PALACKY UNIVERSITY IN OLOMOUC
Faculty of Science
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Study of mechanical injury of leaves by UPE

Bachelor thesis

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Field of study: B1501 Experimental biology
Form of study: Full-time teaching
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Bibliografická identifikace

Jméno a příjmení autora: Marek Vinkler

Název práce: Studium mechanického poranění listů pomocí UPE

Typ práce: Bakalářská

Pracoviště: Laboratoř růstových regulátorů

Vedoucí práce: Mgr. Marek Rác, Ph.D.

Rok obhajoby: 2023

Abstrakt:

Mechanické poranění listu rostliny vyvolává širokou škálu dějů, jedním z nich je ultraslabá fotonová emise (UPE). V této práci jsme se soustředili na to, jakým způsobem probíhala UPE z listu rostlin, kterým byl list mechanicky poraněn. Jako výchozí rostlinu jsme zvolili *Arabidopsis thaliana,* protože byla v minulosti experimentům zahrnujícím UPE již vystavena. Následně jsme zvolili další dva druhy rostlin, a to *Nicotiana rustica* a *Hordeum vulgare*. Poranění u všech tří druhů rostlin bylo zaznamenáváno pomocí chlazené CCD kamery. Druhým cílem této práce bylo zhodnotit, zdali je vhodné sledovat změny UPE vyvolané mechanickým poraněním u studovaných druhů rostlin, přičemž bylo zjištěno, že *A. thaliana* a *H. vulgare* je vhodné takto studovat. U *N. rustica* je studium UPE vyvolané mechanickým poraněním také vhodné, ovšem s jistými limitacemi. Třetím cílem této práce bylo navrhnout ideální možnost optimalizace nové CCD kamery pro budoucí měření, jelikož budoucnost uplatnění UPE je právě jako neinvazivní metoda pro screening poškození ať už rostlinné nebo živočišné tkáně.

Klíčová slova: Ultraslabá fotonová emise, mechanické poranění, *Arabidopsis thaliana, Nicotiana rustica, Hordeum vulgare*

Počet stran: 55

Počet příloh: 0

Jazyk: Anglický

Bibliographical identification

Author's first name and surname: Marek Vinkler

Title of thesis: Study of mechanical injury of leaves by UPE

Type of thesis: Bachelor

Department: Laboratory of Growth Regulators

Supervisor: Mgr. Marek Rác, Ph.D.

The year of presentation: 2023

Abstract:

Mechanical injury to a plant leaf induces a wide range of processes, one of which, is ultraweak photon emission (UPE). In this work, we focused on how UPE from a mechanically injured plant leaf proceeded. We chose *Arabidopsis thaliana* as a starting plant because it has been subjected to experiments involving UPE in the past. Subsequently, we chose two other plant species, namely *Nicotiana rustica* and *Hordeum vulgare*. The injury in all three plant species was recorded using a cooled CCD camera. The second aim of this work was to assess whether it is appropriate to monitor changes in UPE induced by mechanical injury in the plant species studied, finding that *A. thaliana* and *H. vulgare* are appropriate to study in this way. *N. rustica* is for the study of UPE induced by mechanical injury is also appropriate, but with certain limitations. The third aim of this work was to suggest the ideal optimization of a new CCD camera for future measurements, since the future application of UPE is precisely as a non-invasive method for screening damage to either plant or animal tissue.

Keywords: Ultraweak photon emission, mechanical injury, *Arabidopsis thaliana*, *Nicotiana rustica*, *Hordeum vulgare*

Number of pages: 55

Number of appendices: 0

Language: English

Acknowledgement

I would like to thank my supervisor Mgr. Marek Rác, Ph.D. for his professional help during the preparation of the thesis, for his incredible patience and always helpful approach throughout the work.

Statement

I declare that I myself have written this bachelor thesis under the direction of Mgr. Marek Rác, Ph.D., and with the help of the literature mentioned at the end of the thesis.

In Olomouc dated May 8th, 2023

Contents

List of abbreviations

AA	Ascorbic Acid
APX	Ascorbate Peroxidase
CAT	Catalase
DHA	Dehydroascorbate
DHA	Docosahexaenoic Acid
DHAR	Dehydroascorbate Reductase
ETSs	Electron Transport Systems
GPX	Guaiacol Peroxidase
GR	Glutathione Reductase
GSH	Glutathione
GSSG	Glutathione Disulfide
H_2O_2	Hydrogen peroxide
MDHA	Monodehydroascorbate
MDHAR	Monodehydroascorbate reductase
PUFAs	Polyunsaturated Fatty Acids
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
UPE	Ultraweak Photon Emission

1 Introduction

Chemiluminescence is one of the basic types of luminescence, which is characterized as electromagnetic radiation caused by a chemical reaction. The principle of light generation in these chemical reactions is the emission of photons during the transition of excited molecules to the ground state, namely the decay of unstable intermediates between the excited and ground state of the molecule, which is manifested by the aforementioned emission of light (Raval et al., 2023). Chemiluminescence is widely used in laboratories for determination by chemiluminescent immunoassays (Kamyshny & Magdassi, 1998) beside others.

The best-known example of chemiluminescence is bioluminescence. Bioluminescence as such is also characterized as electromagnetic radiation, which is caused by an enzymatic reaction, the oxidation of luciferin under catalysis by luciferase. These reactions do not always occur spontaneously, but are often conditioned by a presence of other molecule or a group of molecules which supply the necessary components or energy for the oxidation (Kaskova et al., 2016). As far as the scientific use of bioluminescence is concerned, it is widely used as reporter system in transgenic organisms throughout different biological species (bacteria (Monica et al., 2021), plants (Jakšová et al., 2021), mice (Tung et al., 2016)).

Another lesser-known subtype of chemiluminescence is ultraweak photon emission (UPE). All metabolically active organisms are sources of very weak light that can be measured with sensitive light detectors. In biological systems at all developmental stages, including microorganisms, plants and animals, oxidative processes occur during metabolism. These chemical processes produce electronically excited species, which emit photons when they move from the excited to the ground state (Pospíšil et al., 2014). Perhaps the most important reason why scientists are looking at this emission today is its potential use as a non-invasive method of detecting higher metabolic activity, such as the occurrence of damage due to oxidation of macromolecules in plants (Winkler et al., 2009), or oxidative damage to tissues affected by cancer (Scordino et al., 2014).

2 Theoretical Part

2. 1 UPE

UPE was first discovered by a Russian histologist A. G. Gurwitsch in 1923, while he tried to understand what triggered the cell division. Nowadays we know that Gurwitsch measured just the UV component of the UPE, which he named mitogenetic radiation (Volodyaev & Beloussov, 2015). There is still not uniform nomenclature and therefore we can encounter different names that refer to the same thing, namely UPE, such as autoluminiscence (Sardarabadi et al., 2020), meaning that energy needed for the emission generates automatically without any stimuli, biophoton emission or biophotons , which means that energy needed for the emission comes from processes inside of a living cell (Wijk & Wijk, 2005), or just chemiluminescence, whereas mentioned energy comes from chemical reactions. (Cifra & Pospíšil, 2014; Kawabata et al., 2004).

UPE is characterized as a non-thermal radiation that occurs from near-ultraviolet (200 nm) through visible region to the near-infrared region (1300 nm) of the electromagnetic spectrum (Burgos et al., 2017). The energy needed for the non-thermal radiation comes straight from the excited molecules formed during chemical reactions (Cifra & Pospíšil, 2014).

UPE is usually divided into spontaneous and induced. Spontaneous UPE is generated without any outside or inside stressor influence, whereas the induced UPE happens due to biotic or abiotic stress. Biotic stressors are viruses, fungi or bacteria. Abiotic stress factors can be for example pH, ionizing radiation, or heat. In normal states such as cellular respiration or photosynthesis UPE may consist of barely tens or hundreds of photons per square centimeter per second whereas when induced UPE may consist of tens of thousands or hundreds of thousands of photons per square centimeter per second (Cifra & Pospíšil, 2014).

UPE is a common phenomenon that occurs due to an oxidative metabolism in all living cells, varying from single bacteria (Devaraj et al., 1997), through various plant cells (Yoshinaga et al., 2006) to human skin cells (Khabiri et al., 2008).

The source of UPE are reactive oxygen species (ROS), which are various oxygen derivatives that are very strong oxidants that cause oxidative stress. This oxidative stress then sets off a cascade of chain reactions that result in the formation of excited molecules such as triplet excited carbonyls or other high energy intermediates. The subsequent decay of these excited molecules results in the formation of UPE (Gutterridge & Halliwell, 2000; Pospíšil, 2014).

