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Changes in ultra-weak photon emission from drying leaves

BACHELOR THESIS

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Abstrakt:

Tato bakalářská práce zkoumá změny v ultra-slabé fotonové emise (UPE) ze zasychajících listů se zaměřením na roli reaktivních forem kyslíku (ROS). Pomocí CCD kamery byla měřena UPE na různých rostlinných druzích, včetně *Arabidopsis thaliana*, *Hordeum vulgare* a *Medicago sativa*. Cílem studie bylo sledovat dynamiku UPE s tvorbou ROS během vysychání listů. Mezi klíčová zjištění patří, že listy *M. sativa* mohou i po delší době vysychání částečně obnovit příjem vody. Dalším zjištěním bylo, že k tvorbě ROS dochází před viditelnými strukturálními změnami. Rychlé vlny ROS byly pozorovány bezprostředně po vyjmutí listů z vody, což naznačuje stresovou reakci a tvorbu ROS. U některých rostlinných druhů jako *C. avellana*, *C. orientalis* a *P. americana* byl zjištěn specifický průběh zasychání.

Klíčová slova: reaktivní formy kyslíku, ultraslabá fotonová emise, zasychání listů, *Arabidopsis thaliana*, *Hordeum vulgare*, *Medicago sativa*

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Abstract:

This bachelor thesis investigates changes in ultra-weak photon emission (UPE) from drying leaves, focusing on the role of reactive oxygen species (ROS). Using a CCD camera, UPE was measured in various plant species, including *Arabidopsis thaliana*, *Hordeum vulgare* and *Medicago sativa*. The aim of the study was to correlate UPE dynamics with ROS production during leaf desiccation. Key findings suggest that *A. thaliana* leaves can partially recover water uptake after desiccation. Another finding was that ROS formation occurs before visible structural changes. Rapid waves of ROS were observed immediately after the leaves were removed from water, suggesting a stress response and ROS production. A specific drying process was observed in some plant species such as *C. avellana*, *C. orientalis* and *P. americana*.

Keywords: reactive oxygen species, ultra-weak photon emission, leaf drying, *Arabidopsis thaliana*, *Hordeum vulgare*, *Medicago sativa*

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Statement

I declare that I have prepared the submitted bachelor thesis independently using the cited literature listed at the end of the thesis under the supervision of Mgr. Marek Rác, Ph.D.

In Olomouc dated 21st May 2024

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CONTENT

List of abbreviations

ATP	adenosine triphosphate
CCD	charged-couple devices
CO_2	carbon dioxide
H_2O_2	hydrogen peroxide
mtETC	mitochondrial electron transport chain
$OH^{\bullet}$	hydroxyl radical
$^1O_2$	singlet oxygen
PSII	photosystem II
ROS	reactive oxygen species
UPE	ultra-weak photon emission

1 Introduction

Water is essential for the viability of plants, playing a critical role in their growth, development, and physiological processes. One of the most significant factors influencing water content in plants is water potential, which drives the transpiration flow from roots to leaves. The majority of water exits through the leaf stomata, some exits through the epidermis of leaves (Pallardy 2008). Understanding the dynamics of water content and its movement within plants is fundamental to comprehending how plants respond to various environmental stresses, such as drought.

Leaf drying, a consequence of water deficit, triggers various physiological responses in plants, including changes in leaf structure. These responses aim to minimize water loss and protect cellular integrity. Reactive oxygen species (ROS) are key players in plant responses to drought stress, formed as a by-products of metabolic processes and signalling molecules during stress conditions. While low levels of ROS are part of normal cellular functions, elevated levels can cause oxidative damage, necessitating the development of antioxidative defence mechanisms in plants (Yang et al. 2021; Du et al. 2023; Baxter et al. 2014).

Ultra-weak photon emission (UPE) is a phenomenon where biological systems emit low-intensity light as a result of metabolic activities and ROS formation. UPE can be spontaneous or induced by external stimuli, and its measurement provides insights into the oxidative state and physiological changes within plants. As UPE is a non-invasive method to study the internal state of a leaf under stress conditions, it is an important procedure applicable in several biological fields (Cifra and Pospíšil 2014).

2 Theoretical part

2.1 Water content in plants

2.1.1 Water potential

To understand the process of leaf drying and the changes in plants we need to understand the water flow in general. One of the most important factors describing water content and its movement in plants is water potential. To maintain the balance of moisture content, water is transported throughout the plant transpiration flow. The flow is based on water potential gradient (from the point where the water potential is highest – the roots, to the point where it is the lowest – leaves) (Procházková 2021). It is measured in units of pressure, typically in megapascals, is influenced by many factors and has 4 basic components that complement each other. The components of water potential are osmotic potential, matric potential, pressure potential and gravitational potential (Slavík 1965).

Another important process for the water uptake and conduction in plant is osmosis. It is a process in which the concentration is equilibrated between two solutions of different concentrations. The equilibration occurs through a semi-permeable membrane, when the water molecules are transferred from a lower dissolved content environment to a higher dissolved content environment. Water regulation in plants is related to the concept of osmoregulation. It is the osmotic adjustment of the cell to control its water content by increasing or decreasing the cytosolic and vacuolar concentrations of osmotically active molecules (Beauzamy et al. 2014).

2.1.2 Transpiration

Water intake is ensured by the root system with following transport by transpiration stream. It is a complex process dependent on many factors such as root pressure, capillary action and cohesion and adhesion of water molecules.

Water circulation in plants is influenced by transpiration, which is defined as release of water through the surface of the plant, especially the leaves. Molecules of water are evaporating from the leaf surface and creating tension in the column of water in the plant. Individual water molecules are bound together by hydrogen bonds, a phenomenon known as cohesion. The main purpose of transpiration is exchange of gas, transport of water and minerals and also prevents leaf overheating. Transpiration takes place mainly through leaf stomata, then through the epidermis and can be regulated by opening and closing the stomata (Pallardy 2008; Ben-Yehoshua and Rodov 2002).

Leaf stomata are one of the most important elements in transpiration process. They allow the exchange of gasses between the plant and the surrounding environment. The main process is the uptake of carbon dioxide (CO_2), which is further used in photosynthesis, and the release of oxygen from photosynthesis. The opposite process is respiration, in which oxygen is taken in and CO_2 is released. Stomata consist of two ventilation cells with a ventilation slit between them.

The mechanism of opening and closing the stomata is influenced by many factors, such as daytime, air humidity, CO_2 concentration and the internal condition of the plant. Thanks to the possibility of regulating the transpiration flow, plants are able to control water consumption and limit excessive water loss. It is known that the regulation is due to differences in turgor between the ventilation cells and companion ventilation cells, that when the turgor in ventilation cells increases, the stomata open, while when the turgor decreases, they close (Penka 1965).

2.2 Leaf drying

Leaf drying is a process that occurs due to lack of water, which is essential for the viability of the plant. Whereas this process can be caused by both internal and external conditions, certain defensive systems had to be established to cope with critical conditions. Guard cells have evolved different mechanisms to sense and integrate various environmental as well as internal signals to optimize the balance between CO_2 exchange for photosynthesis and water loss via transpiration (Kollist et al. 2019). During exposure to stress stimuli, internal signals in the plant are transmitted between individual cells to the point where the plant is able to develop a defence mechanism. Drought is a meteorological term; but the majority of plant physiology papers use it to describe how plants respond to the stress of increasing water deficits (Farrell et al. 2017).

Water content in plants is also related to leaf shrinkage, which occurs in situations when a plant is in water deficit. Leaf shrinkage is the combination of dimensional shrinkages following the three dimensions of the leaf (thickness, width and length) (Essaghi et al. 2016). Thickness of leaves can be decisive in different situations, research has been caried out on mediterranean plant species and how leaf shrinkage is related to water content, leaf thickness and leaf flammability to prevent wildfires. For more information see (Essaghi et al. 2016).

In the shrinkage process, the water content is lost and therefore the water potential is dropping and internal mechanisms such as photosynthesis, metabolism and respiration are reduced. The water loss rate can be affected by the plant itself for example by early closure of the stomata and with shrinkage the plant is reducing the size of the surface available for transpiration and there are changes in stability and strength within the leaf (Fig. 1) (Scoffoni et al. 2014).

The drying mechanism is mainly related to changes in turgor (the internal pressure of the plant) otherwise also the leaf turgor loss point (Blackman 2018). At the cellular level, turgor pressure pushes the plasma membrane against the cell wall and causes in-plane mechanical tension within the cell wall, which provides structural integrity to each cell and to the tissue as a whole (Beauzamy et al. 2014). During the process of drying a decrease in turgor pressure occurs resulting in a collapse of the cell. This can lead to a possible destruction of organelles such as chloroplasts and therefore to a reduction in processes necessary for a viable cell, such as photosynthesis. With the loss of rigidity, the leaf becomes limp and wilted. The correlation between leaf thickness and water status is so strong that utilizing leaf thickness as a guide for irrigation has resulted in water savings of up to 45% (Seelig et al. 2012).

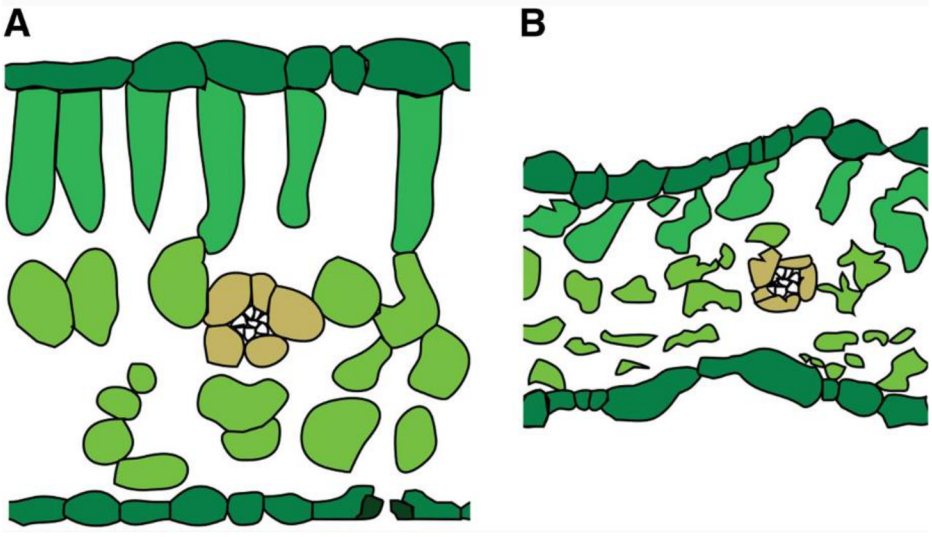


Figure 1. A sketch of a fully hydrated leaf (A) and strongly dehydrated leaf (B; drawing based on leaf cross sections of sunflower in Fellows and Boyer 1978). A significant decrease in leaf thickness, cell size and intercellular air spaces are observed, leading to a reduction in the overall leaf area (B) (Scoffoni et al. 2014).

The obvious symptoms of water deficit during vegetative period are plant height decreased, leaf wilting, number and area of leaves changed (Yang et al. 2021). When plants are subjected to drought conditions, a series of reactions occur in order to minimize water loss and cellular damage. However, prolonged or severe drought can negatively affect plant growth, development and productivity. Leaf drying is directly related to leaf deformation due to distortion of the internal structure and mechanical support.

2.2.1 Leaf deformation

Leaf deformation is a visible response to various types of damage and can take several forms. A dehydrating leaf develops wrinkles on its surface due to buckling instead of compressing as a way to reduce the stress caused by uneven shrinkage. Typically, leaves curl upwards as the top surface, which is more directly exposed to sunlight, dries faster (Fig. 2). During the leaf curling, which is the most common response to drought, the leaf margin or the entire leaf blade curves inwards. (Jeong et al. 2013). For more information regarding this topic see (Jeong et al. 2013).

Figure 2. Leaf deformation in leaves of *Shellbark Hickory,* as dehydration progresses, areas further from the centre of the leaf lose their internal strength and leaf curling occurs. Taken from (Jeong et al. 2013).

Similar to leaf curling is leaf rolling, where the leaf blade is folded to reduce the surface area on the leaf and retain water (Fig. 5). During periods of severe drought some plants roll their leaves tightly, forming a tube-like structure that retains water in the cells (Fig. 1). Another form of leaf deformation is wilting, when plants cannot maintain sufficient internal pressure to keep the leaves in straight position (Fig. 4, right). Wilting helps to reduce leaf surface area and thus the water loss. The final stage of desiccation is necrosis, in which the cell wall is irreversibly damaged, resulting in necrotic lesions or brown spots on the leaves (Jeong et al. 2013; Merrium et al. 2022).

