whole experiment.

# 7. Discussion

A small collection of four moss species, *Atrichum undulatum, Conocephalum conicum, Funaria hygrometrica* and *Physcomitrium patens* were screened for their tolerance to dehydration and rehydration. The described experiments were conducted to determine a potential variation in the behaviour to dehydration and water stress in the tested mosses.

## 7.1 Behaviour of the tested moss species

The dehydration and rehydration behaviour of the mosses differed significantly from each other. Two species, *Conocephalum conicum* and *Physcomitrium patens*, were suppressed in their ability to recover after being dried out for at least 20 min. In the tested rehydration of the mosses, those two species also regained the least amount of its former leaf area (*Figure 8 B)*. It is interesting that those two species *Conocephalum conicum* (liverwort) and *Physcomitrium patens* (*true Bryophyta*) which are morphologically very different are behaving similar to each other in that simple rehydration test. Although other experiments, such as the photosynthetic activity measurement showed that there are significant differences between the dehydration/rehydration behaviour of those two species.

"Compared to other plants like *Arabidopsis thaliana, P. patens* exhibits a high degree of abiotic stress tolerance, making it a valuable source for the identification of genes effecting the stress adaptation" (Frank et al., 2005). This statement is only partially confirmed by my data (*Figure 8)* since *P. patens* had the smallest differences in terms of area between its hydrated/dehydrated and rehydrated state. Although the photosynthetic activity test showed good recovery in *P. patens* when rehydrated (*Figure 22 & 23 B*). But *P. patens* wasn't able after 20 min of dehydration to grow again in the growth chamber (Chapter: 6.5 Survival of the mosses after dehydration). *P .patens* still is a very stress tolerant moss species in terms of water stress and environmental stress such as heavy metals (Petschinger et al., 2021) and easy to handle in the practical work.

But it also must be said that the results of *chapter 6.5 Survival of the mosses after dehydration* experiments especially with *P. patens* were in terms of its meaningfulness limited, as the tested group was very small, and all individuals came from the same agar plate. For this experiment to be more meaningful a much bigger group of individuals and moss cultures would have been needed.

Since the genome of *P. patens* is well understood, (dehydration) stress related genes are known. Genes with regulatory functions within the dehydration-induced signal transduction pathways and genes performing putative protective functions within the plant cell are known in the genome of *P. patens* (Frank et al., 2005). Genes of *P. patens* which show the highest expression levels during the dehydration process code for proteins that are linked to the cold adaptation of seed plants (Frank et al., 2005). Some of the relevant genes are homologs of WPM-1, which codes for a transmembrane polypeptide from wheat (Koike et al., 1997), COR47 which codes for a LEA-like protein from A. thaliana (Gilmour et al., 1992), and COR TMC-AP3 from barley, which codes for a channel protein within the chloroplasts (Baldi et al., 1999). This shows that the genes and proteins which are relevant for moss species to cope with dehydration stress are conserved in vascular plants. The study of mosses and the usage

of mosses as a model system could reveal mechanisms of how plants can cope with drought. This shows the relevance of mosses as a model system.

The figures shown in *6.6 Effective PS II quantum yield, Y(II)* substantiate that *F. hygrometrica*, *A. undulatum* and *P. patens* are very resilient to de- and rehydration. *A. undulatum and P. patens* seems to have a mechanism which allows it to do not lose much water and have high photosynthetic activity also when dried out. *F. hygrometrica* deals with water stress differently, as it has a very low photosynthesis activity when it dries out. Because of their different behaviour in terms of photosynthesis activity, it can be concluded that these species must have different mechanisms to cope with water stress.

Although *P. patens* is the most used experimental model bryophyte species, these experiments confirm that *A. undulatum* is more resilient and is therefore more suitable, when researching dehydration stress, this statement is confirmed by (Hu et al., 2016).
*Funaria hygrometrica* and *Atrichum undulatum* proved to be the most dehydration stress resilient species in all experiments.

It has been found while working with the mosses that some species did not grow very well under these conditions (*Chapter 5.1 Planting moss cultures*). *C.conicum* grows under natural conditions in areas with calcareous substrates (Borovichev et al., 2009), maybe because of that this species grew very slow and calcareous substrates should have been added in this species. There could be huge differences in which moss species prefers which medium, in the literature sometimes very different mediums for mosses are used e.g., Sphagnum medium (Knop medium with ME,0.3% sucrose, and 1.25 mM ammonium nitrate) (Heck et al., 2021). A lot of experimenting with different medias could have been done, to improve the growth and health of each individual species, but nevertheless every species grew in the experiments.