2. 2 ROS in plants

2.7 billion years ago, thanks to the development of photosynthetic organisms, the composition of the atmosphere changed in favor of oxygen. Together with increased oxygen levels, ROS became to appear. Since then, ROS have been persistent companions to any aerobic life (Bhattacharjee, 2012; Gill & Tuteja, 2010).

As for the role of ROS in the plants, they mainly serve as a secondary messenger in cell signaling (del Río et al., 2006). This role is strictly tied to a very low and narrow range of concentrations in which they must be present in the cell. Plant cells are equipped with a complex system of proteins and ions that enable the detection or translation of the ROS-mediated signals, which can lead, to protein phosphorylation, mobilization of the cell's calcium ion pool, or even an increase or decrease in gene expression. From another perspective, ROS can also control the opening or closing of plant stomata, apoptosis or even gravitropism (Sharma et al., 2012).

2. 2. 1 ROS generation in plant organs

The generation of ROS is mainly caused by electron transport systems (ETSs), which is using oxygen as a powerful electron acceptor, meaning the main producers of ROS are namely chloroplasts (Li & Kim, 2022), mitochondria (Starkov, 2010) and peroxisomes (del Rio & Lopez-Huertas, 2016), the other producers of ROS are also plasma membrane, endoplasmic reticulum and the cell wall (Sharma et al., 2012).

Chloroplasts are linked to the process of photosynthesis and to the electron transfer by their very nature. Under common conditions, the flow of electrons through the chloroplast is normal and electron transfer to the final CO_2 acceptor occurs. In contrast, during degraded conditions induced by drought, increased salinity, or extreme excess light, the ECTs become overwhelmed and CO_2 fixation is limited. It is the lack of CO_2 as an electron acceptor that results in electron flow to O_2 causing ROS formation (Elstner, 1991).

In mitochondria, ROS production also occurs to a greater extent only under extreme conditions, as in chloroplasts. In mitochondria, these conditions lead to overcrowding of ETCs and inhibition of ATP synthesis, leading to a reduction of electron carriers and ROS formation (Blokhina & Fagerstedt, 2010).

Peroxisomes are perhaps the largest producers of ROS, specifically hydrogen peroxide (H_2O_2), in the cell, which follows from the very nature of their oxidative function. Peroxisomes mediate cellular biochemical pathways such as β-Oxidation, glycolate oxidase reaction and superoxide radical disproportionation (Sharma et al., 2012).

2. 2. 2 Physical formation of ROS

ROS can be divided into two groups, non-radical and radical. Non-radical ROS such as H_2O_2 and singlet oxygen have no unpaired electron, whereas radical ROS such as superoxide radical and hydroxyl radical have one or two unpaired electrons on the oxygen atom. The formation of ROS in living organisms follows two different pathways, either Type I reaction, which is connected with leakage of electrons within ETSs, or Type II reaction, which is connected with triplet-singlet energy transfer and singlet oxygen formation (Pospíšil et al., 2014).

Both of those reactions use a structure called photosensitizer, cluster of molecules, which can absorb light and transfer its energy into another nearby molecule (Gómez Alvarez et al., 2012). After the photosensitizer is excited via light absorption it can undergo either type I or type II reaction. The type I reaction is associated with the formation of a superoxide radical through formation of an anion-cation complex between photosensitizer and substrate. H_2O_2 and hydroxyl radical can be later formed due to the presence of superoxide radical. The type II reaction is associated with the formation of a singlet oxygen through the transfer of energy between the excited photosensitizer and a substrate, which is triplet oxygen. (Pospíšil et al., 2014).

2. 2. 3 Antioxidants

At higher concentrations, ROS are harmful to the plants or other living cells in general. This fact caused any aerobic living organism to evolve and form a machinery that would be responsible for scavenging and efficiently degenerating radical and non-radical ROS from their body (Mittler, 2002), preventing oxidative stress-induced damage and also maintain homeostasis. The ROS defense machinery could be distinguished into two branches of antioxidants, which are the enzymatic antioxidants and the non-enzymatic antioxidants. (Das & Roychoudhury, 2014).

2. 2. 4 Enzymatic antioxidants

The enzymatic antioxidants are found within the subcellular compartments and the arsenal of enzymes consist of Superoxide Dismutase (SOD), Catalase (CAT), Ascorbate Peroxidase (APX), Monodehydroascorbate reductase (MDHAR), Dehydroascorbate reductase (DHAR), Glutathione reductase (GR) and Guaiacol Peroxidase (GPX) (Das & Roychoudhury, 2014).

SOD is an enzymatic antioxidant which belongs to the family of metalloenzymes. It is present within any aerobic organism, but in plants the main three isoenzymes, which are classified by the metal they are bonded with are localized in mitochondria (Mn-SOD), chloroplasts (Fe-SOD) and cytosol (Cu/Zn-SOD). SOD plays a key role in reducing stress induced damage, because it prevents hydroxyl radical from forming by the removal of the superoxide radical by its dismutation into oxygen and later into oxygen peroxide (Mittler, 2002). Also, the activity of SOD was found to be up regulated by abiotic stress (Boguszewska et al., 2010).

CAT is a tetrameric heme-enzyme that catalyzes the dismutation of H_2O_2 into water and later into oxygen with great efficiency. CAT is found in peroxisomes, because of the high production of H_2O_2 due to intense metabolic activity that is present, such as photorespiration or purine metabolism (Mittler, 2002).

APX is an enzyme that could be found mainly in the peroxisomes, but also in the cytosol and chloroplasts, depending on its isoform, based on various bonded amino acids. APX scavenges H_2O_2, with even greater efficiency than CAT, and reduces it into water and docosahexaenoic acid (DHA), using Ascorbic acid (AA) as a reducing agent (Mittler, 2002; Sharma & Dubey, 2004). MDHAR and DHAR are helper enzymes that procure sufficient quantities of AA for APX usage. MDHAR regenerates AA from monodehydroascorbate (MDHA) using NADPH. DHAR reduces dehydroascorbate (DHA) to AA by reduced glutathione (GSH) while also producing glutathione disulfide (GSSG) (Eltayeb et al., 2007).

GR is an enzyme found in chloroplast and in small amount is also found in mitochondria and cytosol. Its function is to reduce GSSG to GSH, so that GSH could be used to regenerate AA by DHAR. One of the main functions of GR is to maintain the GSH/GSSG ration in cellular environment so that there would not occur a shortage of AA for reducing H_2O_2 in the first place (Das & Roychoudhury, 2014).

GPX is another key enzyme for degenerating H_2O_2 found both intercellularly and in the cell wall. GPX eliminates excess H_2O_2 both in normal conditions and stress conditions. GPX has a secondary function which is a synthesis of lignin and also it plays a role in defense against biotic stress by utilizing H_2O_2 and degrading indole acetic acid (IAA) (Mittler, 2002). It uses guaiacol, which is an aromatic compound, as an electron donor (Asada, 1999).

2. 2. 5 Non-enzymatic antioxidants

The non-enzymatic antioxidants make up the second part of the antioxidant apparatus, and consist of AA, GSH, α-tocopherol, carotenoids, phenolics and flavonoids. Their function is preventing oxidative damage, but they also regulate many vital functions such as cell division, elongation, senescence and cell death (de Pinto & de Gara, 2004).

AA is an abundant substance present in plant cells, it is also extremely powerful because of its ability to donate electrons to a wide variety of reactions both enzymatic and on-enzymatic. AA in cells is generated via two pathways the first one is the Smirnoff-Wheeler pathway in mitochondria with the presence of the L-galactano-γ-lactone dehydrogenase. The second pathway uses the D-galactouronic acid. AA is stored in the cell guaranteeing quick response to ROS (Barnes et al., 2002). But it is not exactly the AA that reacts with ROS, AA oxidates into MDHA which later breaks apart into AA and DHA which react with H_2O_2, hydroxyl radical and superoxide radical, while also regenerating α-tocopherol. (Shao et al., 2005).

GSH is a thiol tripeptide found in most of cellular compartments. It is present in cytosol, mitochondria, chloroplasts, vacuoles and even in the apoplast. Its functions are ranging from scavenging ROS, to cell differentiation, growth, death, it also regulates a variety of molecular transports and detoxification, lastly regulates stress gene expression and enzymatic activity (Mullineaux & Rausch, 2005). The structure of GSH with its cysteine residue is mainly responsible for its reducing ability. GSH scavenges all ROS, meaning H_2O_2, singlet oxygen, hydroxyl radical and superoxide radical and also protects biomolecules by adducting or reducing them in the presence of ROS or other radicals. GSH is also important when generating AA for GSSG as mentioned (Roychoudhury et al., 2012). It is crucial that GSH a GSSG stays in balance in order to achieve the redox balance in cell environment (Das & Roychoudhury, 2014).