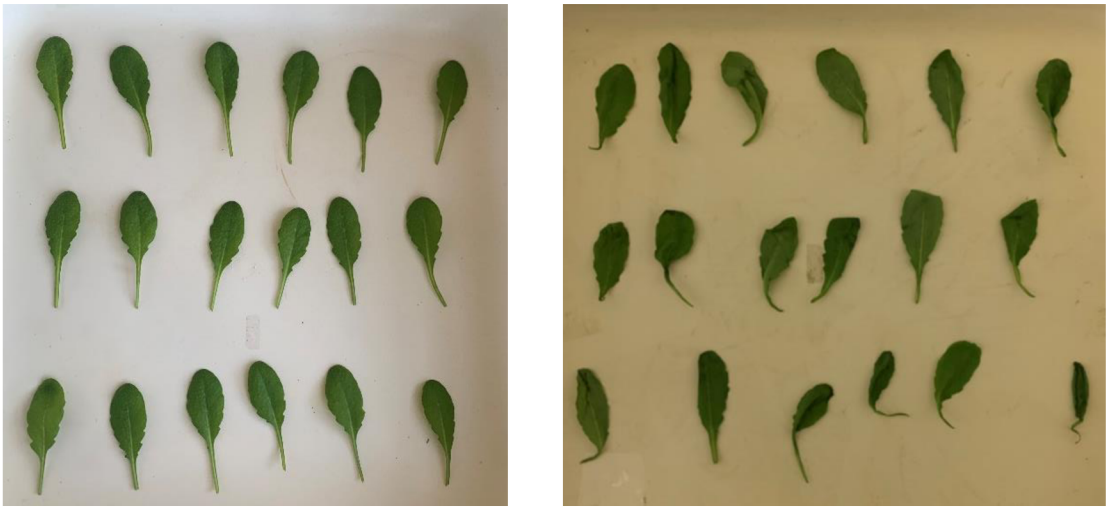


Figure 3. Leaves of *A. thaliana* freshly cut of the mother plant (left), same leaves after prolonged time period of drying (right). Leaf wilting as a type of deformation is shown as a result of drying. Leaves lost their original elasticity, they are not dry to the touch and they cannot hold an upright position.

Figure 4. Leaf of grass showing leaf rolling as a type of deformation shown as a result of drying. Gradual drying from the edges to the centre of the leaf is shown (Jeong et al. 2013).

2.3 Reactive oxygen species

Reactive oxygen species (ROS) are considered to be compounds that contains molecules of oxygen and unpaired electrons such as peroxide anion (O_2^{2-}), superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) peroxyl radical ($LOO^{\bullet}$) and hydroxyl radical ($OH^{\bullet}$) (Li, Jia, a Trush 2016). Furthermore, we can include the singlet molecular

oxygen ($^1O_2$), which can be formed by various mechanisms. The excited state of $^1O_2$ is also reported to be one of the important sources of the ultra-weak photon emission (UPE) observed in biological system (Miyamoto et al. 2014). These compounds are highly reactive due to unpaired electrons creating an unstable configuration, and therefore they have been mistaken in the past to be only toxic.

The source of ROS is a series of redox reactions such as the reduction of oxygen during electron transport in mitochondria or photolysis of water by chloroplast electron transport chain (Piterková et al. 2005). They are formed either during the metabolic processes linked to life-sustaining enzyme-catalysing reactions or during the response to stress reactions when microorganisms, plants and animals including humans are exposed to biotic and abiotic stress factors (Fig. 6) (Pospíšil et al. 2014).

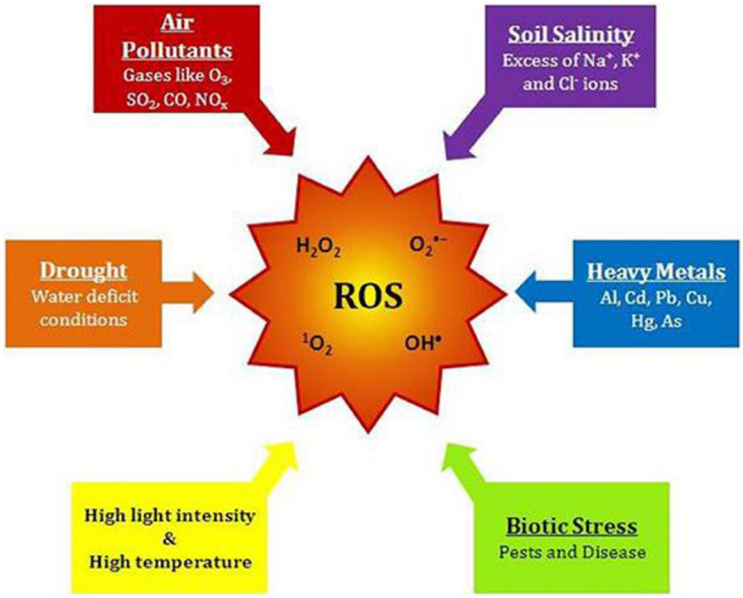


Figure 5. Various causes responsible for the generation of ROS (Das et al. 2022).

Biotic stress factors include damage by pathogens, ageing or exposure to environmental organisms. Abiotic factors include for example intense light, heat, cold or drought. Under optimal growth conditions, ROS are mainly produced at a low level in organelles such as chloroplasts, mitochondria and peroxisomes, which are organelles with high metabolic activity. However, during stress, their rate of production is dramatically elevated (Miller et al. 2010).

The plants had to develop a system for dealing with increasing levels of ROS such as the formation of antioxidants. They play a crucial role in protecting cells from oxidative stress caused by various environmental factors and metabolic processes. In plants, they can take different forms, such as enzymatic or non-enzymatic antioxidants.

In the process of photosynthetic electron transport, chloroplasts consistently produce oxygen, which becomes eliminated by reduction and assimilation (Mansoor et al. 2022). However, in certain situations, incomplete oxygen elimination can occur, resulting in the formation of ROS. High concentrations of ROS can cause additional damage to photosynthetic apparatus and impairment of cellular components, DNA or aminoacids (Zimmermann a Zentgraf 2005).

ROS play crucial roles as signalling molecules in regulating plant growth and response to environmental stress factors. This involves complex interactions with other signalling molecules and pathways. The interplay between ROS and other signals like calcium fluxes, mitogen-activated protein kinases (MAPKs) and hormones is critical (Baxter, Mittler, a Suzuki 2014; Mohiuddin et al. 2023).

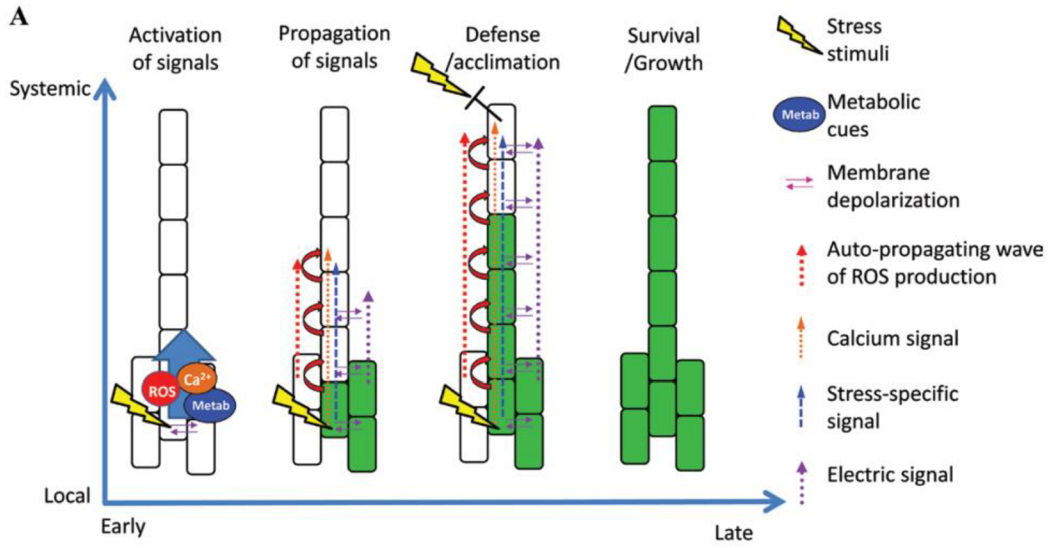


Figure 6. A hypothetical model of ROS mechanism on plants during stress stimulation. In a rapid answer to stress, calcium and ROS wave are generated, which propagate signalling between neighbouring cells to form a defence mechanism (Baxter et al. 2013).

Drought stress triggers the production and accumulation of ROS in plants. In general, it affects photosynthesis by disrupting the photosynthetic apparatus and reducing the efficiency of light capture and limits the CO_2 entry by closing stomata as a response to water deficit. The low concentration of CO_2 has a direct effect on increased $^1O_2$ production in photosystem via Mehler reaction. (Wang et. al 2019; Impa, Nadaradjan and Jagadish 2012; Das et al. 2022).

$$2O_2 + 2Fd_{red} \rightarrow 2O_2^{\bullet -} + 2Fd_{ox}$$

Figure 7. Mehler reaction describing reduction of O_2 to superoxide anion by donating electron with ferredoxin, which is a protein performing oxygenic photosynthesis.

Aerobic metabolism constantly generates ROS which are confined to the different plant cellular compartments, like the chloroplast, mitochondria and peroxisomes. These organelles produce ROS under both normal and stress conditions, chloroplast and peroxisomes ROS production is dependent on a presence of light, while mitochondria is a producer of ROS under the dark conditions (Das et al. 2022; Mattos and Moretti 2016).

With aerobic respiration the electrons are transferred through a series of complexes known as the electron transport chain, located on the inner mitochondrial membrane. Many enzymes are involved in this process, such as lipoxygenases, peroxidases, nicotinamide adenine dinucleotide phosphate hydrogen oxidase and xanthine oxidase (Mattos a Moretti 2016). In mitochondrial electron transport chain (mtETC) the main components responsible for regular production of ROS are Complex I and Complex II, regulated by nicotinamide adenine dinucleotide dehydrogenase. Important role plays adenosine triphosphate (ATP) as an energy-carrying molecule. During stress conditions mtETC and ATP synthesis is directly affected, which results in excessive reduction of electron carriers such as the ubiquinone pool, thus leading to the production of ROS (Rhoads et al. 2006; Das et al. 2022; Blokhina and Fagerstedt 2010).

In chloroplast, which contain a meticulously structured thylakoid membrane system that houses every component of the light-capturing photosynthetic apparatus and ensures all necessary attributes for optimal light absorption. (Pfannschmidt 2003). In normal conditions the photosystem II (PSII) produce $^1O_2$, but under stress conditions it accumulates in chloroplast causing peroxidation of membrane lipids especially

polyunsaturated fatty acids resulting in severe damage of PSII (Das et al. 2022; Ali et al. 2005).

2.4 Oxidative stress

During plant development and aging, ROS levels naturally increase during aging-related processes. This leads to oxidative damage of cellular components and ultimately to aging. Oxidative stress is known as a plant condition in which ROS outnumber antioxidants, disrupts the internal balance and can lead to severe damage. The inconsistency between the formation and the elimination of ROS causes the oxidation of lipids, proteins and nucleic acids (Pospíšil et al. 2014).

To limit elevated ROS levels, the plant produces enzymes such as peroxidase and catalase, which contribute to plant organelle and metabolic protection. It also contains non-enzymatic substances such as glutathione, ascorbic acid, carotenoids and flavonoids which target $OH^{\bullet}$ and $^1O_2$ (Mansoor et al. 2022). The role of antioxidants is to prevent rising levels of ROS or balance their quantity by donating free electrons. By stabilizing them it can prevent serious cellular damage.

Several factors can influence the induction of oxidative stress. Environmental factors such as intense light, extreme temperatures, drought or air pollution can be mentioned first. To keep up with drastic environmental conditions, plants have evolved various stress-responsive genes encoding their respective proteins required for activation as well as regulation of ROS (Mansoor et al. 2022). Other factors include pathogen attack and increased respiration and metabolism. The importance of oxidative stress in physiopathology makes it imperative to find an analytical method that can continuously and non-invasively monitor oxidative metabolic status *in vivo* (Du et al. 2023).

2.5 Fomation of ROS during drought stress

ROS production is increased under drought stress due to stomatal closure and limited CO_2 fixation. As high ROS levels can lead to oxidative damage, controlled ROS production acts as a signal that activates defence mechanisms in plant response to drought. H_2O_2 is considered as the most stable and diffusive ROS, with role as a secondary messenger in stress-response pathways. With controlled production of H_2O_2 and scavenging enable rapid signalling which is important for plant adaptation to drought stress (Cruz de Carvalho 2008).To regulate high ROS levels the crucial role play the antioxidant system including enzymes such as ascorbate peroxidase and superoxide

dismutase, which help to maintain cellular redox homeostasis during water deficiency (Noctor et al. 2014).

ROS production occurs at various intracellular sites, including chloroplasts and peroxisomes. Drought-induced stomatal closure leads to increased photorespiration and H_2O_2 production in peroxisomes. In chloroplasts, restricted ATP consumption supports ROS generation via the Mehler reaction (Fig. 7) and $^1O_2$ production on PSII. Mitochondria also have a specific process by which they avoid excessive ROS production during drought stress, involving alternative oxidase pathway along with other energy-dissipating systems, which reduce ROS production by diverting electron flow and effectively manage energy dissipation (Cruz de Carvalho 2008; Noctor et al. 2014).

In the context of desiccation and internal changes there can be an accumulation of specific protective compounds such as non-reducing sugars, di- and oligosaccharides, compatible solutes, and protective proteins like late embryogenesis abundant proteins and heat shock proteins. These compounds replace water in the cellular structure to maintain integrity and function during water shortage, but in can be described as a temporary solution as this accumulation can result in undesirable processes such as protein denaturation (Hoekstra et al. 2001; Kaiser et al. 1985).

With ROS signalling during drought stress, the plant hormones triggered by oxidative stress play a possible role. A small number of drought-induced genes respond to $^1O_2$ and abscisic acid, suggesting a link between ROS and hormone signalling under drought conditions (Noctor et al. 2014).

2.6 Ultra-weak photon emission

UPE is a phenomenon concerning the emission of low intensity photons in the range of 200 to 800 nm. It can also be referred to as bioluminescence, one of the functional characteristics of biological organisms, characterized by specialized, low-energy level luminescence (Du et al. 2023). Their formation occurs in most living organisms as a part of metabolic processes and the formation of ROS. UPE observed from various biological samples has been surveyed starting from the subcellular level, cellular level up to the individual organism including plants, animals and humans (Pospíšil et al. 2014). The bioluminescence phenomenon is usually associated with the activity of the enzyme luciferase, which catalyses the binding of luciferin to oxygen, resulting in the production

of light. UPE is slightly different from classical bioluminescence because it can occur spontaneously.