Another factor how the tested mosses could give other results is the role of colony morphology in bryophytes. The dehydration rate is heavily influenced by size and form of a moss colony (Cruz de Carvalho et al., 2019). In the experiments a bud of a few gametophytes was analysed on its dehydration/ rehydration behaviour.
Other studies differed between fast drying and slow drying (Pressel and Duckett, 2010) also this could be a factor were the results of the different moss species could differ again.

## 7.2 Strategies to cope with dehydration stress

The survival ability of bryophytes to cope with extreme water stress for short periods, exceeds the range tolerated by most crop plants (Proctor and Pence, 2002). The ability to withstand desiccation in mosses is outstanding.

The measurement of the leaf/cell area and the photosynthesis activity over time was essential to monitor the dehydration and rehydration progress in the tested mosses. Fm and $F_0$ values of the photosynthesis activity measurement differ from vascular plants. The mean values of the mosses are shown in *chapter 6.6 Photosynthesis activity and physiological effects*. These values are much lower than those of vascular plants, e.g., *A. thaliana* has a hydrated Fm value of 0.8 (Sherameti et al., 2008) and a $F_0$ value of 0.24 (Pfündel et al., 2013).

There are some known strategies of vascular and non-vascular plants to cope with dehydration stress. For example to be able to get water from deeper soil layers, but this mechanism can be excluded in the tested moss species, (Chaves et al., 2002) as the tested mosses neither have access to soil nor they possess roots.
The following mechanisms apply to the tested moss species. Stomatal control of water losses is a possibility of plants to cope with water stress, this limits the carbon uptake of the leaves, in response to a decline in leaf-turgor or water potential, the stomata are closing (Chaves, 1991).
The next known mechanism to cope with dehydration is based on low CO2 availability, which leads to changes in the cell carbon metabolism (Meyer and Genty, 1999). This low CO2 availability could happen due to stomatal closure (Chaves et al., 2002). The alteration of the metabolism maintains the osmotic pressure within photosynthetic cells, this increases the nitrate concentration and decreases the carbohydrate export (Chaves et al., 2002).
In terms of their morphology, there were visible differences between *A. undulatum* and *F. hygrometrica*, with *A. undulatum* having thicker and bigger leaves than *F. hygrometrica*.

*A. undulatum* seems to have a mechanism which allows a minimal loss of water and a normal photosynthesis activity even when dehydrated for a long time. Curled leaves, cytorrhysis and plasmolysis were observed during dehydration and in many cases reversed during rehydration. These observations suggest that the dehydration stress induced cell and membrane damage was not irreparable. At the beginning of the dehydration *A. undulatum* has a condensed state of chromatin and increased cytoplasmic viscosity this is probably also very important for the survival of this species in drought conditions (Hu et al., 2016).
*F. hygrometrica* deals with water stress differently, as it has a very low photosynthesis activity when it dries out. But when rehydrated *F. hygrometrica*, was the most successful in gaining its former leaf area and its photosynthesis activity.

Those different coping mechanisms/approaches of these two species have the same outcome, which is the survival of the plant. This raises the question if the reasons for these differences are of physiological or metabolic origin. Therefore, a genetic screening approach and field research would be justified at least in *F. hygrometrica* and *A. undulatum*. To determine the metabolic pathways and genes which are responsible for this behaviour. Field research would be interesting to determine the exact ecological circumstances under which each species lives. Unfortunately, the exact locations from where the used moss stem colonies came from were unknown.

The results were surprisingly different between the four randomly the tested moss species as there was an unexpected variation in the reaction of the mosses to dehydration/rehydration. No single feature of these moss species could be pinned down to determine which moss is more tolerant to dehydration. This result would justify the research on a bigger group of mosses, to find more similarities between the dehydration tolerant/intolerant mosses.