The α-tocopherol is a lipophilic antioxidant which efficiently scavenges ROS by incorporating them into biological membranes (Holländer-Czytko et al., 2005; Kiffin et al., 2006). It is also found only within photosynthetic organisms, and is localized in green tissues of plants. The α-tocopherol has four different isomers from which the α-isomer has the greatest antioxidant capacity. The synthesis of α-tocopherol begins at γ-tocopherol molecule which is then changed to the α-isomer using γ-tocopherol-methyl-transferase. Tocopherols generally always protect lipids and photosystem II, preserving its function and structure. After the interaction with lipid radicals are tocopherols recycled using GSH and AA (Igamberdiev et al., 2004).

Carotenoids are lipophilic antioxidants localized in plastids of all of the plants, and also in micro-organisms (Liu et al., 2020). They are a part of the antennae apparatus, which means that they are absorbing the light and transferring its energy to the chlorophyll molecule. Carotenoids have a wide variety of antioxidant abilities, they are scavenging singlet oxygen and generate heat as a by-product, they are reacting with the lipid peroxidation products so that they would end their chain reaction, they prevent the formation of singlet oxygen by reacting with excited chlorophyll molecules and lastly, they dissipate excess excitation energy by xanthophyll cycle (Das & Roychoudhury, 2014)

Flavonoids are common in the leaves and reproductive organs of the plant. They consist of four classes: flavonols, flavones, isoflavones and anthocyanins. Their main function is providing the pigmentation of the plant, fruit or the seed. Flavonoids also provide a defense system against pathogens, and their ROS scavenging ability activates after the plant experiences damage to the photosynthetic apparatus due to high excitation energy (Fini et al., 2011).

2. 3 ROS induced oxidative damage to biomolecules

ROS may be beneficial to the cell, but only up to a certain concentration. As soon as the concentration of ROS increases, complications arise that are associated with the destruction of biomacromolecules found in the cell, mainly lipids, proteins and DNA. It is the damage to these molecules that causes a number of secondary complications, which will be discussed below (Sharma et al., 2012).

2. 3. 1 Lipid peroxidation

Lipid peroxidation occurs at elevated concentrations of ROS in the cell and is particularly severe because it itself produces lipid-derived radicals that pose an additional threat to the cell. The main product of phospholipid peroxidation is malondialdehyde, which is responsible for damaging the cell membrane. Polyunsaturated fatty acids (PUFAs) are another major target of ROS . PUFAs are also part of the cell membrane and if they are peroxidized, the chains are torn and the cell membrane is damaged (Sharma et al., 2012).

2. 3. 2 Protein peroxidation

Proteins are modified by ROS either directly or indirectly. Direct modification includes nitrosylation, carbonylation, beside others. Indirect modification of proteins occurs by conjugation of proteins with degraded fatty acids (Yamauchi et al., 2008), which results in a change in their activity. Thus, the interaction of ROS with proteins is generally responsible for protein modification, fragmentation or aggregation. In the worst cases, they can even cause so much damage that proteins are subsequently tagged and undergo proteolysis (del Río et al., 2006).

2. 3. 3 DNA peroxidation

The interaction of ROS with DNA is particularly dangerous for the cell, as ROS can destroy any type of DNA in the cell, including nuclear, mitochondrial and chloroplast DNA. This damage generally causes subsequent disruption of protein function. ROS, when interacting directly with DNA, cause strand breaks, modification or removal of bases or promote conjugation of proteins to DNA (Evans et al., 2004; Sharma et al., 2012).

2. 4 UPE experiments on plants

2. 4. 1 Why UPE is tested on plants

Most plants tend to grow in habitats where they experience ideal conditions for life and reproduction and where they can reach their full potential. However, such an idyllic habitat is not always without complications. A plant has to cope with all sorts of complications in its life that can cause it to stress. Stressful stressors are brought on by various non-ideal conditions, such as too much heat, too much cold, drought, lack of light or, on the contrary, too much light. Also, salinity or some pollution of the habitat. If instead of habitat conditions we focus on different stressors for the plant, we could also name, various pests, pathogens, fungi, bacteria, viruses or, nibbling by herbivores (Atkinson & Urwin, 2012). As soon as a plant is exposed to a stress situation, it naturally produces UPE in higher intensities (Cifra & Pospíšil, 2014), which can be analyzed in various ways.

2. 4. 2 Plant organs tested by UPE

Plants go through different phases during their lifetime, with different organs at different stages of life. As small seedlings they have only a root and then a stem, during growth they then acquire leaves which gradually differentiate until the plant has different types of leaves with different functions. During maturity, some plants also acquire flowers or fruits. There are many organs on the plant body that have the potential to be measured for UPE emission, but not all possibilities have been or are being explored.

Seeds are essential for plant development, and seeds have been found to emit UPE from its shells. Where the UPE and therefore also ROS probably mediate the transducer function between the external and internal environment of the seed (Footitt et al., 2016).

Plant roots are a complex system that provides water and nutrients to the plant. Previous research on radish roots using UPE found that the roots emit spontaneous UPE. Subsequently, upon application of an oxidant, H_2O_2, induced UPE was produced. Thus, this research not only confirmed that roots also emit spontaneous UPE and induced UPE, but that it is possible and worthwhile to continue studying roots using UPE (Rastogi & Pospíšil, 2010).

Leaves are the most frequently examined part of a plant in general, measuring UPE from leaves is no exception. Leaves make up a large part of plant biomass, so they are widely available for testing, also in different sizes and ages. Also because of their handling capabilities. The leaves can be tested as long as they are intact on the plant, or also when they are separated. The leaves can be tested after the plant has been stressed by any type of stress, such as water stress (Kamal & Komatsu, 2015; Pónya & Somfalvi-Tóth, 2022).

2. 4. 3 Plant species studied by UPE

UPE was measured on different plant species and types. In terms of use, we can divide them into scientific model plants: green algae such as *Chlamydomonas reinhardtii* (Prasad & Pospisil, 2011) or higher plants as *A. thaliana* (Prasad & Pospíšil, 2013). Then biotechnologically interesting flowers as *Isatis indigotica fort* (Chen et al., 2005). And finally, on agricultural plants and similar such as radishes, *Raphanus sativus* (Rastogi & Pospíšil, 2010), soy, *Glycine max* (Kamal & Komatsu, 2015), kidney beans, *Phaseolus vulgaris* (Kawabata et al., 2004), red beans, *Phaseolus angularis* (Kai et al., 1995), potatoes, *Solanum tuberosum* (Floryszak-Wieczorek et al., 2011), and sunflowers, *Helianthus annuus* (Pónya & Somfalvi-Tóth, 2022).

2. 4. 4 Effect of different stress factors measured by UPE

The plants were subjected to a wide range of experiments that tested different types of stressors, whether biotic or abiotic, and as a result, the intensity of UPE was measured as a response to stress.

Isatis indigotica fort was tested for exposure to microwaves and He-Ne laser, and it was found that the adult plant subsequently produced UPE at significantly higher intensities than plants grown from control seeds (Chen et al., 2005).

Isolated cells of radish, *Raphanus sativus* roots were tested for the addition of H_2O_2, and it was found that after the addition of H_2O_2, ROS is formed, which correlated with an increase in UPE (Rastogi & Pospíšil, 2010).

On soybean plants, *Glycine max* the impact of flooding was measured, with mitochondrial proteins isolated from the root cells, on which the intensity of UPE was subsequently tested, and it was found that flooding had no significant impact on the intensity of UPE (Kamal & Komatsu, 2015).

For bean seeds, *Phaseolus vulgaris* it was tested whether changing environments, i.e., changing temperature and humidity, have an impact on when the seed starts to sprout, specifically the seed envelope and its role in this process were then tested. In the experiment, the UPE of seed packages was measured under changing humidity, temperature, and it was found that with increasing temperature and decreasing humidity, the UPE grew (Footitt et al., 2016).

In potato leaves, *Solanum tuberosum* that were inoculated with *Phytophthora infestans* in this experiment, it was found that UPE also increased (Floryszak-Wieczorek et al., 2011).

In bean leaves, *Phaseolus vulgaris* that were tested for Kanzawa spider mites', *Tetranychus urticae* infestation, a strong increase in UPE was found in the area of veins that were heavily infested with mites, at the same time, an overall increase in UPE was noted due to the systematic reaction of the plant to the attack (Kawabata et al., 2004).