2.6.1 Mechanism of formation of UPE

The formation of UPE involves a combination of biochemical and biophysical processes within living organisms and is usually related to formation of ROS. The oxidative reaction of ROS on biomolecules initiates the decomposition of the unstable high-energy intermediates 1,2-dioxetane and tetroxide, thereby starting the formation of triplet excited carbonyls (Du et al. 2023). Followed by energy transfer between excited carbonyls and chromophores resulting in transition from the excited state to the ground.

2.6.2 Spontaneous UPE

Spontaneous UPE is a type of radiation emitted without any enzymatic systems. It also can be defined as the one which is generated in the course of the oxidative metabolism without any influence of external stressors or stimuli (Cifra and Pospíšil 2014). The emission of photons is therefore directly related to the ROS formation during biochemical reactions, which can serve as an indicator of the internal state of the organism.

2.6.3 Induced UPE

Induced UPE refers to deliberate stimulation or manipulation of biophoton emission events in living organisms. This can be achieved in a variety of ways, including exposure to external stress stimuli such as light, electromagnetic fields, chemicals, temperature or pressure changes. Formation of UPE is closely related to formation of ROS. When ROS are produced during the stress reactions, the ultra-weak photons are emitted by the relaxation of electronically excited species formed during the oxidative stress processes (Pospíšil et al. 2014). The UPE can also provide valuable information about the state of oxidative stress without invasion of a monitor (Kobayashi 2014).

2.6.4 UPE imaging

Because of the ultra-weak intensity in cannot be detected by the naked eye, but in can be measured by highly sensitive apparatuses, such as photo-multiplier tube (PMT) and charged-coupled devices (CCD) (Du et al. 2023). The UPE imaging is a technique to visualize and analyse photons emitted at extremely low levels. It allows us to observe the spatial distribution and dynamics of photon emission withing living organisms, tissues or cells. To identify the properties of UPE for extracting valuable information, visualisation

as two-dimensional (2D) images is necessary for non-invasive diagnosis (Kobayashi 2014). To capture these low signals, the detection devices should be placed in a dark room with as little access to external light as possible.

3 Aims of work

The aim of this work is to investigate the correlation between ultra-weak photon emission and leaf drying and formation of reactive oxygen species. Another aim of the bachelor thesis is to conduct literature research on physiological changes in drying leaves, their causes and consequences. Special attention will be paid to formation of reactive oxygen species as a result of drying of leaves. Another objective is to measure the drying mechanism on the model of *A. thaliana* and other plant species using CCD camera.

4 Material and methods

4.1 Material

4.1.1 *Arabidopsis thaliana wt*

The most used plant material was *A. thaliana* wild type, which is a model organism used for its relatively short life cycle and ease of cultivation. Wild type is a form without mutations or alterations in the internal or external structure.

Seedling trays with small cross section were used for planting. First of all they were completely filled with substrate, which was subsequently sterilized and dried in the Memmert UF 110 (Memmert GmbH + Co. KG, Schwabach, Germany) dryer for 40 minutes at 70°C. The substrate was then rehydrated with water and left to cool off. This was followed by planting the seeds *A. thaliana*, which were soaked in water for 24 hours before planting, using an automatic pipette so that there is one grain in each hole of the seedling tray.

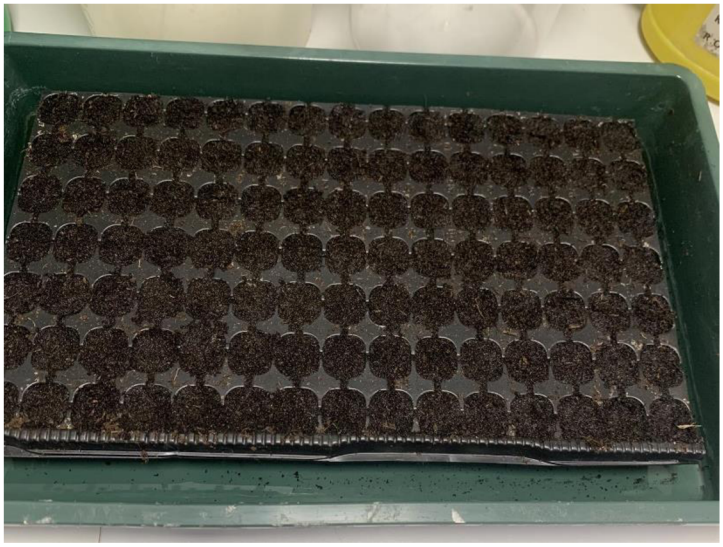


Figure 8. Seedling trays with small cross sections filled with rehydrated substrate before planting the *A. thaliana* seeds.

In the next step the seedling trays were placed in the PhytoScope phytotron (PSI, Drásov, Czech republic) which is used to induce natural conditions ideal for plant growth. Constant conditions were maintained, temperature 21°C, relative humidity of 60%, light with intensity 100 µmol/m^2/s, with repeating cycle 8 hours of light and 16 hours of dark for two to three weeks. After that the small plants were transplanted into bigger seedling trays.

The bigger seedling trays were prepared the same way as the small ones, filled with substrate, sterilized and dry in the Memmert UF 110 dryer for 45 minutes at 70°C. Followed by rehydration of the substrate and again left to cool off. After cooling off, holes were made in the sections of the larger seedling plate to match the size of the section in the small seedling plate by using a planting pin. The substrate with seedlings were removed from small seedling plate and transferred to the holes in the sections of the bigger seedling plate.

Figure 9. Small plant of *A. thaliana* in a small seedling tray in the PhytoScope phytotron before transplanting to bigger seedling trays (left), old *A. thaliana* in a bigger seedling tray (right).

4.1.2 *Hordeum vulgare*

For seeds of *H. vulgare* were used planting pots filled with perlite to about ¾ of their volume, then the seeds were placed on the top and covered with a thin layer of perlite all the way to the edge. Prepared pots were placed in the rectangular low container, watered with Knop´s solution and placed in the photocompartment in the same conditions as *A. thaliana*. Constant conditions were maintained, temperature 21°C, relative humidity of 60%, light with intensity 100 µmol/m^2/s, with repeating cycle 8 hours of light and 16 hours of dark for two to three weeks. The plants were left to grow for over a week.

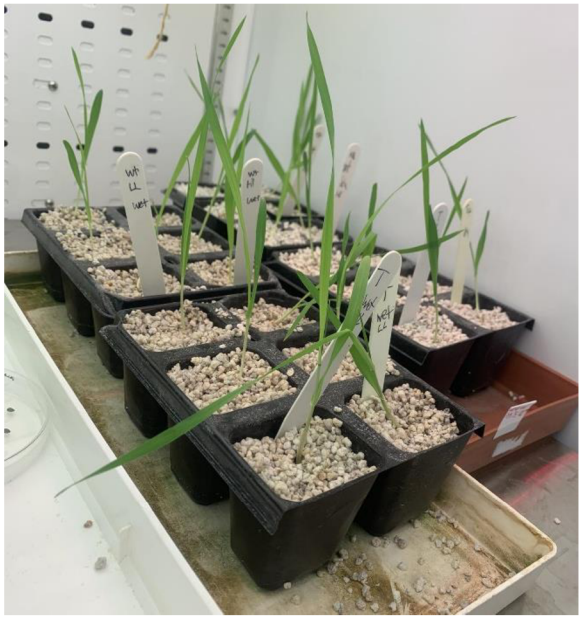


Figure 10. Planting pots filled with perlite with mature plants of *H. vulgare* placed in the PhytoScope phytotron.

4.1.3 *Medicago sativa*

Medium seedling trays featuring were utilized for planting. Initially, these trays were thoroughly packed with substrate, which was then sterilized and dried in a Memmert UF 110 dryer for 40 minutes at 70°C. After drying, the substrate was re-moistened with water and allowed to cool. This was followed by planting the seeds *M. sativa*, which were soaked in water for 24 hours before planting, using an automatic pipette so that there is one grain in each hole of the seedling tray.

For cultivation the PhytoScope phytotrone was used with temperature 21°C, relative humidity of 60%, light with intensity 100 µmol/m^2/s, with repeating cycle 8 hours of light and 16 hours of dark for two to three weeks.

4.1.4 Other plant material

Other plant species surveyed included leaves of *Carpinus orientalis*, *Corylus avellana* and *Persea americana*, which were taken from the external environment.

4.2 Methods

4.2.1 CCD camera

The VersArray 1300B camera (Princeton Instruments, Trenton, NJ, USA) equipped with a 50-mm focal distance lens with a f-number of 1.2 (Nikon, Tokyo, Japan) to enhance the light collecting efficiency. Spectral sensitivity of CCD camera was within the range of λ = 200–1000 nm with almost 90% quantum efficiency in the visible range of the spectrum. The spectral sensitivity was limited to λ = 350–1000 nm by the lenses. CCD camera parameters were as follows: scan rate, 100 kHz; gain, 2. Photons were captured in photon-counting mode. It required cooling to temperatures below -100°C, necessitating pre-cooling at least two hours prior to use. This was achieved by manually filling the camera with liquid nitrogen using a polystyrene container and a funnel. Apart from the first experiment, all of the measurement were performed by using the CCD camera in the dark room. Exposure time of every picture was 0.5 hour. Unfortunately conditions in the dark room could not be ensured at all times, for example humidity could play a major role in the drying process.

Figure 11. The CCD camera VersArray 1300B with a container for liquid nitrogen placed in the dark room.

4.2.2 Measuring

For the experiments the VersArray 1300B camera was used. The exposure time for every picture was 30 minutes and before the measurement started, leaves were put in the dark room for a certain amount of time to adapt to dark and reduce potential delayed luminescence. The number of pictures varied depending on the length and character of the experiment.

Firstly, the ability to restore water flow in the leaves of *A. thaliana* was investigated. For this experiment 24 leaves were used. In the first part of the experiment twelve leaves were taken, weighted and placed in the dark for over 6 hours, which is the time period in which we think there should not be permanent damage to the internal structure. After the drying period leaves were weighted again and put in Eppendorf tubes filled with water for 18 hours. After this period of time leaves were weighted for the third time to discover if the leaves were able to absorb water after drying.

In the second part of the experiment additional 12 leaves were taken, weighted and placed in the dark for over 18 hours, which is the period of time when there was clear permanent damage caused by drought stress. After that the leaves were weighed, put in the Eppendorf tubes filled with water for another 18 hours and weighed again.

The aim of the next experiment was to capture the rapid ROS wave that should occur when the leaves lose contact with water and the drying process begins. In order to capture the exact moment of drying, a cardboard stand with Eppendorf tubes (1,5 ml) was constructed. Leaves were placed in Eppendorf tubes filled with water and after the measurement started, the leaves were removed from the tubes simply by moving the tubes downwards.

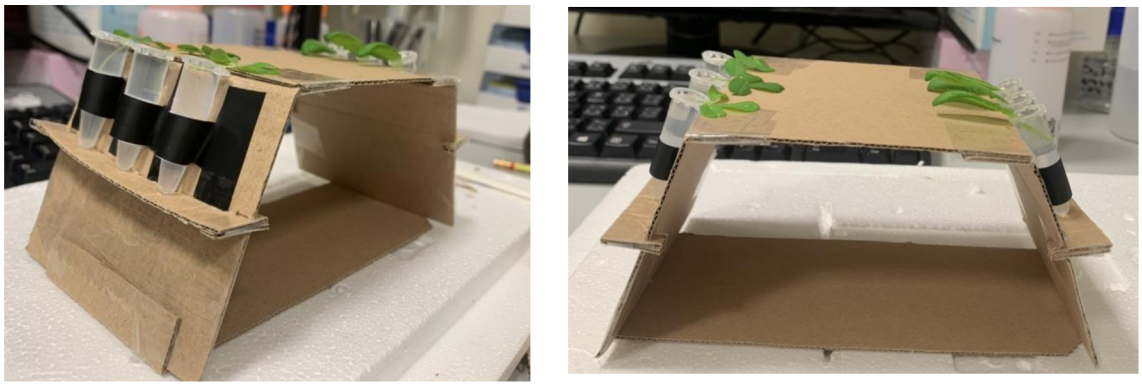


Figure 12. Cardboard stand with Eppendorf tubes (1,5 ml) filled with water used for capturing the exact moment of drying. There are small holders under the tubes that were taken away during the measurement and the tubes could move downwards.

For some plant species, glass was used to fix leaves on the stand. The aim of this experiment was to determine whether the change in UPE is related to mechanism of desiccation itself or it occurs after a physical change in leaf structure associated with desiccation.

Experiments were performed with different numbers of leaves, the number of leaves is indicated in each graph (n=number of leaves).

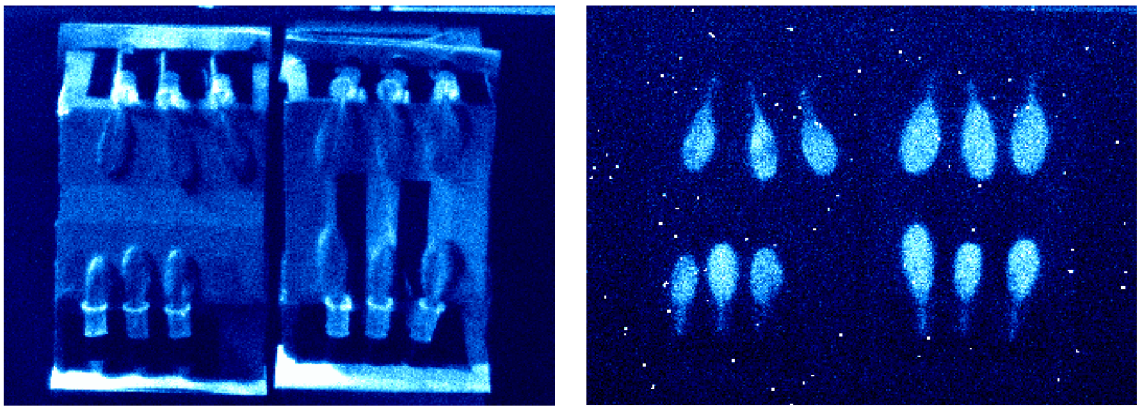


Figure 13. Picture from CCD camera of *A. thaliana* leaves taken with minimal light (left), picture from CCD camera taken in complete darkness showing UPE from *A. thaliana* leaves (right).