## 7.3 Evolutionary and ecological aspects

Bryophytes are basal land plants and are a paraphyletic group (Groth-Malonek and Knoop, 2005). This group includes liverworts, mosses and hornworts (Petraglia et al., 2014). Bryophyte fossils are much rarer than those of vascular plants (Bomfleur et al., 2014) because

of that their systematic is still in debate. Yet from the systematic of the tested species *P. patens* and *F. hygrometrica*, which both belong to the clade of *Funariaceae* are the most basal species while C.conicum which is a part of the *Marchantiophyta* clade (Troitsky et al., 2007).
Because of similarities in the primary cell wall between charophytes and primitive bryophytes morphological evidence is provided which states that bryophytes and all land plants derived from charophytes (Popper and Fry, 2003).

The tested moss species are widespread in Europe and have a similar and wide natural distribution ("Atlas-of-British-and-Irish-Bryophytes-V1-69.pdf," n.d.), yet they behave very differently in this study.

As it has been stated in the introduction*, Atrichum undulatum* is a generalist and a desiccation-tolerant basal plant (Hu et al., 2016). *A. undulatum* prefers acid to neutral clay and loamy soils in woodland (Büscher et al., 1990).

*Conocephalum conicum* is a represent of the liverwort and the only multi cell layered species which has been tested. *Marchantia polymorpha*, which is also a liverwort species similar to *C.conicum*, showed high resistance in extreme water-limiting conditions and suffering from drought-induced stresses (Godinez-Vidal et al., 2020). C. conicum appears to be morphologically very similar to *M. polymorpha*, yet it lacks the expression of prominent roots as it is the case in *M. polymorpha*. Under natural conditions hornworts and liverworts grow on moist substrata, while other primitive mosses grow submerged in water (Popper and Fry, 2003). It was expected at the beginning of the experiment that *C. conicum* would be very dehydration tolerant since it had multiple cell layers and therefore it must be easier for the plant to keep hydrated. But *C. conicum* had a very poor dehydration performance. This leads me to the conclusion that maybe with higher complexity of the bryophyte species, the (drought) adaptability suffers.

*Funaria hygrometrica* was used in the experiment because it seemed very fragile from its structure, although later in the experiments, it proved to be a very robust species. *F. hygrometrica* occurrence is temporary and therefore not quite as abundant in nature, it occurs in disturbed, open habitat ("Atlas-of-British-and-Irish-Bryophytes-V1-441.pdf," n.d.). The problem with this species was, that it grew very slow and was often infected with fungi.

The moss *Physcomitrium patens* is a well-known model plant and is widely used for genetic studies (Rensing et al., 2020). *P. patens* grows in seasonally wet areas such as flood plains of lakes and edges of rice fields or in moist soil along paths or fields, always at low to moderate elevations (Rensing et al., 2020).

## 8. Conclusion

As seen in the chapters above, dehydration and rehydration affected the mosses differently. The experiment has proven that some moss species are more resistant to dehydration than others. It can also be concluded that there exists a resistance mechanism or structure in moss species against drought stress (Frank et al., 2005).

Based on my experiments the most suitable moss to use as a model system was *A. undulatum*, as it has been proven to be easy to propagate, did not have problems with fungi, grew fast and was a very robust species in terms of environmental stress. But it is not fully sequenced and therefore, we cannot look for potential genes or proteins that would help in desiccation tolerance to crop plants. In contrary to that *P. patens* is fully sequenced and therefore the better choice when it is used as a model plant and very robust when compared to other species.

*F. hygrometrica which* has proven to be a very drought resistant species, grew very slow and was very prone to fungal contamination under laboratory conditions. There was no obvious reason for the slow growth of *F. hygrometrica*, first we thought it was because of the medium, then we thought because of bad ventilation inside the petri dish. But when we changed it there was only very little impact on its growth speed. This species was no matter how sterile we worked always infected with fungi after a while. This fact led me to the hypothesis that maybe fungi is somehow necessary for it to grow or maybe this fungal contamination was the problem why it grew so slow.

A screen with more moss species would be interesting.
Different moss species could be picked based on their complexity, maybe it could be stated if, with higher complexity of the plant, the adaptability of the moss increases or decreases. There could be other advantages in having a multicellular physiology, as in *C. conicum* where experiments to test for those, had to be designed first. Also, much more basal moss species e.g., *Sphagnum ssp*. could also be tested for their drought response. This could also answer evolutionary questions how basal mosses developed mechanisms which in the evolutionary context, led into higher plants.