2. 5 Mechanical injury and UPE

Mechanical injury to plants is irretrievably related to damage to the plant tissue and to the penetration of oxygen into the plant in a way that is not inherent in the plant. Although the plant is equipped with a wide range of mechanisms that can prevent the spread of oxygen-induced damage, such as oxidative burst, which has the task of strengthening the cell wall, or the formation of phenols. These measures are mediated mainly by means of jasmonic acid, or salicylic, or abscisic acid, but these mechanisms have only limited functionality (Prasad et al., 2020; Sharma et al., 2012).

When oxygen penetrates into the plant tissue, several reactions occur at once. First, there is a massive formation of ROS, which subsequently react with biomolecules of plant cells. The reactions of ROS and biomolecules that take place first include the peroxidation of lipids and proteins, since they are exposed to ROS immediately (Prasad et al., 2020).

When measuring UPE on plants *A. thaliana* it was found that UPE has increased in the area of damage on the leaf of the plant compared to undamaged leaves. The increase in UPE was due to the excessive formation of ROS and the subsequent reaction between ROS and plant biomolecules, resulting in the formation of excited molecules and thus an increase in UPE (Prasad et al., 2020).

Another examples of a mechanical injury tests on plants were experiments on potato (Floryszak-Wieczorek et al., 2011) and bean leaves (Kawabata et al., 2004). It should be noted, however, that herbivore damage to a plant falls within the characteristics of mechanical damage, but herbivore also adds a chemical factor to the mechanical damage itself, such as various enzymes that it can excrete through the oral organs, which can influence the impact of the mechanical damage itself (Bricchi et al., 2010). It was found that even in the case of herbivore damage, there is a massive increase in UPE at the sites of damage (Floryszak-Wieczorek et al., 2011), and even a systemic reaction of plants, which resulted in an increase in the total UPE of the plant (Kawabata et al., 2004).

3 Aim of work

The aim of this work is to focus on UPE originating from three different plant species, namely *Arabidopsis thaliana*, *Nicotiana rustica* and *Hordeum vulgare. N. rustica* and *H. vulgare* were chosen because the relationship between mechanical injury and UPE has not yet been studied on them. The second aim of this work is to determine whether it is appropriate to study these species using UPE. The third aim of this work is to transfer the method for UPE detection from plants established on older CCD camera to new CCD camera including all the optimization steps.

4 Material and methods

4. 1 Material

4. 1. 1 *Arabidopsis thaliana* wt.

The variation of *A. thaliana* wild-type was used in the experiments, it is a variation of the organism that is not modified or mutated in any way.

Before sowing the plants, the substrate was sterilized and dried, for this purpose a Memmert UF 110 (Memmert GmbH + Co. KG, Schwabach, Germany) dryer was used in which the planting plates with the substrate spent 40 minutes at 70 °C. This step was carried out both for sterilization and to speed up the subsequent hydration of the substrate with normal water.

A. thaliana seeds were hydrated in normal water for at least 24 hours before sowing. Subsequently, one seed at a time was transferred to a single plating plate chamber on the substrate using a pipette (Fig. 1).

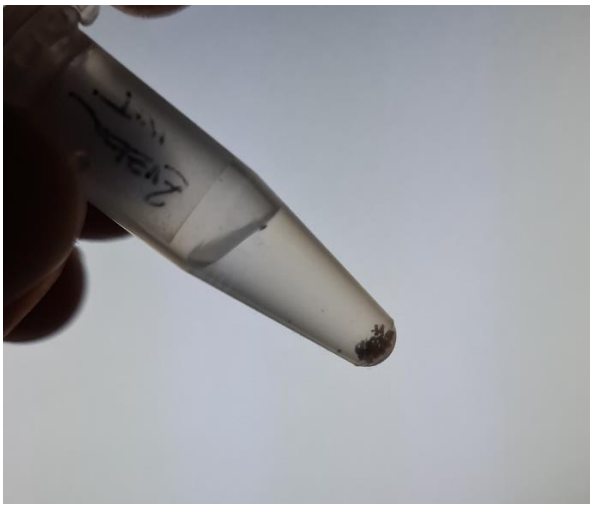

Figure 1. *A. thaliana* seeds rehydrated in normal water in a small test tube

Planting plates with seeds were then transferred to the PhytoScope phytotron where they were allowed to grow to an adequate age (Fig. 2). The conditions in the phytotron were as follows, 21 °C, 8 hours of light and 16 hours of darkness. The light intensity was 100 μmol/m^2/s with a relative humidity of 60 %. The small plants were originally planted in small planting plates, but they spent only two to three weeks in them. Subsequently, the seedlings had to be transplanted into larger planting plates.

Figure 2. The cultivation process of *A. thaliana*, visible plants at various ages, young seedlings are also covered with plastic wrap to prevent initial drought.

4. 1. 2 Repotting *A. thaliana*

Before transplanting, it was necessary to fill the larger planting plates with substrate. Like the smaller plates, these were also left to dry and sterilize in Memmert UF 110 dryer in which the planting plates with the substrate spent 45 minutes at 70 °C.

Subsequently, the soil had to be rehydrated with normal water and left to cool again, as it retained more heat due to the larger volume of the chambers. When the plates reached laboratory temperature, an adequate hole the size of the small chamber on the previous planting plate was made in each chamber using a planting pin. Then, using a spatula, the entire small seedling with soil was removed from the previous plate and inserted into the hole in the chamber on the new plate.

4. 1. 3 *Nicotiana rustica*

Before sowing the plants, the substrate was sterilized and dried, for this purpose a Memmert UF 110 dryer was used in which the bigger planting plates with the substrate spent 40 minutes at 70 °C. This step was carried out both for sterilization and to speed up the subsequent hydration of the substrate with normal water. Subsequently, tobacco seeds were sown in the prepared soil. They were then transferred to the phytocompatment, where they were left to grow in the same conditions as *A. thaliana* for three months.

4. 1. 4 *Hordeum vulgare*

Before planting, planting pots were prepared and filled with perlite (Fig. 3). Subsequently, the perlite was watered with Knop's solution to make it very moist. Next, the *H. vulgare* seeds were placed on the perlite and then covered with perlite so that they were under about a centimeter thick layer of perlite. Finally, the filled and seeded pot was re-watered with Knop's solution and placed in the phytocompartment, where they were left to grow in the same conditions as *A. thaliana* for a week.

Figure 3. Preparing seedling pots for sowing *H. vulgare*, the pots were filled with dry perlite to 3/4 of their volume. Seeds were freely distributed on this perlite, which was then overlaid with a subsequent centimeter layer of perlite.

4. 2 Methods

4. 2. 1 CCD camera

Two CCD cameras were used in our experiments. First, the older camera VersArray 1300B (Princeton Instruments, Trenton, NJ, USA) on which measurements of biological aspects of plants in relation to UPE were made. Subsequently, a new CCD camera iKon-XL (Oxford Instruments plc, Tubney Woods, Abingdon, United Kingdom) (Fig. 4) was used on which the optimization for possible future measurements was performed.

Figure 4. The new CCD camera, with visible water-cooling pipes and attached objective and a stand construction.

The cameras always needed to be cooled down before the actual measurement in order to reduce the dark current and thus improve the signal/noise ratio. The VersArray 1300B camera was cooled down with liquid nitrogen, which was manually added to the camera using a polystyrene can and funnel. The cooling of the camera had to be started at least 2 hours before the measurement to reach the required temperature below - 100°C. The iKon-XL camera was also cooled down to the same temperature, but the difference was the cooling technique. The chip was cooled down to -100°C by Peltier cells which transferred heat from the chip to the water circuit cooled down to 13 °C by an additional thermostat Cole-Parmer RHC-800 Digital Plus Refrigerated Circulator, 15 L, -40 to 200°C; 240 VAC (Cole-Parmer Instrument Co. Europe, UK)

4. 2. 2 Preparation of the plant

Before starting the actual measurement, it was necessary to leave the plant in a dark room to avoid delayed luminescence. The plant needed to be adapted to dark for at least an hour.

4. 2. 3 Focusing

The focusing was carried out as soon as possible after placing the plant in the dark room. The focusing was carried out in very low light, which was achieved by slightly opening the door to the dark room. The adjacent room, where the data processing on the PC took place, had to be illuminated by green LED light only. The green light is used here because very low light intensities are needed for relatively sufficient illumination and, the plants absorb the green light least from whole visible spectrum, so there is very low light retention in the plants and thus minimal delayed luminescence.

4. 2. 4 Measuring

The measurements on the VersArray 1300B camera were carried out using WinView/32 (Princeton Instruments, a division of Roper Scientific, Inc., Trenton, NJ, USA), in which all the settings of the measurement parameters were made. The exposure time, which was 20 minutes per frame for all experiments. Furthermore, the number of frames, which was nine - two frames were reserved for each damaged leaf, so with four damaged leaves this is eight frames plus one frame illustrating the spontaneous emission of the plant. Next, the binning, which was chosen to be 2x2 on this camera, and the readout rate, which here was 100 kHz at Gain 2 at High sensitivity setting. Before starting the second image, the leaf of the plant was mechanically damaged, it was cut using scissors so that about a third of the leaf was removed and so that the wound hit the midvein. The signal was recorded on the next and subsequent images. All four leaves were damaged in the same way.