4.2.3 Data analysis

Data analysis was performed using the Andor Solis software, which convert pictures files from .SPE format to .TIF format. This .TIF format of pictures taken by the camera were then processed in the ImageJ software, where the area of leaves was selected using the freehand tool. Additionally, the “measure” tool in ImageJ was used to calculate various metrics within the designated area, with “mean” values being used for subsequent analysis. This process was applied to the entire series of images. The “mean” values were then exported to MS Excel for the following analysis.

5 Results

5.1 Restoration of water flow in *A. thaliana*

In the first part of the experiment all of the 12 leaves lost almost half of their original weight. However, after 18 hours in the water, not only were all the leave able to recover their water intake, but they all weighed more than their original weight. Leaf n. 7 was excluded from the evaluation due to an interruption in measurement during the second and third weighing.

In the second part of the experiment all of the additional 12 leaves lost most of its weight. After 18 hours in the water four out of twelve leaves showed minimal change in their weight and two out twelve leaves weighed less than their weight after drying. Five out of twelve leaves showed a significant increase in weight compared to their weight after drying. One out of twelve leaves showed the highest increase in weight, almost up to its original weight.

Table 1. Overview of the leaf weights right after separation from the plant, after 6.5 hours of drying and after 18 hours on the water.

Leaf [*Arabidopsis thaliana*]	**Original weight [mg]**	**Time of drying [hours]**	**Weight after drying [mg]**	**Time in water after drying [hours]**	**Final weight [mg]**
Leaf n. 1	43.46	6.5	27.44	18	49.96
Leaf n. 2	39.12	6.5	20.58	18	47.11
Leaf n. 3	42.00	6.5	27.38	18	53.64
Leaf n. 4	36.17	6.5	22.82	18	46.98
Leaf n. 5	48.46	6.5	28.27	18	54.88
Leaf n. 6	41.06	6.5	26.15	18	48.56
Leaf n. 8	41.23	6.5	32.16	18	50.76
Leaf n. 9	50.33	6.5	34.73	18	58.80
Leaf n. 10	48.82	6.5	26.98	18	53.32
Leaf n. 11	41.82	6.5	30.84	18	52.21
Leaf n. 12	45.37	6.5	30.80	18	51.92

Table 2. Overview of the leaf weights right after separation from the plant, after 18 hours of drying and after 18 hours on the water.

Leaf [*Arabidopsis thaliana*]	**Original weight [mg]**	**Time of drying [hours]**	**Weight after drying [mg]**	**Time in water after drying [hours]**	**Final weight [mg]**
Leaf n. 1	56.73	18	5.36	18	7.8
Leaf n. 2	54.74	18	9.06	18	9.5
Leaf n. 3	54.48	18	19.64	18	45.5
Leaf n. 4	58.27	18	7.64	18	8.6
Leaf n. 5	57.14	18	13.68	18	19.2
Leaf n. 6	52.65	18	9.06	18	7.4
Leaf n. 7	46.96	18	6.59	18	15.9
Leaf n. 8	54.07	18	12.68	18	25.3
Leaf n. 9	54.07	18	12.26	18	24.6
Leaf n. 10	50.20	18	13.54	18	11.3
Leaf n. 11	48.18	18	10.77	18	11.4
Leaf n. 12	45.57	18	11.04	18	37.3

5.2 Effect of leaf fixation on UPE formation

Effect of leaf fixation was investigated on the leaves of *H. vulgare*. The leaves covered with glass fixation and leaves without fixation were compared. In both courses the UPE increased after pulling the leaves out of the water after 0.5 hour from the measurement start. Leaves without fixation (orange) showed a further increase in UPE between 1.5 and 2.5 hours. The leaves with fixation showed increase in UPE later between 2.5 and 3.5 hours. There was a further increase in UPE in both measurements, in the case of leaves without fixation the increase occurred earlier.

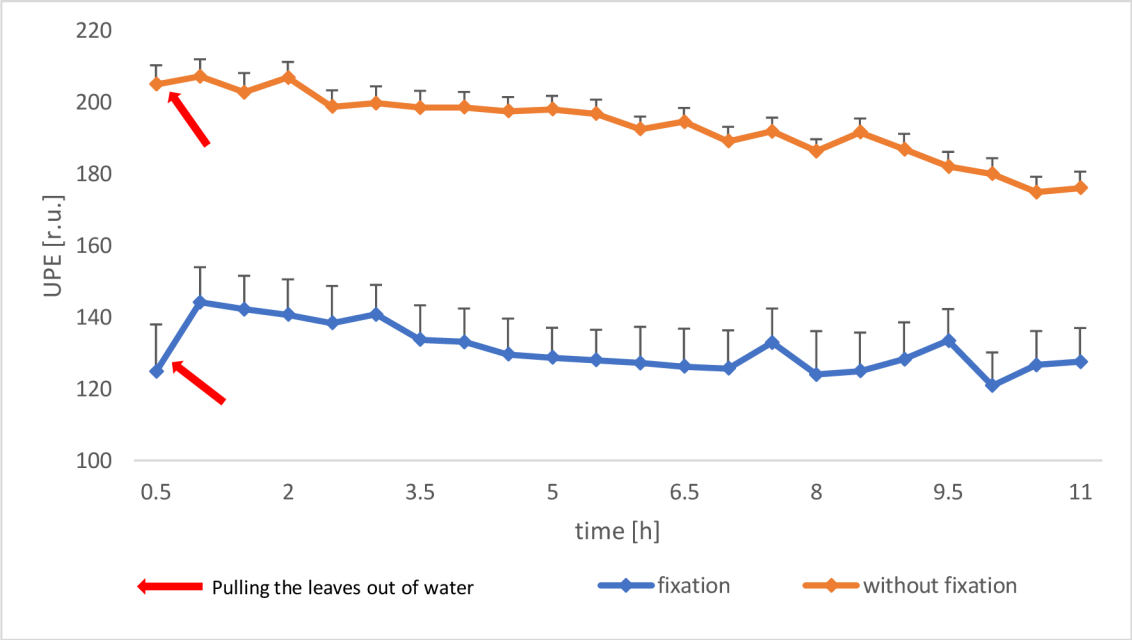


Figure 14. The course of UPE of the *H. vulgare* leaves without glass fixation (orange) and with glass fixation (blue). In both courses the increase in UPE occurred after pulling the leaves out of the water after 0.5 hour after the measurement start, n=6.

The effect of leaf fixation was performed also on *M. sativa* leaves. The UPE increased immediately after pulling the leaves out of the water. This results in an increase in UPE between the 0.5 and 1 hour of the measurement. After that, no other significant increase in UPE occurred, but it decreased till hour 4.

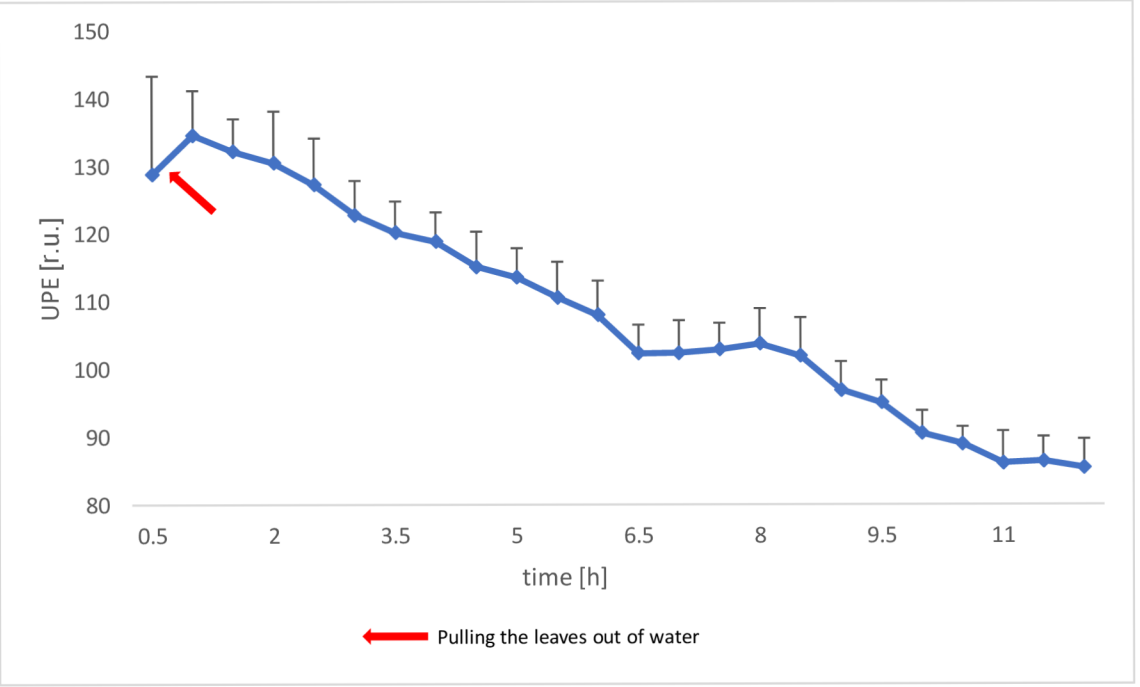


Figure 15. The course of UPE signal of the *M. sativa* leaves, the dark adaptation was 0.5 hour. After pulling the leaves out of the water, the UPE increased, n=6.

5.3 Rapid ROS wave capture

Leaves of *A .thaliana* were not cut under water in this experiment. When the leaves are separated in the air, the water flow was interrupted and no rapid ROS wave occurred after pulling them out of the water, so no increase in UPE occurred after pulling them out of the water and the UPE decreased gradually with no significant increase.

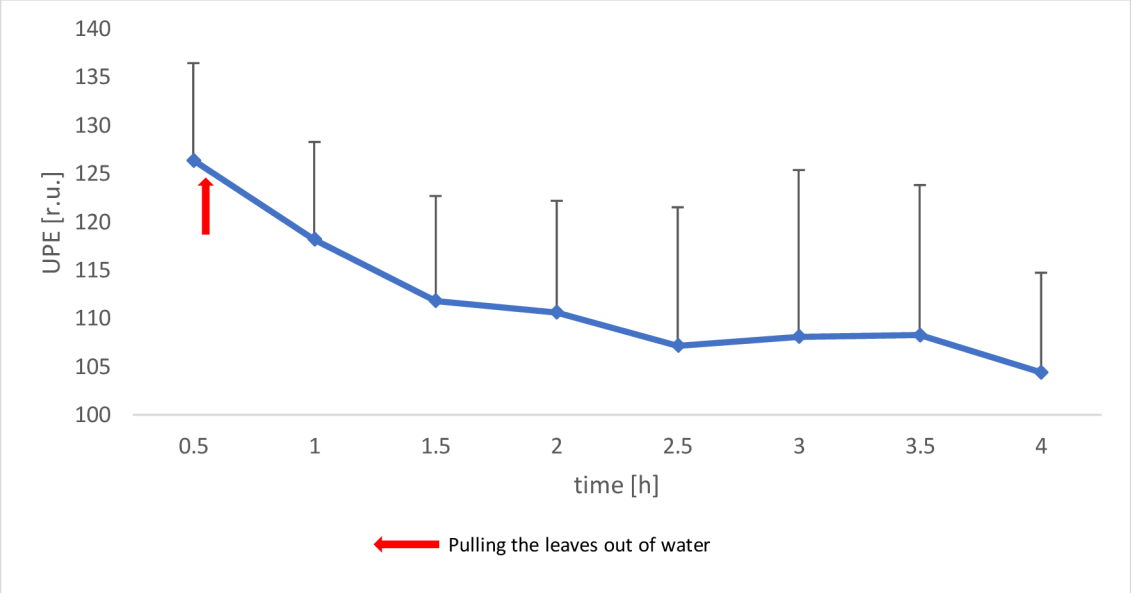


Figure 16. The course of UPE signal of *A. thaliana* leaves, the dark adaptation was 0.5 hour. The leaves ware taken out of the water after 0.5 hour from the measurement start, followed by decrease in UPE, n=3.

The subject of this part was to separate the leaves under the water and capture the ROS wave right after pulling the leaves out of the water. A significant increase in UPE occurred immediately after pulling the leaves out of the water between 0.5 and 1 hour of the measurement (Fig. 16, 1 hour). After that the UPE decreased for 30 minutes and increased again for another hour. Then the UPE decreased until the end of the measurement.