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## 10. Video (Phaidra) Permalinks

### 10.1 Visualization of the de- and rehydration process of the gametophyte tip and leaves

Dehydrating gametophyte of *Atrichum undulatum*
https://phaidra.univie.ac.at/view/o:1594990

Rehydrating gametophyte of *Atrichum undulatum*
https://phaidra.univie.ac.at/view/o:1595035

Dehydrating gametophyte of *Conocephalum conicum*
https://phaidra.univie.ac.at/view/o:1595036

Rehydrating gametophyte of *Conocephalum conicum*
https://phaidra.univie.ac.at/view/o:1595037

Dehydrating gametophyte of *Funaria hygrometrica*
https://phaidra.univie.ac.at/view/o:1595038

Rehydrating gametophyte of *Funaria hygrometrica*
https://phaidra.univie.ac.at/view/o:1595039

Dehydrating gametophyte of *Physcomitrium patens*
https://phaidra.univie.ac.at/view/o:1595040

Rehydrating gametophyte of *Physcomitrium patens*
https://phaidra.univie.ac.at/view/o:1595041

## 10.2 3D visualization of the selected moss species on a cellular level:

Hydrated cells within the leaf of *Atrichum undulatum*
https://phaidra.univie.ac.at/view/o:1595044

Dehydrated cells within the leaf of *Atrichum undulatum*
https://phaidra.univie.ac.at/view/o:1595043

Hydrated cells within the leaf of *Conocephalum conicum*
https://phaidra.univie.ac.at/view/o:1595046

Dehydrated cells within the leaf of *Conocephalum conicum*
https://phaidra.univie.ac.at/view/o:1595045

Hydrated cells within the leaf of *Funaria hygrometrica*
https://phaidra.univie.ac.at/view/o:1595047

Dehydrated cells within the leaf of *Funaria hygrometrica*
https://phaidra.univie.ac.at/view/o:1595048

Hydrated cells within the leaf of *Physcomitrium patens*
https://phaidra.univie.ac.at/view/o:1595049

Dehydrated cells within the leaf of *Physcomitrium patens*
https://phaidra.univie.ac.at/view/o:1595048

## 11. Appendix I Power Test calculations of the cell area measurement done via R

*A. undulatum*

```
-Two Sample t-test
data:  A_undulatum_hydrated_measurements$"Area [µm²]" and A_undulatum_dehydrated_measurements$"Area [µm²]"
t = 11.533, df = 98, p-value < 2.2e-16
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
 393.0911 556.4749
sample estimates:
mean of x mean of y
 803.7962  329.0132

Two-sample t test power calculation
 n = 50
          delta = 474.783
             sd = 191.7275
      sig.level = 2.2e-16
          power = 0.9801895
    alternative = two.sided
```

*C. conicum*

```
NOTE: n is number in *each* group

-Two Sample t-test

data:  C_conicum_hydrated_measurements$"Area [µm²]" and C_conicum_dehydrated_measurements$"Area [µm²]"
t = 10.219, df = 82, p-value = 2.757e-16
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
 511.5334 758.8262
sample estimates:
mean of x mean of y
1043.2855  408.1057

Two-sample t test power calculation

              n = 42
          delta = 635.1798
             sd = 261.5054
      sig.level = 2.757e-16
          power = 0.7699321
    alternative = two.sided

NOTE: n is number in *each* group
```

*P. patens*

```
-Two Sample t-test

data:  P_patens_hydrated_measurements$"Area [µm²]" and P_patens_dehydrated_measurements$"Area [µm²]"
t = 8.4615, df = 78, p-value = 1.198e-12
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
 315.466 509.586
sample estimates:
mean of x mean of y
 811.7188  399.1927


Two-sample t test power calculation

              n = 40
          delta = 412.526
             sd = 146.8444
      sig.level = 1.198e-12
          power = 0.9996576
    alternative = two.sided

NOTE: n is number in *each* group
```

*F. hygrometrica*

```
-Two Sample t-test

data:  F_hygrometrica_hydrated_measurements$"Area [µm²]" and F_hygrometrica_dehydrated_measurements$"Area [µm²]"
t = 10.698, df = 78, p-value < 2.2e-16
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
 577.9436 842.2279
sample estimates:
mean of x mean of y
1133.3782  423.2925

Two-sample t test power calculation
              n = 40
          delta = 710.0857
             sd = 158
      sig.level = 2.2e-16
          power = 1
    alternative = two.sided

NOTE: n is number in *each* group
```