The data from iKon-XL camera were recorded using AndorSolis 64-bit (Andor Technology Ltd, Oxford Instruments plc, UK) application with almost analogous settings, exposure times, binning. The exact procedure for these measurements was not given. The measurements were carried out experimentally, testing the optimization of different combinations of settings.

4. 2. 5 Data analysis

The data were analyzed using the Andor Solis application, from where the individual series of images formatted .SPE were converted to format .TIF. Subsequently, the images in .TIF format were opened in the ImageJ program, where the area around the injury site was created using the freehand setting. In addition, the "measure" function was used to measure various parameters in the marked area, but means were used for further analyses. This measurement was made on the whole series of images. The individual measured values were transferred to MS Excel, where their subsequent analysis took place.

5 Results

5. 1 *Arabidopsis thaliana* wt.

5. 1. 1 Comparison of spontaneous UPE in experiments

The subject of this experiment was to compare how spontaneous UPE occurs in plants in different experiments. It can be seen from experiments that the spontaneous emission oscillates differently both in time for individual samples and between individual samples (Fig. 5).

It was found that the number one control signal continued to increase until 60 minutes, with a slight decrease in UPE between 60 and 100 minutes. Further, between 100 and 120 minutes, there was an increase in UPE and a decrease in it again, which was measured at a time of 140 minutes. Finally, the UPE continued to increase until the end of the measurement. While the overall trend of the signal of the control number one was an overall slight increase.

It was found that the progress of the spontaneous UPE of experiment number two was first increasing by 40 minutes, then decreasing by 80 minutes. Between 80 minutes and 100 minutes there was a small increase in the UPE. Subsequently, from 100 minutes to 180 minutes, there was a periodic increase and decrease in the intensity of the spontaneous UPE. While the overall trend of the signal of the control number two was an overall slight increase.

It was found that the spontaneous UPE in experiment three increased during the first forty minutes and then decreased until the time of 120 minutes, when there was a re-increase in the next twenty minutes, measured in 140 minutes. Then the UPE went down again. While the overall trend of the signal of the control number three was an overall sharp decline.

Spontaneous UPE was taken as a control in our experiments and, knowing that it oscillated in this way, it was subsequently subtracted from the injury data.

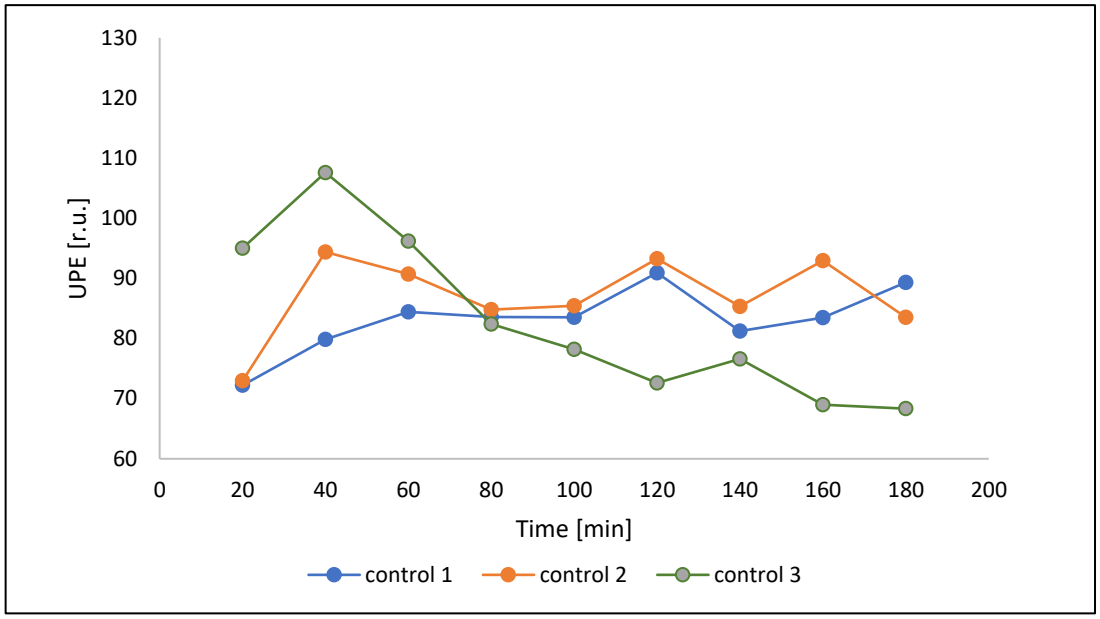

Figure 5. Courses of spontaneous UPE intensities in *A. thaliana* for experiments one (blue), experiment two (orange), and experiment three (Green), showing individual increases or decreases of spontaneous UPE intensities for single experiments. The number one control showed a gradual increase, the number two control showed a somewhat higher increase, and the number three control showed a sharp decrease.

5. 1. 2 Comparison of spontaneous and induced UPE

The subject of this experiment was to determine the average values of the induced UPE from all injured leaves and from all controls to establish the relationship between control and injury and at the same time to prove that after a mechanical injury of the leaf there is an increase in the UPE.

It was found that after mechanical injury occurs in *A. thaliana* an increase in the UPE by approximately twice. In addition, it was found that the course of the averaged graph corresponds to the course of the injury signal in individual experiments, when there was an increase in the UPE after damage and subsequently its decrease over time to a certain level of intensities, which were higher than the control (see Fig. 6).

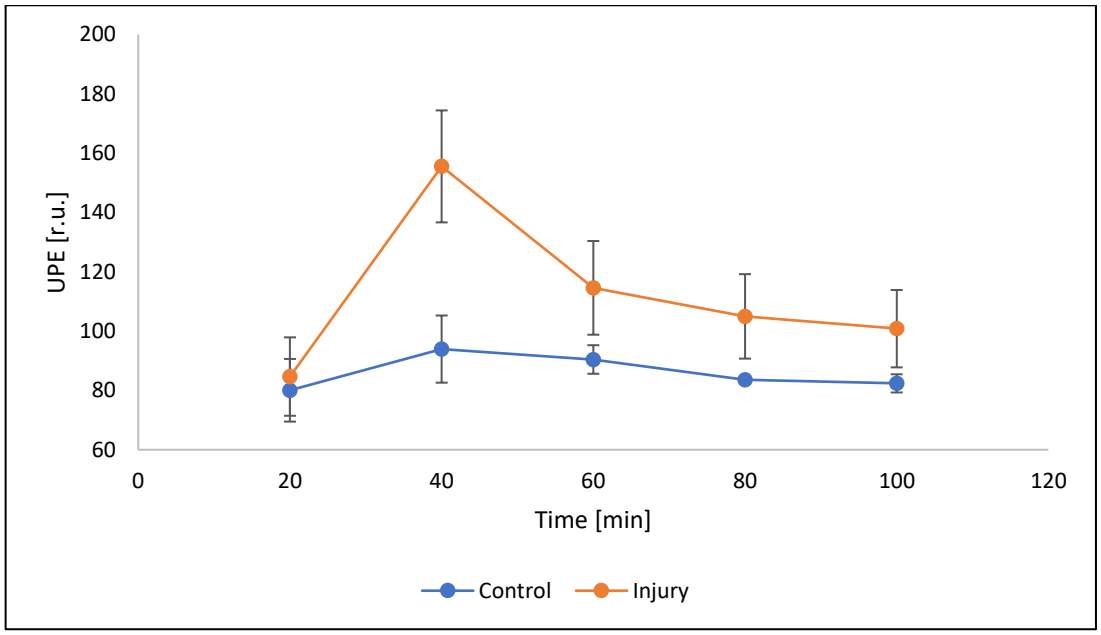

Figure 6. Course of the average signal intensity in *A. thaliana* for injuries (orange) of all leaves with error sections in the form of a standard deviation. Next, the graph shows the progress of the average intensity of the control UPE (blue) again with error segments in the form of standard deviations. The average intensity of the injury UPE is always higher than the average intensity of the control UPE.

5. 1. 3 First, second and third leaf UPE comparison

In this experiment, the increase in the UPE between the first, second and third injured leaves on one *A. thaliana* plant was compared (Fig. 7). The experiment took place over five frames of 20 min, with the first frame recording the leaf in its uninjured state, after capturing the first frame, the leaf was mechanically injured, and the signal of the site of this injury was captured in the next frame, that is, at 40 min. Further, the signal progress of the injured leaf was monitored on 3 additional images, that is, at a time of 60, 80 and 100 min.