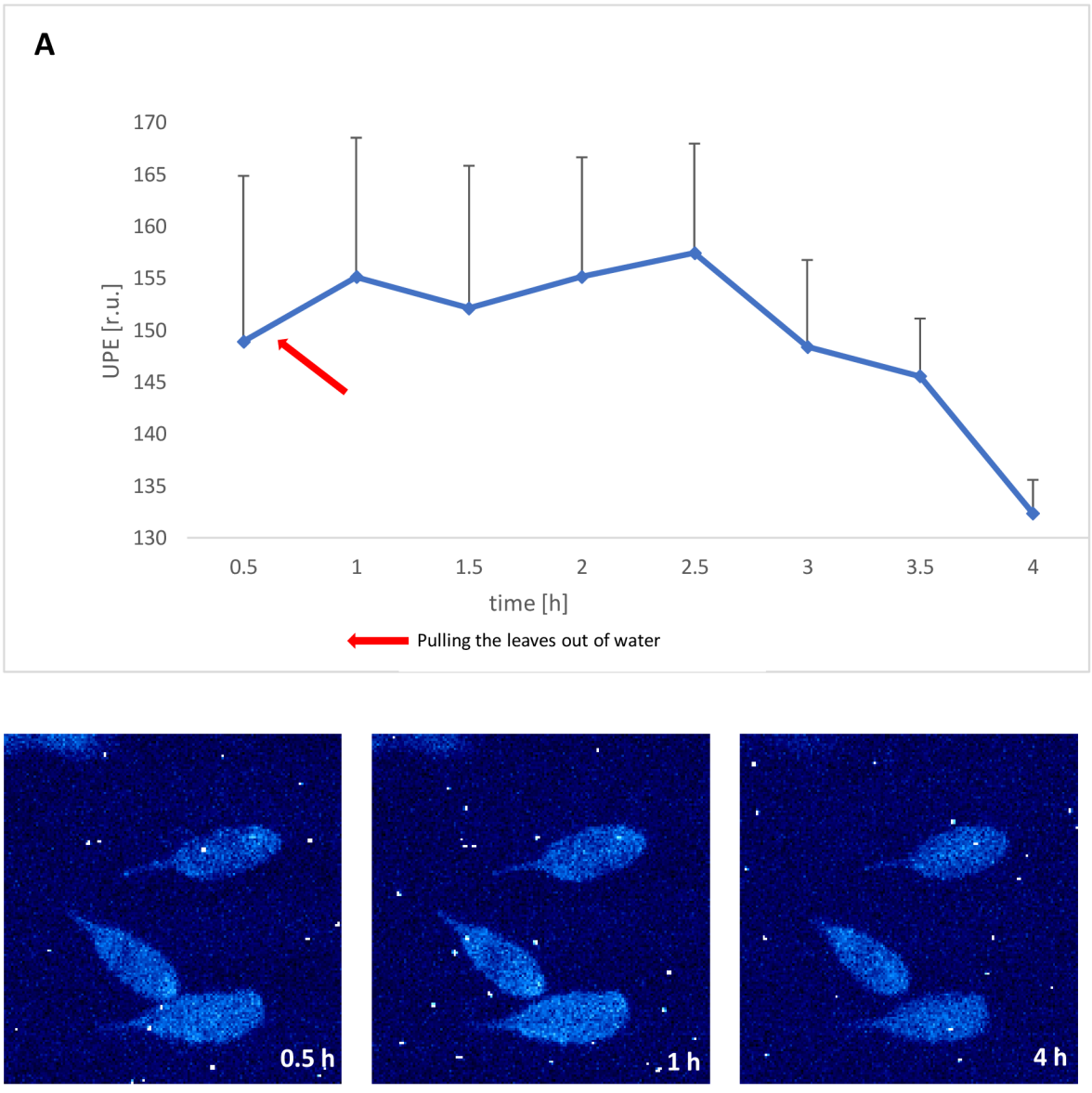


Figure 17. (A) The course of UPE signal of *A. thaliana* leaves, the dark adaptation was 1 hour and the leaves were cut off under the water. The leaves ware taken out of the water after 0.5 hour from the measurement start, followed by 0.5 hour decrease in UPE after which another increase in signal occurred, n=2. (B) Picture from CCD camera of *A. thaliana* leaves in the water (left), the leaves 0.5 hour after taken out of the water (middle) and leaves after 4 hours from the measurement start (right). The middle leaf is not included in the results because it was left in the water.

Leaves of *A .thaliana* were not cut under water in this experiment. No increase in UPE occurred after pulling them out of the water. The UPE decreased gradually with no significant increase.

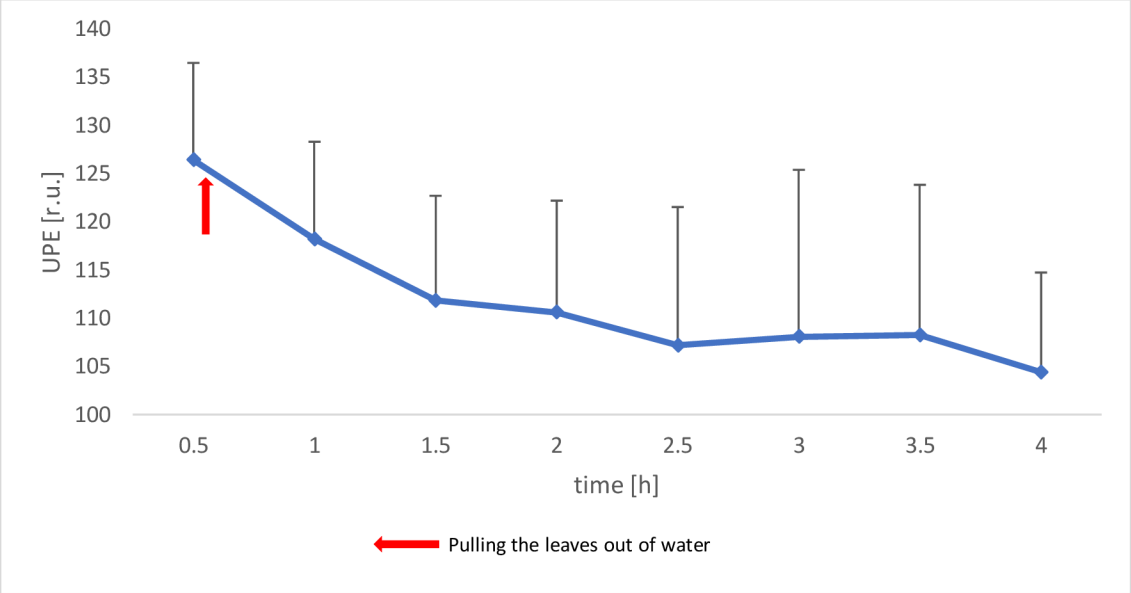


Figure 18. The course of UPE signal of *A. thaliana* leaves, the dark adaptation was 0.5 hour. The leaves ware taken out of the water after 0.5 hour from the measurement start, followed by decrease in UPE, n=3.

Rapid ROS wave was observed on leaves of *H. vulgare*. The UPE increased after pulling the leaves out of the water after 0.5 hour after the measurement start. The increase occurred between the first and second picture, then the UPE decreased for 0.5 hour and after that it increased again. After the second increase, the UPE decreased until the hour 4 with slight increase between 2.5 and 3 hours.

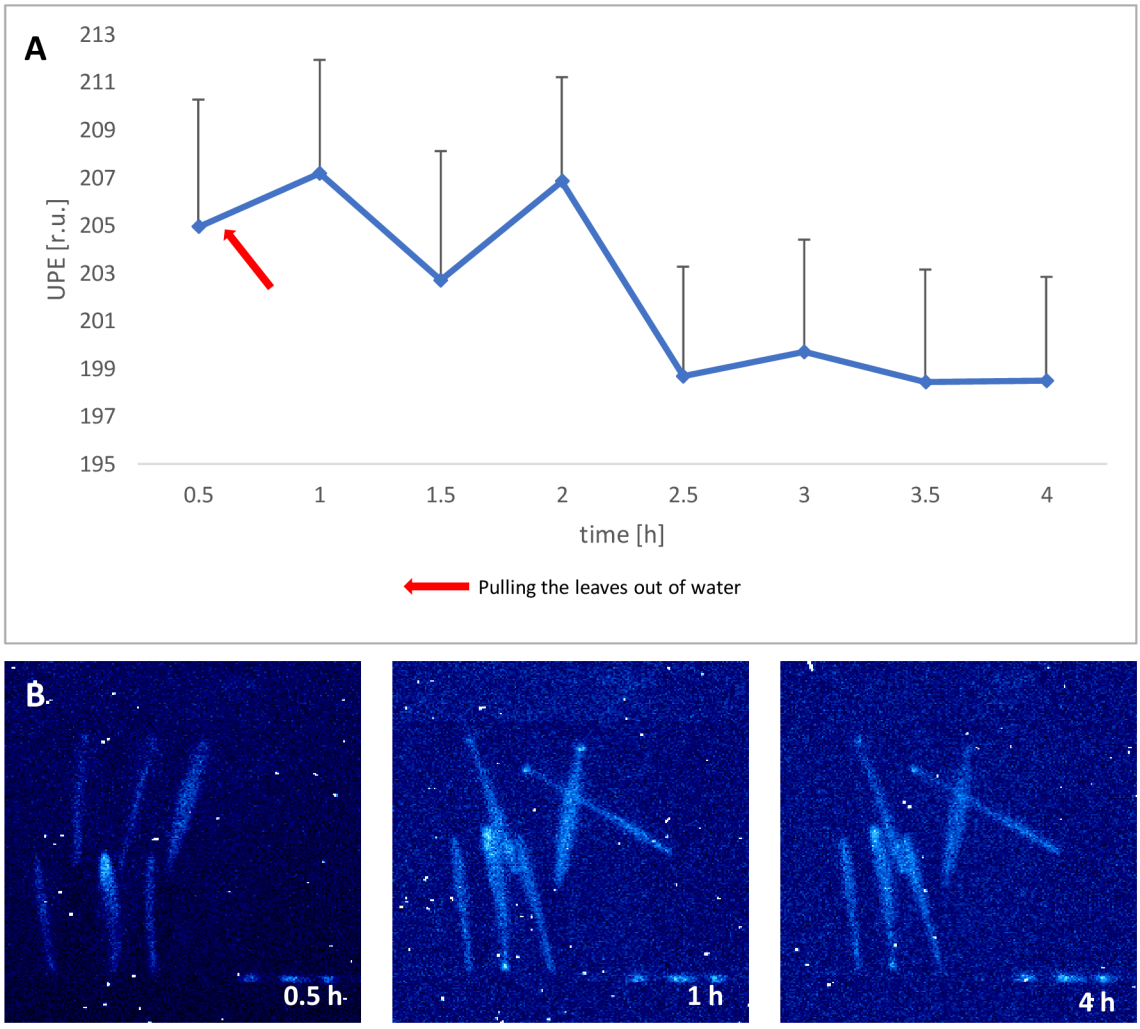


Figure 19. (A) The course of UPE signal of *H. vulgare* leaves, the dark adaptation was 0.5 hour. The leaves ware taken out of the water after 0.5 hour from the measurement start, followed by 0.5 hour decrease in UPE after which another increase in UPE occurred, n=6. (B) Picture from CCD camera of *H. vulgare* leaves in the water 0.5 hour from the measurement start (left), the leaves 0.5 hour after taken out of the water (middle) and leaves after 4 hours from the measurement start (right).

For catching the rapid ROS wave the leaves of *M. sativa* were pulled out of the water after 0.5 hour from the measurement start. Followed by immediate increase in UPE between 0.5 and 1 hour. Then the UPE decreased until the hour 4.