It was found that the differences in the intensity of the induced UPE are very small, nevertheless, the highest increase in the intensity of induced UPE occurred with the first injured leaf, the second highest increase in induced UPE occurred with the third injured leaf, and the lowest increase in UPE occurred after the injury of the second leaf (Fig. 7).

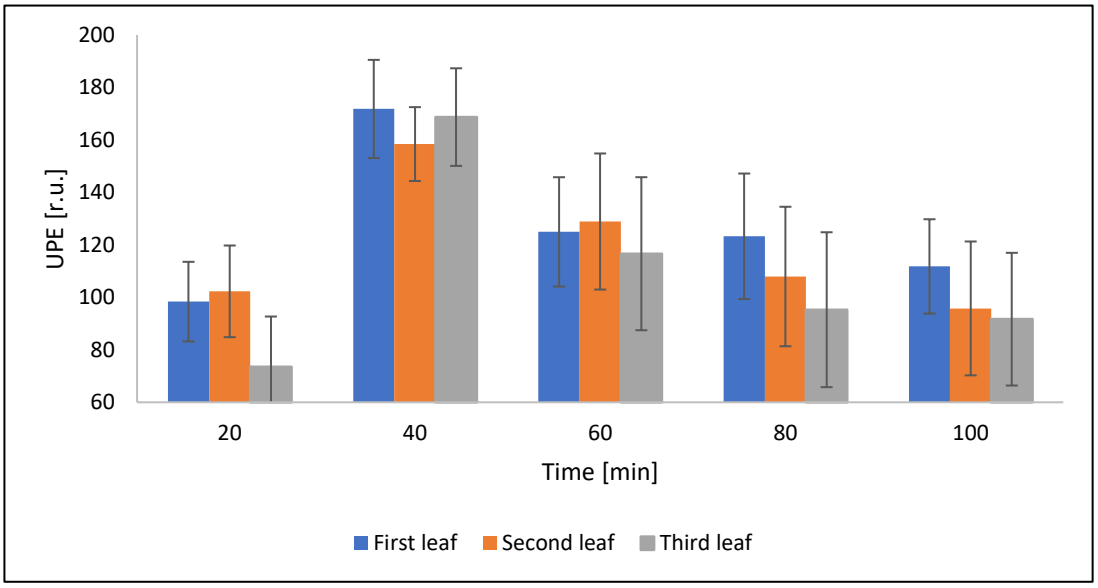

Figure 7. Changes in UPE in injured leaves one, two and three from *A. thaliana*. With leaf number one (blue) providing the highest intensity, leaf number two (orange) providing the lower signal and leaf number three (gay) providing the higher than leaf number two and lower than leaf number one intensity. The subsequent course of the UPE for leaf number one involves a rapid decline first, followed by a gradual decrease at the highest level of the three injured leaves. The subsequent signal progression for leaf number two involves a less radical decrease and a subsequent gradual decrease at levels lower than for leaf one. The subsequent course of the UPE for leaf number three also includes, as with leaf one, a radical decrease, but in this case to the levels of the lowest of all three leaves and a subsequent gradual decrease at this lowest level.

5. 1. 4 Kinetics of UPE from injured leaf

Imaging of the UPE from plant *A. thaliana* was performed over the time period of 180 min, while accumulation time of each frame was 20 min (Fig. 9). In image one, which was captured at a time of 20 min, only spontaneous UPE was observed and white pixels, caused by cosmic radiation, or bad pixel readout from the chip, these white pixels appear in every image. After capturing image one, a mechanical injury to the leaf was performed, resulting in the increase in UPE (Fig. 9, 40 min), followed by slow decrease over the next 60 min (Fig. 9, 60 – 100 min). At 120 min reveals small increase in the UPE from the injured leaf, possibly a result of systemic reaction to the plant injury. Further images (Fig. 9, 140 – 180 min) show a slow decrease in the UPE from injured leaf, while part of the place of injury is still visible even 180 min after the cut. The average intensity of UPE at the place of injury is plotted in graph (Fig. 8).

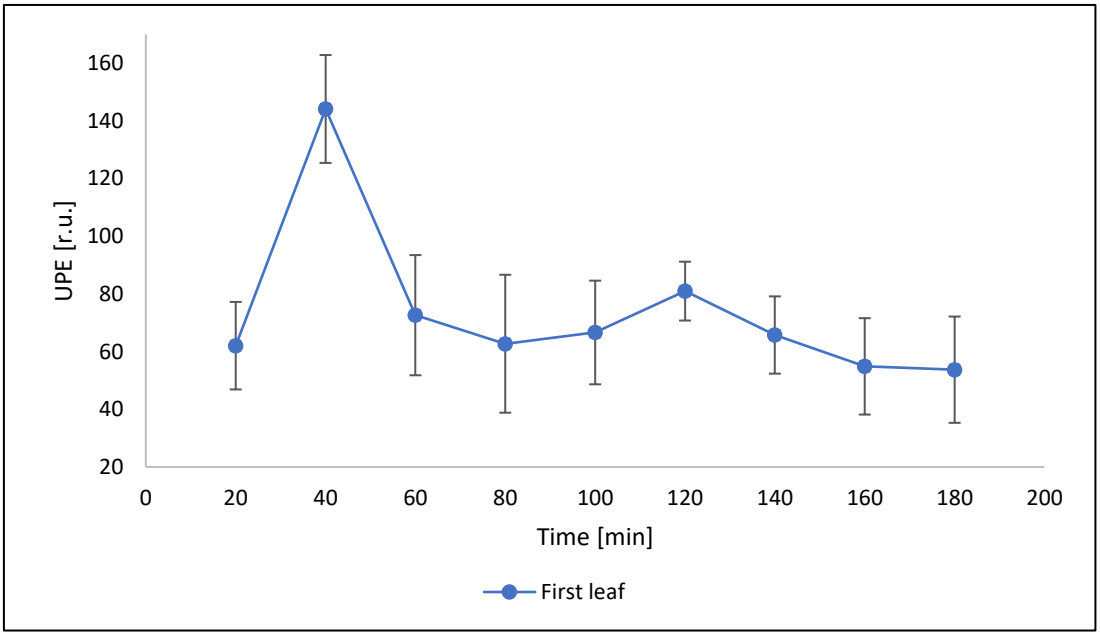

Figure 8. Course of UPE from the injured leaf of *A. thaliana* with reaching a maximum in 40 minutes, that is, after the injury and with a subsequent decrease until 80 min. Subsequently, with a small increase from 100 minutes to 120 minutes due to a probably systematic reaction. Further, a gradual decrease in UPE can be observed.

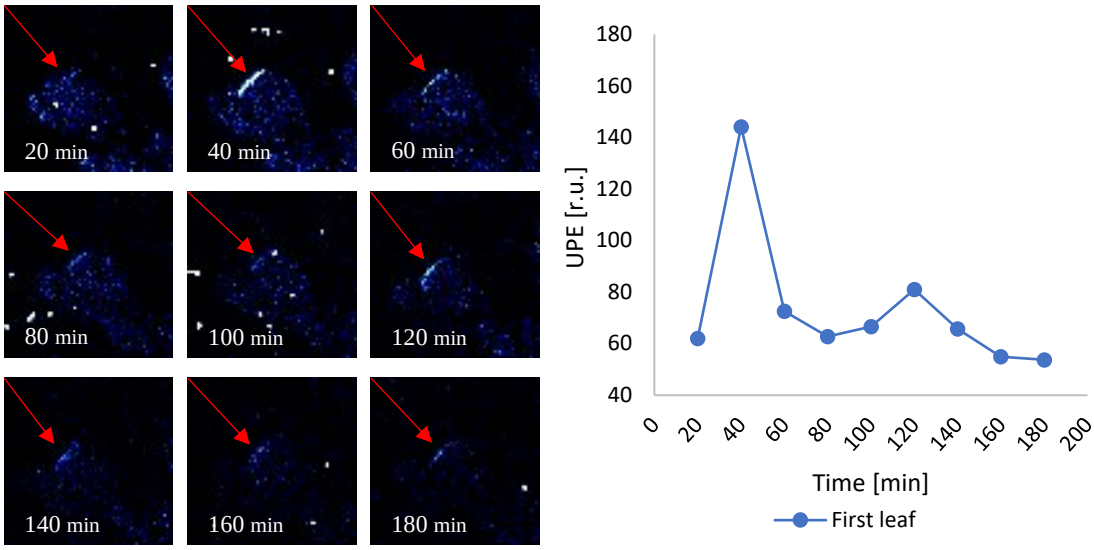

Figure 9. UPE from *A. thaliana* leaf, that was mechanically injured (The site of injury marked by red arrow). Only spontaneous UPE is visible at 20 min and white pixels caused by cosmic radiation, or bad pixel readout. At 40 min a massive increase in UPE is visible, induced by mechanical injury. Followed by rapid decay at 60 min. At 80 min shows a drop in UPE followed by minor increase through 100 and 120 min At 140 – 180 min shows a gradual decline in UPE.