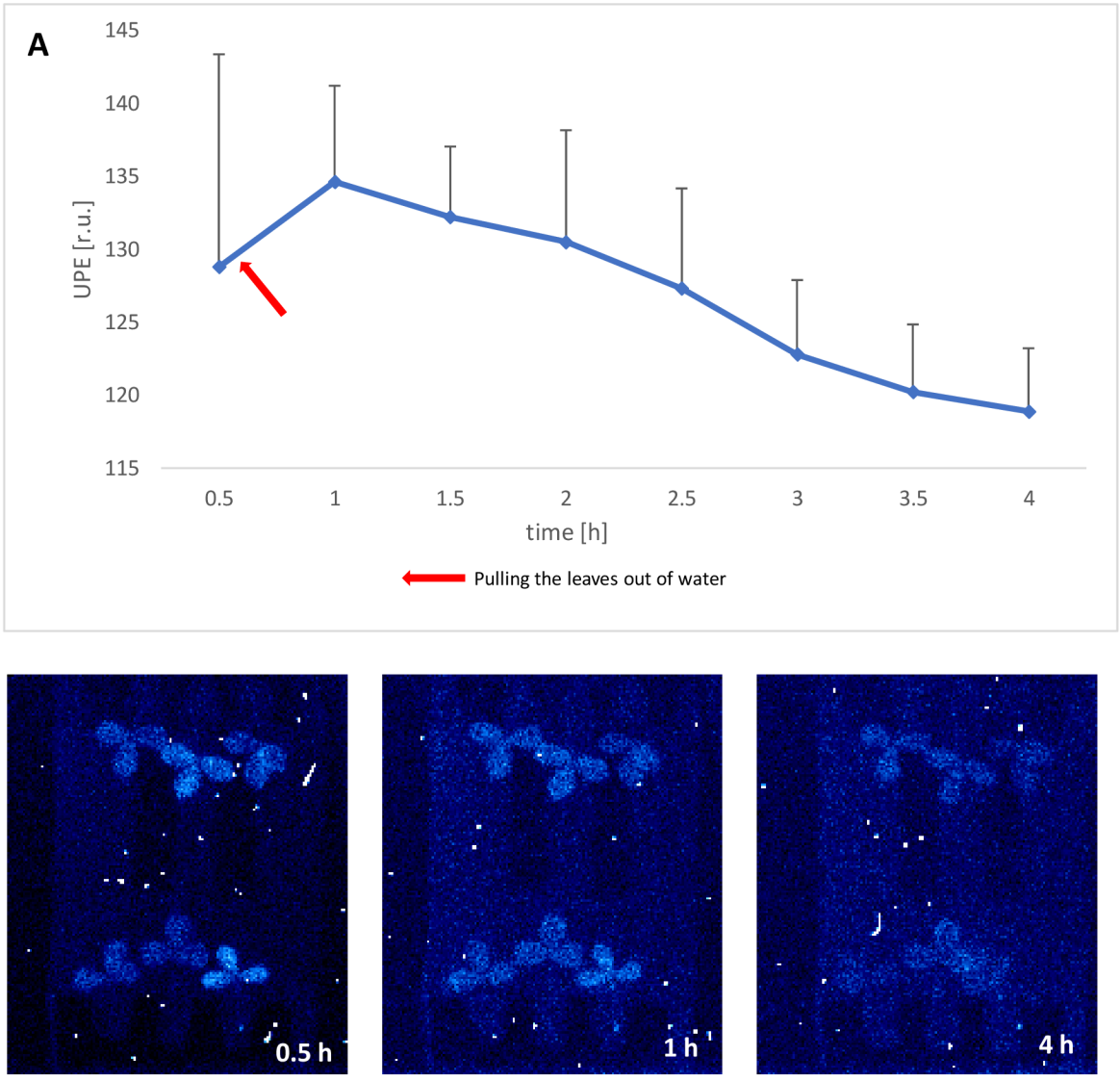
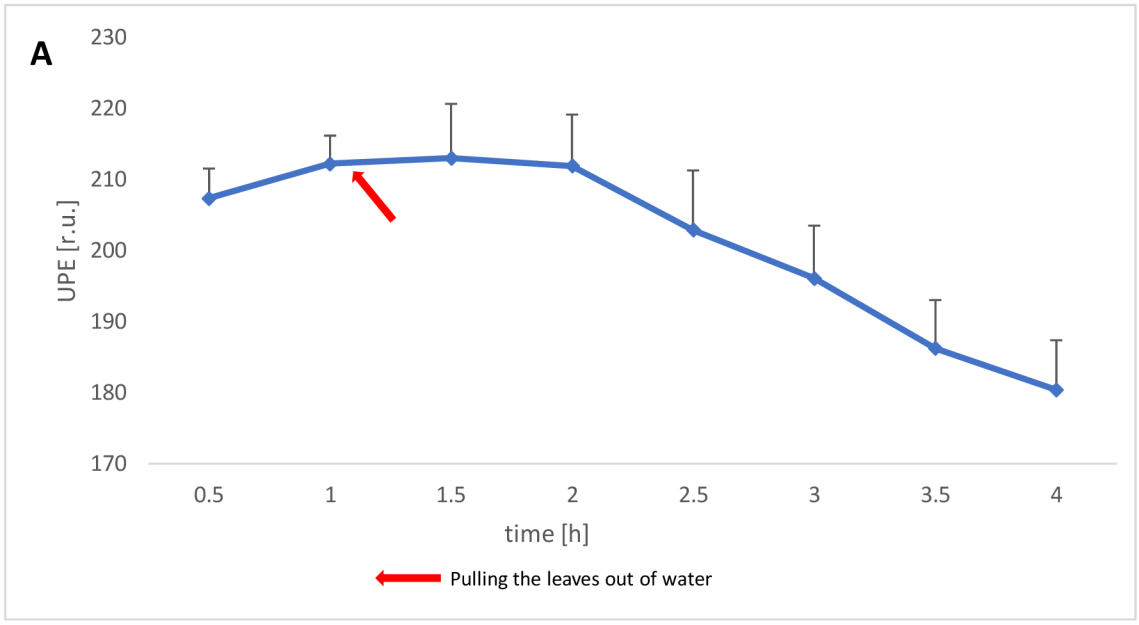


Figure 20. (A) The course of UPE signal of *M. sativa* leaves after 1 hour of dark adaptation and they were cut off under the water. The leaves were pulled out of the water 0.5 hour after the measurement started, which resulted in a significant increase in UPE, n=6. (B) Picture from CCD camera of *M. sativa* leaves in the water 0.5 hour from the measurement start (left), the leaves 0.5 hour after taken out of the water (middle) and leaves after 4 hours from the measurement start (right).

The leaves of *C. orientalis* were used to observe if the rapid ROS wave occurs after pulling them out of the water. The leaves were pulled out of the water 1 hour after the measurement start. This result in an increase in UPE, which in this case was gradual rather than immediate. After 2 hours of the measurement the UPE decreased until the hour 4.



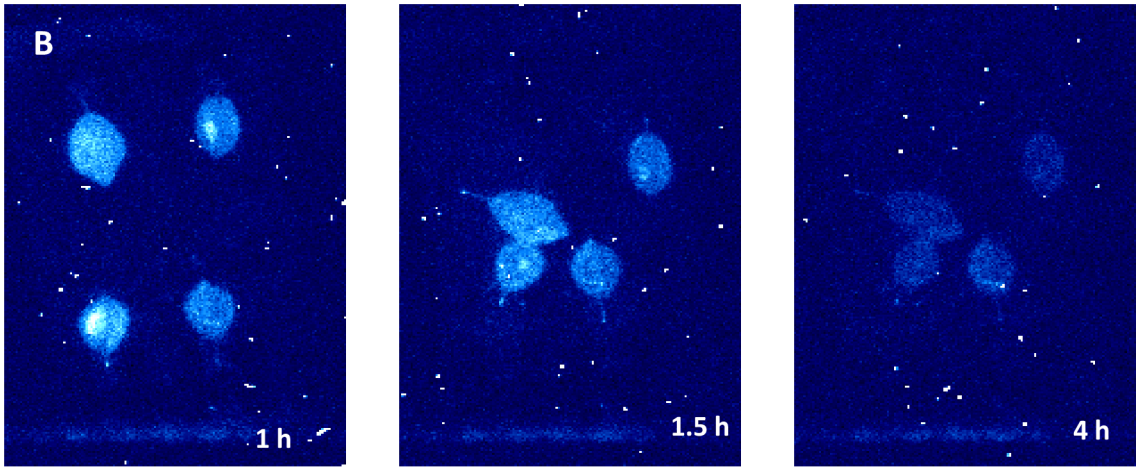


Figure 21. The course of UPE signal of *C. orientalis* leaves after no dark adaptation and they were cut off under the water. The leaves were pulled out of the water 1 hour after the measurement started, n=4. (B) Picture from CCD camera of *C. orientalis* leaves in the water 1 hour from the measurement start (left), the leaves 0.5 hour after taken out of the water and 1.5 hour from the measurement start (middle) and leaves after 4 hours from the measurement start (right).

No rapid ROS wave was observed in *C. avellana* leaves. The leaves were pulled out of the water 1.5 hour after the measurement started and the UPE decreased gradually.

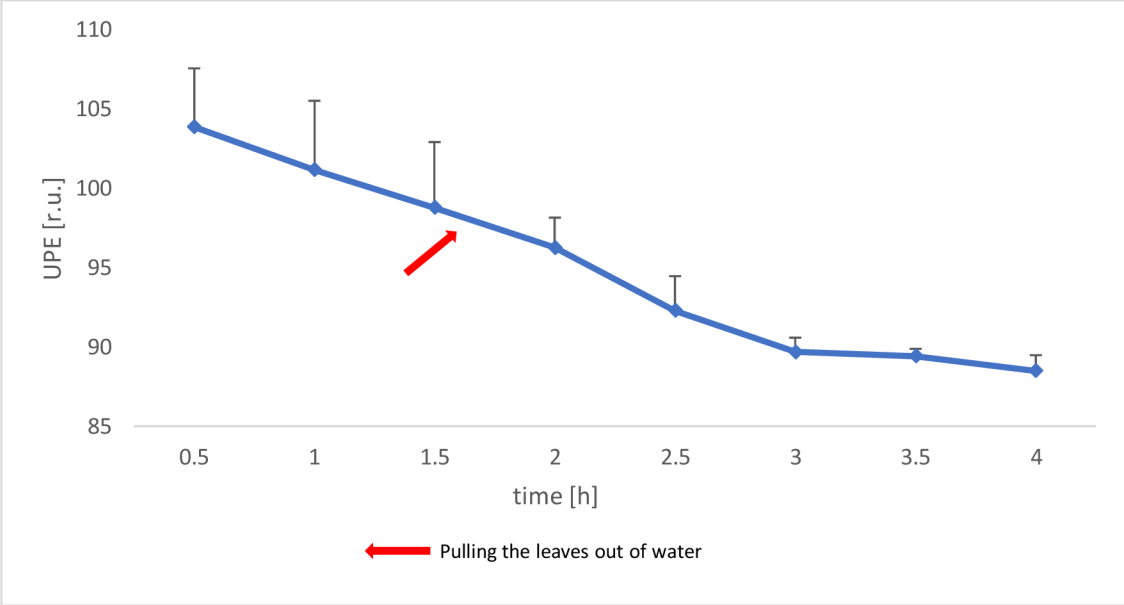


Figure 22. The course of UPE signal of *C. avellana* leaves after 1.5 dark adaptation and they were cut off under the water. The leaves were pulled out of the water 1.5 hour after the measurement started, n=4.

5.4 The drying phenomenon

The long-termed drying effect was monitored. The leaves of *A. thaliana* were pulled out of the water after 0.5 hour of the measurement start, which resulted in the increase in UPE between 0.5 and 1 hour. Followed by 0.5 hour decrease and another increase between 1.5 and 2.5 hour. Then the UPE decreased steadily till the 9.5 hours from the measurement start, where the UPE increased.

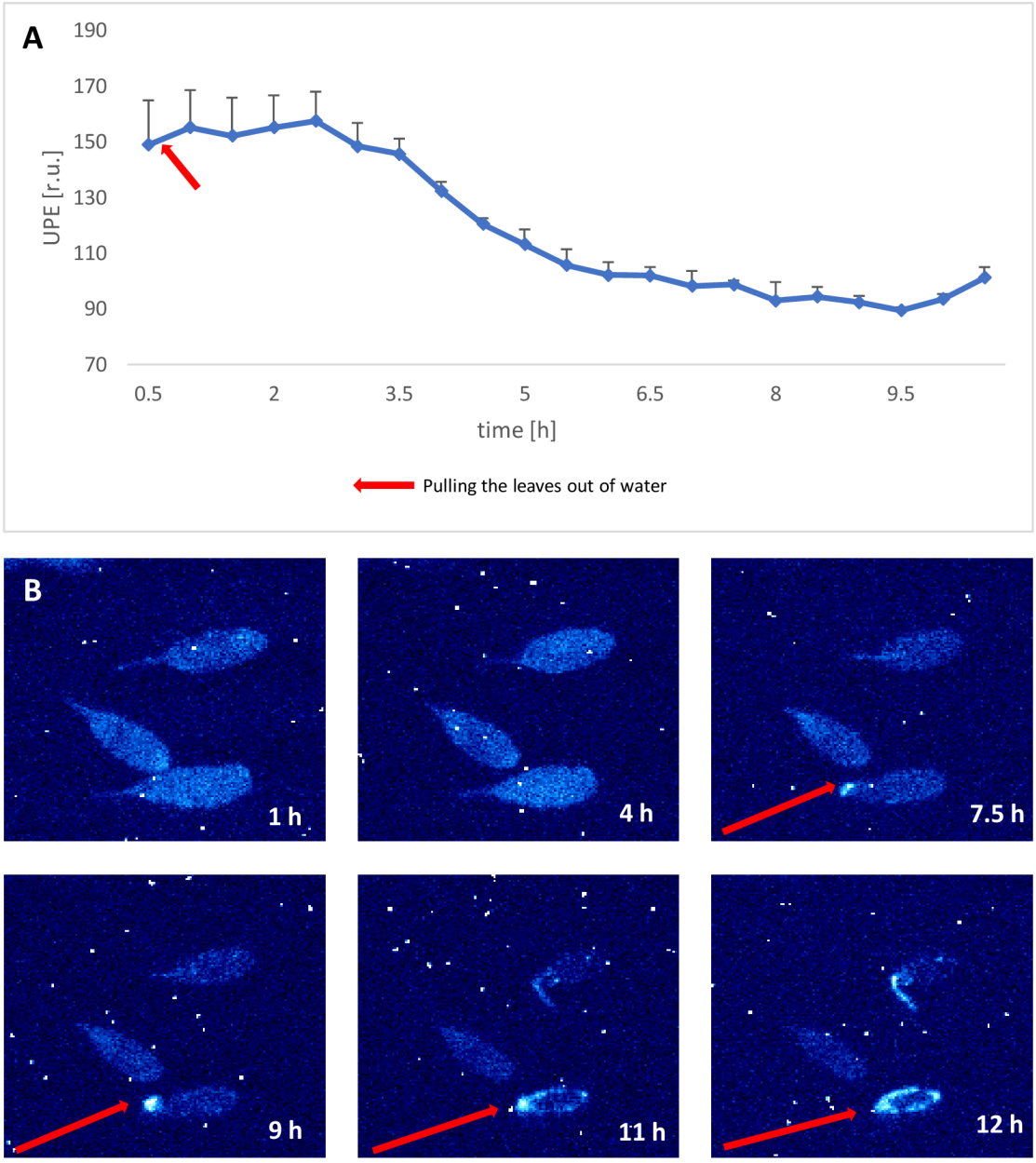


Figure 23. (A) The course of UPE signal of *A. thaliana* leaves after 1 hour of dark adaptation and they were cut off under the water. The leaves were pulled out of the water 0.5 hour after the measurement started, n=2. (B) Pictures from CCD camera of *A. thaliana* leaves, change in UPE over time.