5. 2 *Nicotiana rustica*

5. 2. 1 Comparison of spontaneous UPE in experiments

The subject of this experiment was to compare how spontaneous UPE occurs in plants in different experiments. According to the theory, spontaneous emission should be constant, eventually decreasing slowly with decreasing metabolic activity caused by the movement of the plant in the dark. However, despite this assumption, it can be seen from experiments that the spontaneous emission oscillates differently both in time for individual samples and between individual samples (Fig. 10).

It was found that the number one control spontaneous UPE increased between 20 and 40 minutes, remained at approximately the same level between 40 and 60 minutes, slightly decreased between 60 and 80 minutes, and remained at the same level until 140 minutes. Subsequently, between 140 and 160 minutes, it decreased again, and finally between 160 and 180 minutes, it increased slightly. While the overall trend of the UPE intensity of the control number one was an overall very slight decrease.

It was found that control number two spontaneous UPE decreased almost linearly between the times of 20 minutes to 80 minutes, then between the times of 80 and 100 minutes it increased. Between the times of 100 minutes and 160 minutes, it continued to decrease until finally, between the times of 160 minutes and 180 minutes, it increased again. While the overall trend of the UPE intensity of the control number one was an overall decrease.

It was found that control number three spontaneous UPE increased sharply between times of 20 minutes and 40 minutes, and then gradually decreased between times of 40 minutes and 180 minutes. While the overall trend of the UPE intensity of the control number three was an overall very slight decline.

It was also found that control number one varies in different intensities of the UPE signal compared to controls number two and number three, with these intensities being much lower.

Spontaneous UPE was taken as a control in our experiments and, knowing that it oscillated in this way, it was subsequently subtracted from the injury data.

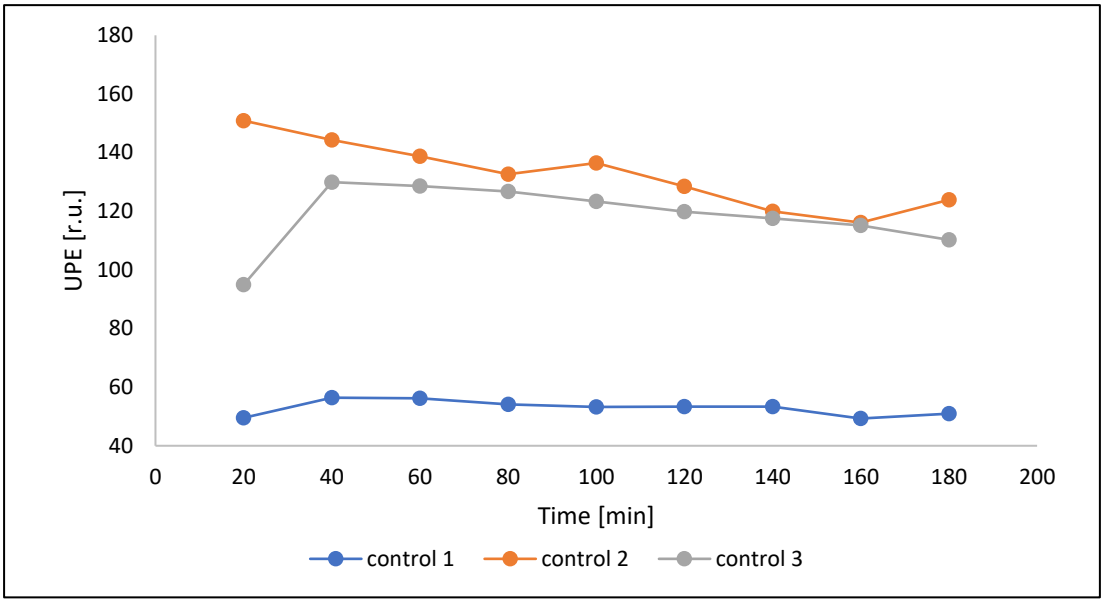

Figure 10 displays a graph of the courses of control spontaneous UPE intensities in *A. thaliana* for experiments one (blue), experiment two (orange), and experiment three (Gray), showing individual increases or decreases of UPE intensities for single experiments. The number one control showed a gradual slight decrease, the number two control showed a steep decrease, and the number three control showed a slight decrease. Control number one varies in different intensities of the UPE compared to controls number two and number three, with these intensities being much lower.

5. 2. 2 Comparison of spontaneous and induced UPE

The subject of this experiment was to determine the average values of the UPE from all injured leaves and from all controls to establish the relationship between control and injury and at the same time to prove that after a mechanical injury of the leaf there is an increase in the UPE.

It was found that after a mechanical injury in *N. rustica* there is a weak increase in the UPE (Fig. 11). Subsequently, its gradual decrease occurs. It was also found that the signal of spontaneous emission, i.e., control, is more intense at many measurement points than the signal of injury.

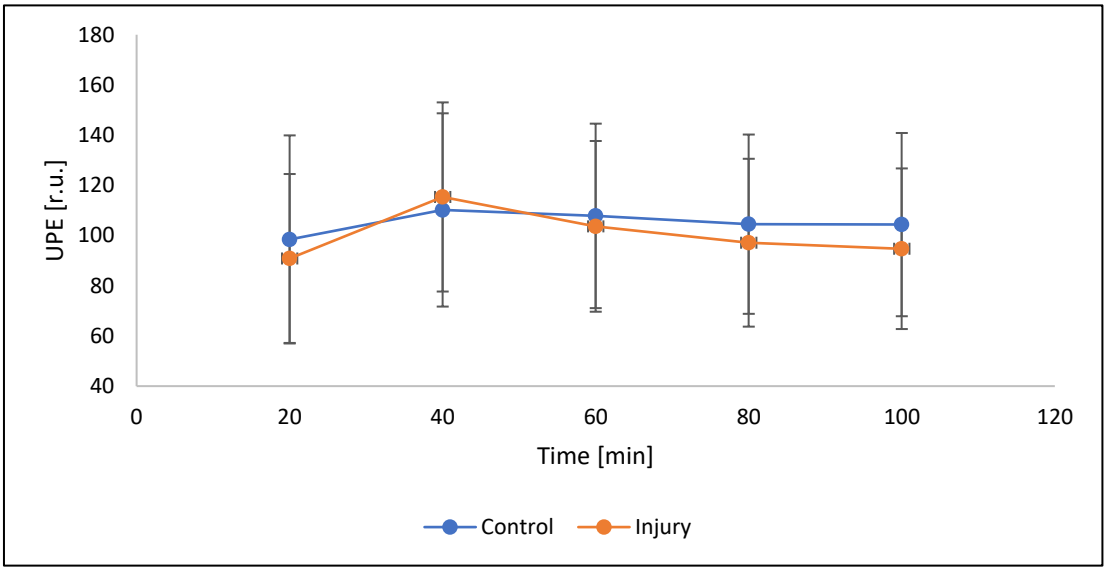

Figure 11 shows the course of the average UPE intensity for injuries (orange) of all *N. rustica* leaves with error sections in the form of a standard deviation. Next, the graph shows the progress of the average intensity of the control spontaneous UPE (blue) again with error segments in the form of standard deviations. The average intensity of the injury UPE is always higher than the average intensity of the control UPE. The average intensity of the injury UPE increases slightly, but subsequently decrease below the values of the control UPE.

5. 2. 3 First, second and third leaf UPE comparison

In this experiment, the increase in the UPE between the first, second and third injured leaves on one *N. rustica* plant was compared in one experiment over five frames of 20 minutes, with the first frame recording the leaf in its uninjured state, after capturing the first frame, the leaf was mechanically injured, and the UPE of the site of this injury was captured in the next frame, that is, at 40 minutes. Further, the UPE progress of the injured leaf was monitored on 3 additional images, that is, at a time of 60, 80 and 100 minutes.

It was found that the differences in the intensity of induced UPE are very small, nevertheless, the highest increase in the intensity of induced UPE occurred with the first injured leaf, the second highest increase in induced UPE occurred with the third injured leaf, and the lowest increase in UPE occurred after the injury of the second leaf (Fig. 12).