The increase in UPE occurred right after pulling the leaves out of the water between 0.5 and 1 hour of the measurement. The long-term drying effect was investigated. Another increase in UPE occurred between 1.5 and 2 hours followed by decrease in UPE until the 5.5 hours, where the UPE increased and decreased three times within 2 hours. Although there was an UPE increase, it was not visible in the CCD camera.

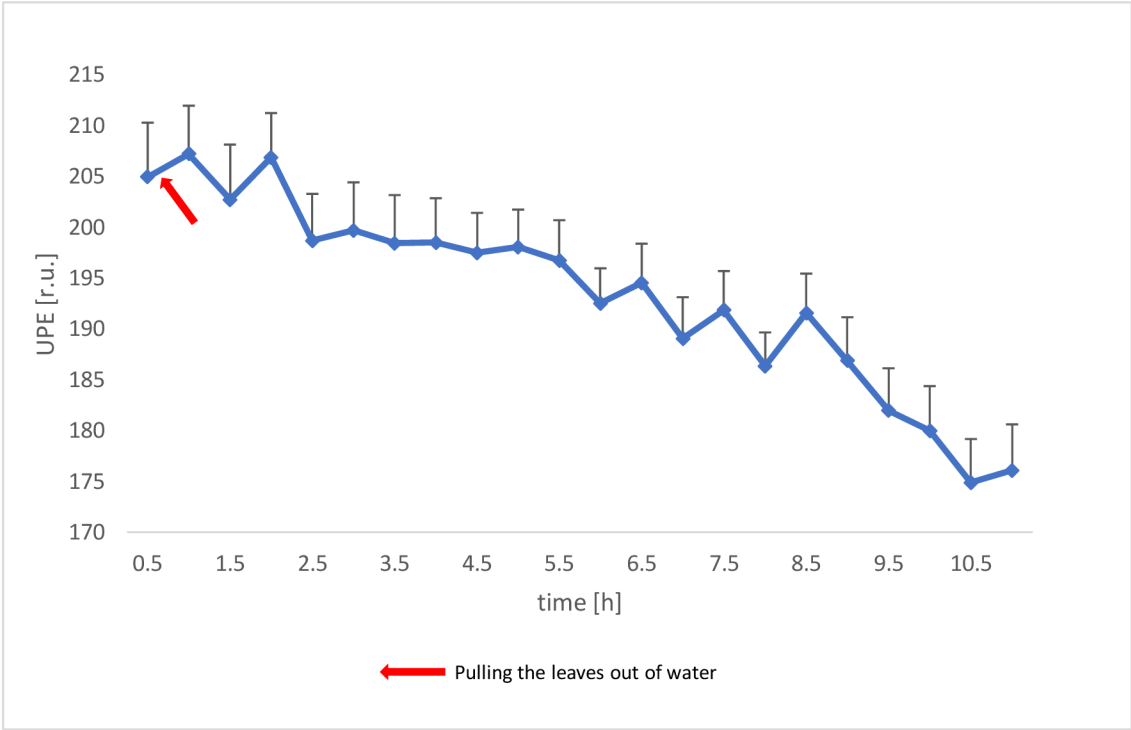
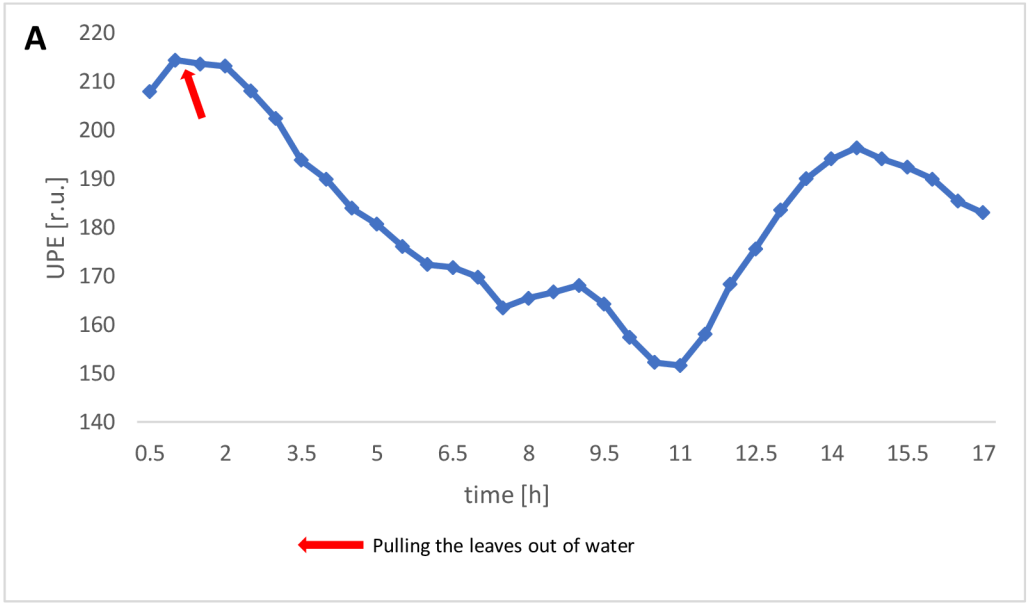


Figure 24. The course of UPE signal of *H. vulgare* leaves after 0.5 hour of dark adaptation. The leaves were pulled out of the water 0.5 hour after the measurement started, n=6.

The increase in UPE occurred immediately after pulling the leaf of *C. orientalis* out of the water between 1 and 1.5 hour of the measurement. Only one leaf was used during data analysis. Following decrease in UPE up until the 7.5 hours from when the UPE increased for 1.5 hours followed by increase in UPE between 9 and 10 hours. From the 11.5 hours of the measurement the UPE significantly increased for over 3 hours.



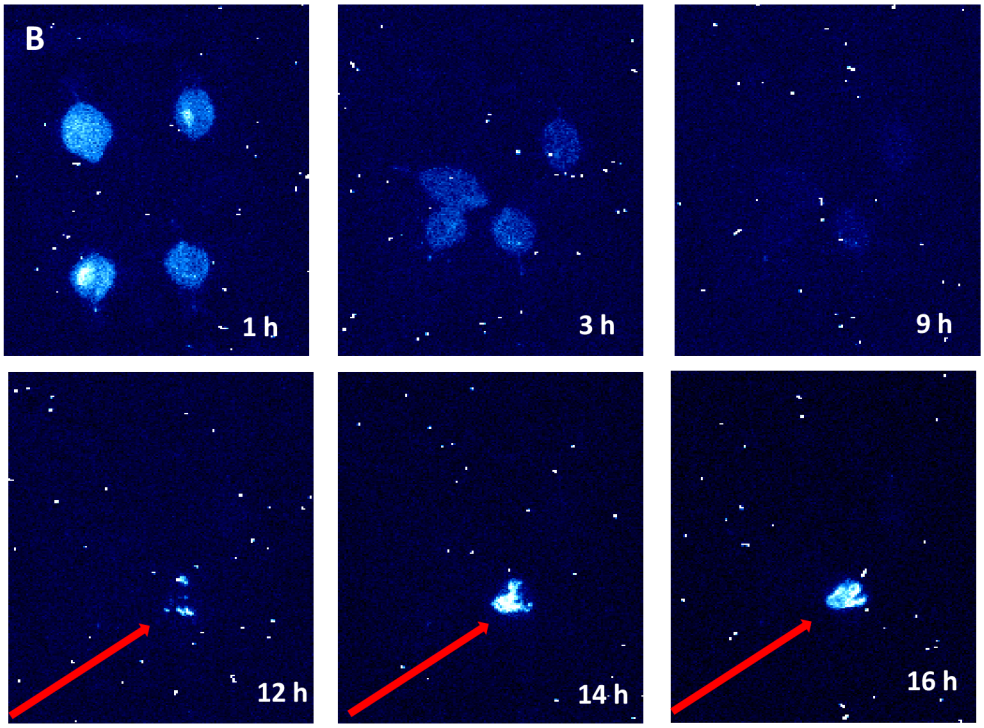


Figure 25. (A) The course of UPE signal of *C. orientalis* leaves after no dark adaptation and they were cut off under the water. The leaves were pulled out of the water 1 hour after the measurement started, n=1. (B) Pictures from CCD camera of *C. orientalis* leaves, change in UPE over time.

In leaves of *C. avellana* there was no increase in UPE after pulling the leaves out of the water. The only increase in UPE occurred after 7 hours of the measurement and steadily increased for 5.5 hours which is clearly visible in Fig. 17.

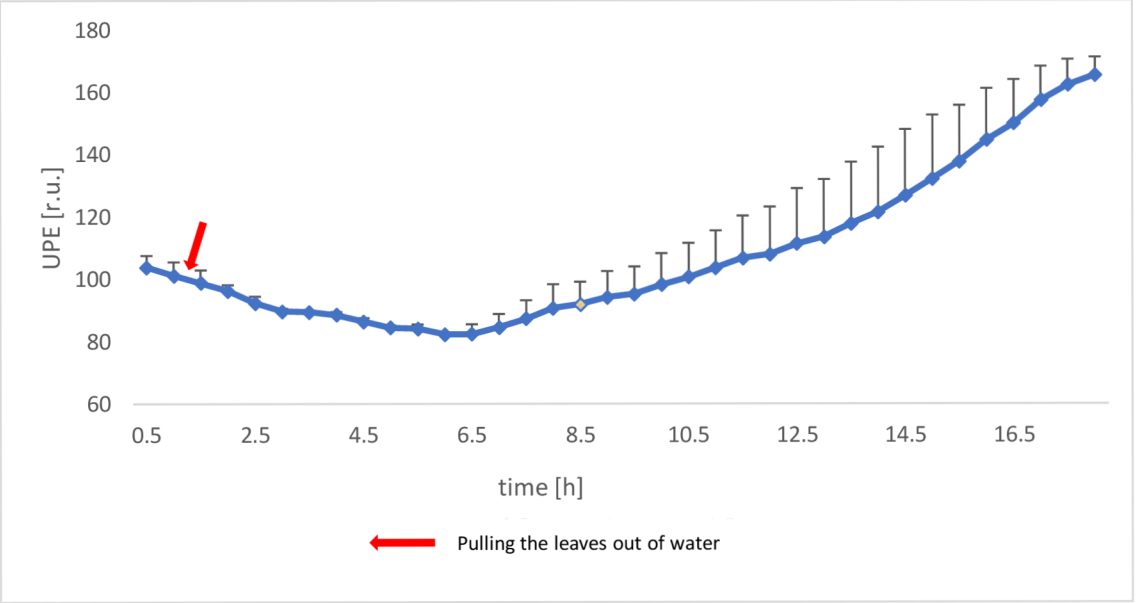


Figure 26. The course of UPE signal of *C. avellana* leaves after 1.5 dark adaptation and they were cut off under the water. The leaves were pulled out of the water 1.5 hour after the measurement started, n=4.

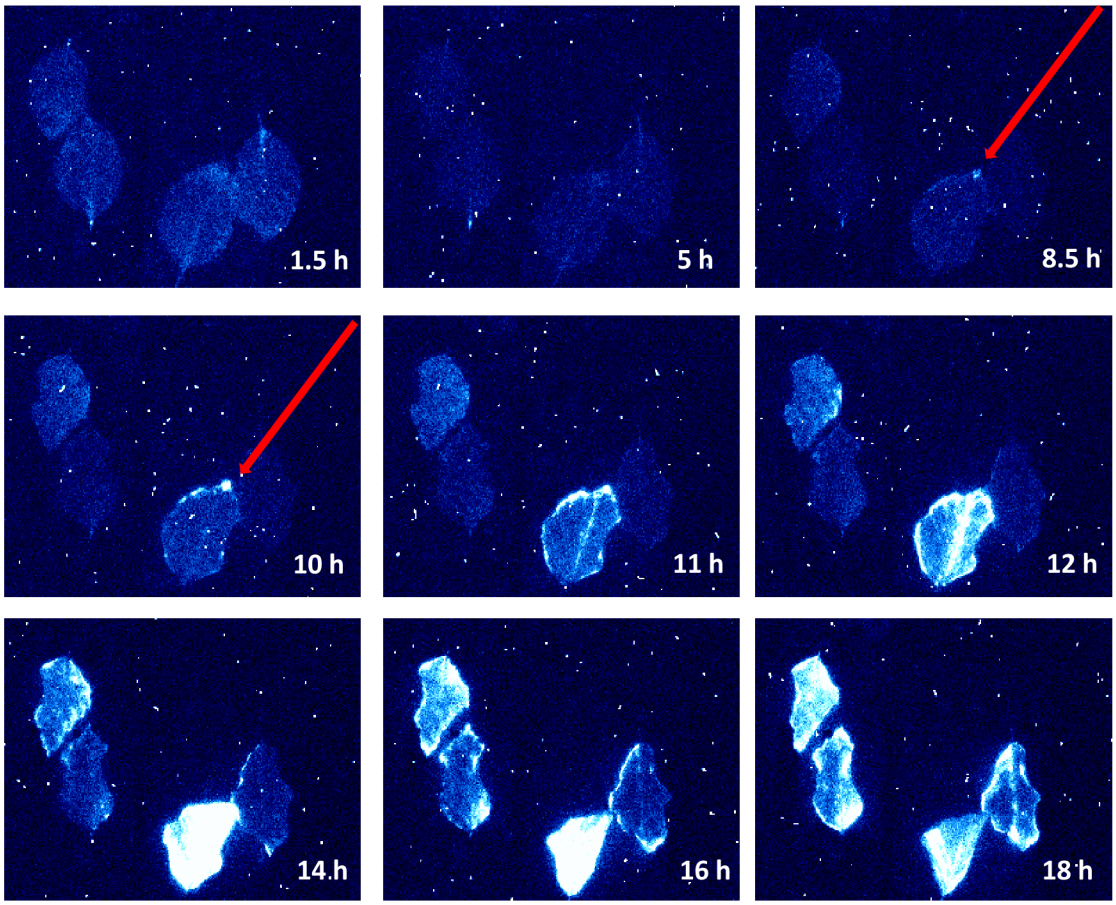
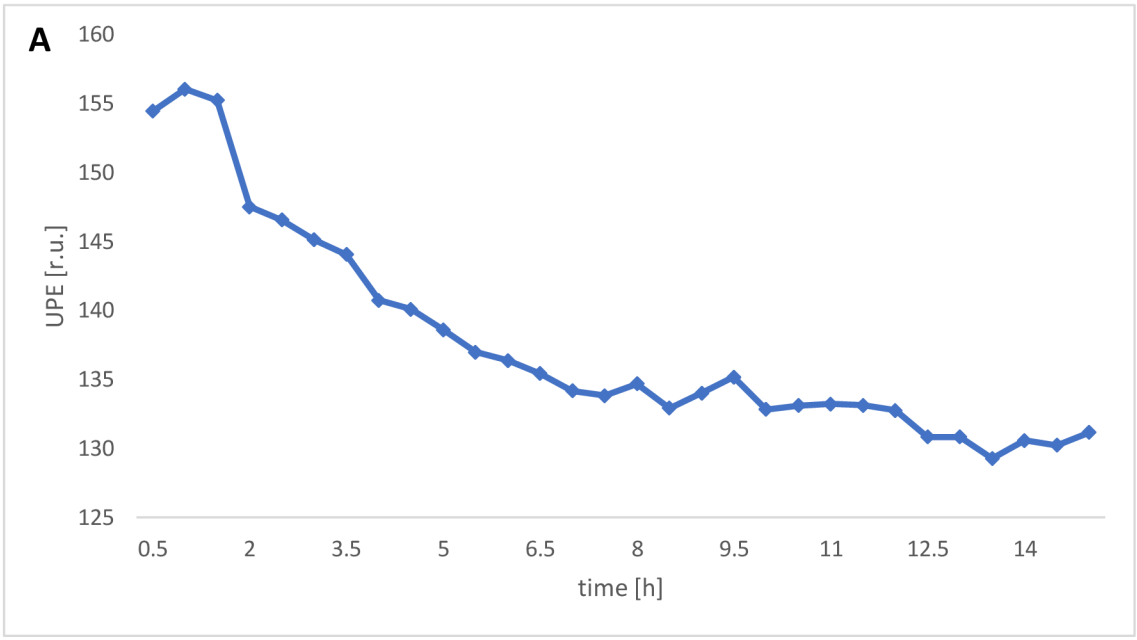


Figure 27. Pictures from CCD camera of C. avellana leaves, change in UPE over time.

The long-term drying effect was observed on the leaf of *P. americana*. The leaves were put directly in front of the CCD camera without water. In the beginning of the measurement there was an increase in signal which could be delayed emission since the leaf was not in the water. After 7.5 hours of the measurement the signal increased again at two consecutive intervals. Even though the changes in UPE were strong, they occurred in a small portion of the leaf only, therefore the changes in UPE looks rather small compared to other species where the UPE was increased throughout the whole leaf.



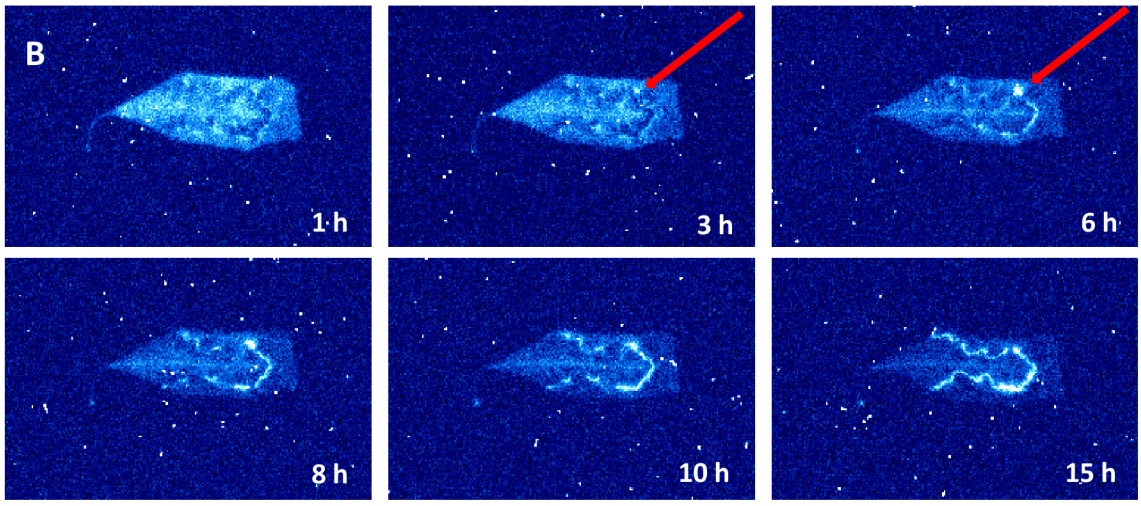


Figure 28. (A)The course of UPE signal of *P. americana* leaves after no dark adaptation. The leaf was not in the water for this experiment, n=1. (B) Pictures from CCD camera of *P. americana* leaves, change in UPE over time. After 6 hours the edges of the internal structure caused increase in emission which continued until the end of the measurement.

Figure 29. The leaf of *P. americana* on visible light. The edges of the leaf are completely dry, in the centre of the leaf there is a green area specifically demarcated.

5.5 Comparison of different long-term drying processes

Different plant species were used to measure the long-term drying effect. When compared, the leaves had different drying mechanisms. In *A. thaliana* the drying occurred from the edges and stem (Fig. 23 - B, Fig. 30 – 1), in *P. americana* the drying occurred only in the centre of the leaf. In *C. avellana* the drying started from the edges and gradually expanded to the centre. The leaves of *C. orientalis* showed increase in UPE caused by drying in the centre of the leaf first, then also on the edges. The leaves of *M. sativa* dried out differently, as the leaf is composed of 3 smaller leaves. Usually it started from the edges and expanded to the centre, but the 3 smaller leaves dried independently of each other.

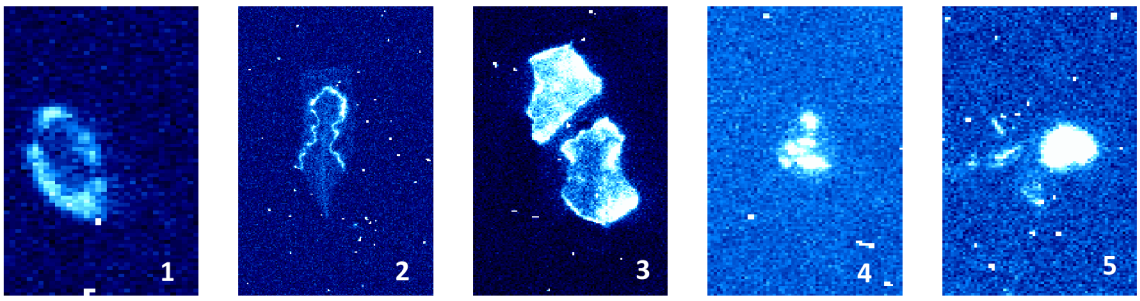


Figure 30. The pictures from CCD camera showing UPE in drying leaves. Leaf of *A. thaliana* (1), leaf of *P. americana* (2), leaves of *C. avellana* (3), leaf of *C. orientalis* (4) and leaves of *M. sativa* (5).

6 Discussion

When the plant is exposed to stress factors such as drought, excessive ROS production occurs (Miller et al. 2010). In general, not much detailed information is known about the relationship between ROS formation and drying. However, the formation of ROS is related to the production of UPE, so this is a suitable method for investigating the kinetics of drying and the temporal-spatial distribution of ROS formation..

In the experiment for restoration of water flow in *A. thaliana* was discovered that the leaves are able to partially recover their water intake even after a prolonged drying period. The leaves that were left to dry for 6 hours were all able to restore their water flow and during final weighing they all weighed more than their original weight (Table 1). This could be caused by the fact that the leaf didn´t excrete water through the stomata or epidermis resulting in water accumulation in the leaf, which led to an increase in its weight. The leaves that were left to dry for over 18 hours left more than half of their weight and after 18 hours in the water some of them were still able to absorb water, even though it was assumed that after such a long drying time it would not be possible. In one case the leaf almost reached its original weight (Table 2, leaf n.7).

We know that mechanical damage causes UPE (Pospíšil et al. 2014; Prasad et al. 2019). To determine whether the UPE from drying leaves is due to ROS formation during drying or as a result of mechanical damage during leaf curling, we covered the leaves with glass. Leaves fixed in this way were not allowed to move, so there was no mechanical damage from leaf curling. In the first experiment (Fig. 14) was discovered, that the leaves under the glass fixation had less UPE intensity than leaves without fixation. This could be caused by the fact that fixation prevented rapid drying through the leaf surface. In both cases, UPE increased immediately after the leaves were removed from the water, but subsequent increases in UPE occurred earlier in leaves without fixation, which could also be the result of faster drying. The main finding was that the formation of ROS over a prolonged drying period is not due to external structural change caused by drying. Simplistically, ROS formation is followed by change in leaf structure causing leaf deformation.

Second part of the thesis was about capturing rapid ROS wave in a form of an increase in UPE, which should occur right in the moment after the leaves were pulled out of the water. This wave occurred throughout the plant species. It was captured in

A. thaliana, where second increase in UPE occurred right after the first one (Fig. 17) which could be the result of a different signalling pathway occurring in the leaf during desiccation (Fig. 6). The rapid ROS wave was captured in leaves that were cut off under the water during preparation. In leaves of *A. thaliana* that were not cut off under the water the rapid ROS wave did not occur (Fig. 16). The rapid ROS wave was observed in *H. vulgare* (Fig. 19 - A), where second increase in UPE occurred right after the first increase after pulling the leaves out of the water. In *M. sativa* leaves the first big increase in UPE occurred as well, but no other significant increase occurred (Fig. 20 - A). Surprisingly, the increase after pulling the leaves out of the water, occurred in *C. orientalis* leaves, where the increase was rather gradual (Fig. 21 - A). In leaves of *C. avellana* no increase in UPE occurred after pulling the leaves out of the water, so this phenomenon is not common to all plant species (Fig. 22).

Next the drying phenomenon was observed, otherwise the long-term course of drying was monitored. In *A. thaliana* there was an increase in UPE after 6.5 hours, where leaf curling started at the stem and continued towards which should be a result of a ROS formation (Pospíšil et al. 2014). This was accompanied by an increase in UPE clearly visible in Fig. 23 – 7.5 hours. In *H. vulgare* prolonged drying was reflected by a signal increase three times (Fig. 24). Even though the signal was visible in the course of UPE, in the pictures of camera the difference was not significant, therefore the picture sequence is not attached in this case. The later increase in UPE could be caused by internal structure of the *H. vulgare* leaf (similar to Fig. 4) as the leaf dries away from the edges causing gradual drying, which may be accompanied by short increases in UPE.

UPE increase after prolonged drying was also discovered in *C. orientalis* leaves. The increased in UPE was rather gradual than immediate (Fig. 25). 15 hours after the start of the measurement, the UPE increased significantly. Even though the leaves of *C. avellana* did not show the rapid ROS wave, a significant increase in UPE occurred 8.5 hours after the measurement started (Fig. 27). Firstly the UPE increase appeared only in one leaf, but was followed by an increase in UPE in all four leaves. According to these findings, the *C. avellana* has a different response to drought than most of the other plant species studied.

An interesting phenomenon was observed in the leaf of *P. americana* where the UPE increased after 6 hours from the measurement start. According to the structure of the leaf

(Fig. 29) the increase in UPE appeared in the centre of the leaf. This leaf was found to have a unique drying mechanism, by drying out from the edges, leaving the centre of the leaf green (Fig. 28 – B).

When comparing desiccation mechanisms between plant species, significant differences can be observed. In *A. thaliana* the leaves dried out from the edges (Fig. 30 – 1) and the stem (Fig. 23 – B). A completely unique drying mechanism has been observed in leaf of *P. americana* where the increase in UPE occurred only in the centre of the leaf in one line, gradually increasing the UPE intensity (Fig. 30 – 2). Another unique mechanism of drying was observed on leaves of *C. avellana*, where no rapid ROS wave occurred but after prolonged period of drying the intensity of UPE increased gradually. The UPE increase was one of the highest compared to other species.

7 Conclusions

It was found that the leaves of *A. thaliana* are able to restore their water uptake after prolonged period of drying. After 6 hours of drying all the leaves were able to restore the water uptake and they even weighed more than their original weight, which could be caused by cumulation of water in the leaf without excretion of water through the stomata or epidermis. And even after 18 hours of drying the *A. thaliana* leaves were able to partially restore the water uptake.

For most monitored plant species, a rapid ROS wave was detected immediately after removal from the water, so the first moment of desiccation was caught. In *A. thaliana* the rapid ROS wave was captured only in the experiment where the leaves were cut off under the water.

One of the key findings was that during drying the ROS formation occurred first, probably causing changes in internal and external structure of the leaf resulting in leaf curling and leaf rolling.

Across plant species a different pattern of drying was observed. Some of them dried form the edges to the centre as *A. thaliana* and *M. sativa*, some of them from centre to edges like *C. orientalis*. Two unique mechanisms were found in *C. avelana* where no rapid ROS wave occurred but the UPE increased gradually to a significant intensity and in *P. americana* leaf where the increase in UPE occurred only in the middle of the leaf.

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