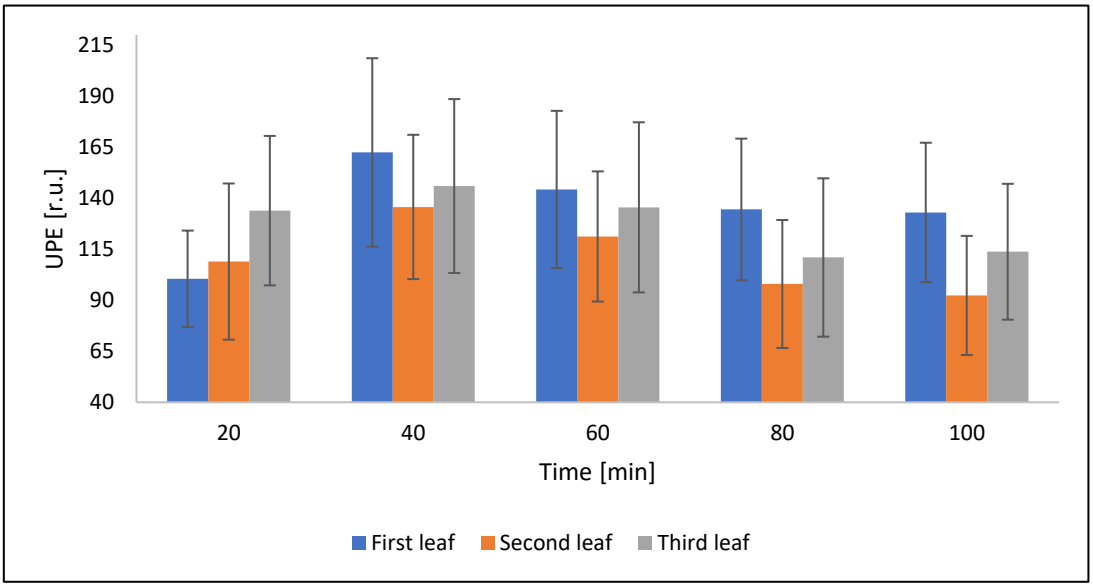

Figure 12. Changes in UPE in injured *N rustica* leaves number one (blue), two (orange) and three (gray). With leaf number one providing the highest intensity, leaf number two providing the lower intensity and leaf number three providing the higher than leaf number two and lower than leaf number one UPE intensity. The subsequent course of the UPE for leaf number one involves a gradual decrease at the highest level of intensities out of the three injured leaves. The subsequent UPE progression for leaf number two involves also a decrease to the levels of the lowest intensities out of all three leaves and a subsequent gradual decrease at this lowest level of intensity. The course of the UPE for leaf number three includes a decrease, but in this case in the level of intensities between the first and the second injured leaf.

5. 2. 4 Kinetics of UPE from injured leaf

Imaging of the UPE from plant *N. rustica* was performed over the time period of 180 min., while accumulation time of each frame was 20 min (Fig. 14). In image one, which was captured at a time of 20 min, only spontaneous leaf emission was observed and white pixels, caused by cosmic radiation, or bad pixel readout from the chip, these white pixels appear in every image. After capturing image one, a mechanical injury to the leaf one was performed, resulting in the increase in UPE on image Fig. 14, 40 min. followed by slow decrease over the next 60 min (Fig. 14, 60 – 100 min.). Figure 13, 120 min. reveals small increase in the UPE from the injured leaf, possibly a result of systemic reaction to the plant injury. Further images (Fig. 14, 140 – 180 min.) show a slow decrease in the UPE from injured leaf, while part of the place of injury is still visible even 180 min. after the cut. The average intensity of UPE at the place of injury is plotted in graph (Fig. 13).

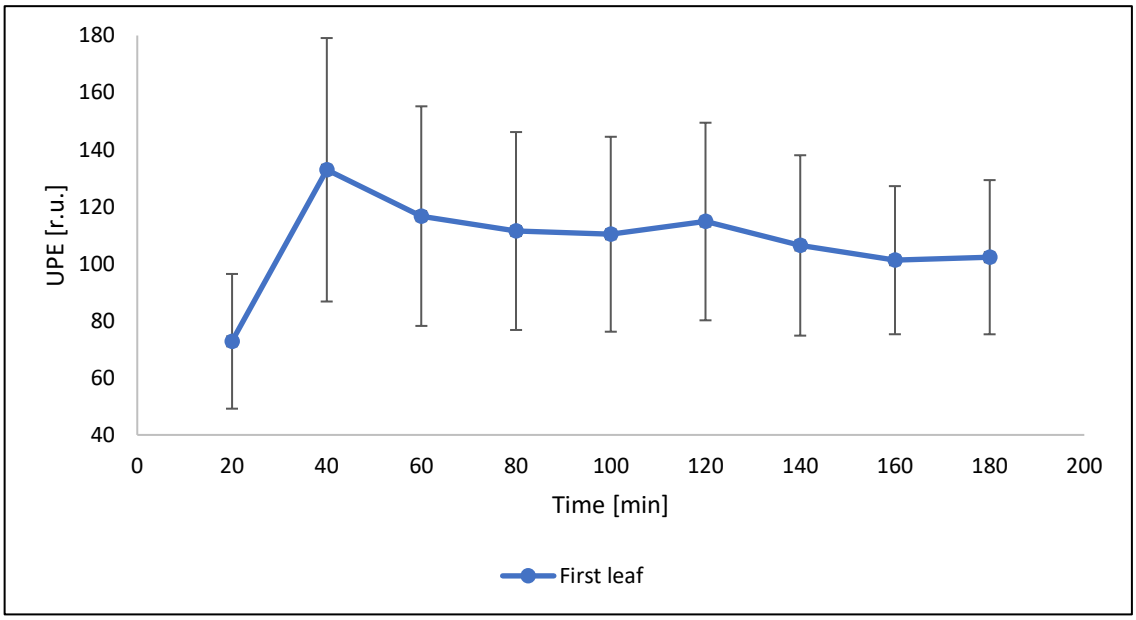

Figure 13. The course of UPE from the injured *N. rustica* leaf with reaching a maximum in 40 minutes, that is, after the injury and with a subsequent decrease until 80 min. Subsequently, with a small increase from 100 minutes to 120 minutes due to a probably systematic reaction. Further, a gradual decrease in UPE can be observed.

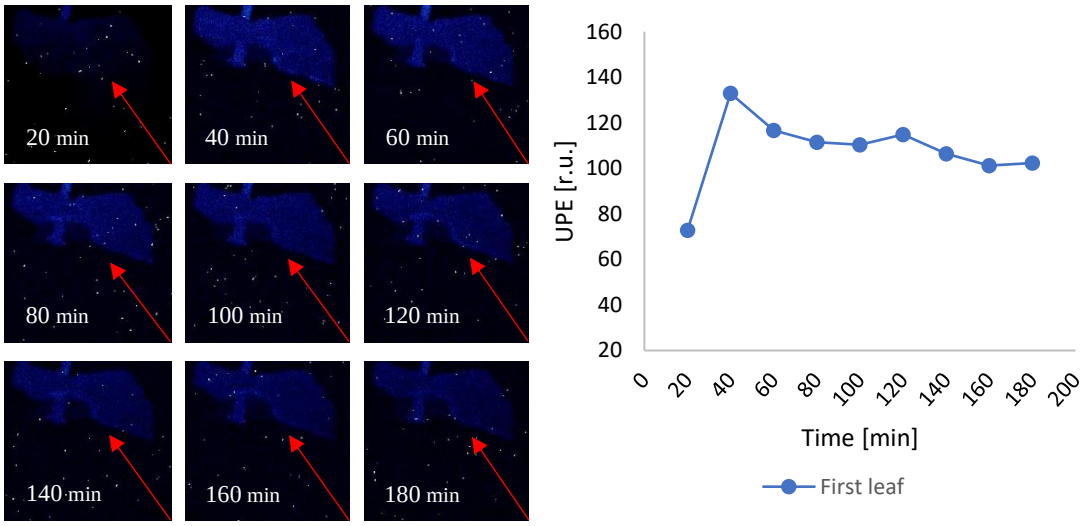

Figure 14. UPE from *N. rustica* leaf, that was mechanically injured (The site of injury marked by red arrow). Only spontaneous UPE is visible at 20 min and white pixels caused by cosmic radiation, or bad pixel readout. At 40 min an increase in UPE is visible, induced by mechanical injury. Followed by decay at 60 – 100 min, followed by minor increase at 120 min, at 140 – 180 min shows a gradual decline in UPE.

5. 3 *Hordeum vulgare*

5. 3. 1 Comparison of spontaneous UPE in experiments

The subject of this experiment was to compare how spontaneous UPE occurs in plants in different experiments. According to the theory, spontaneous emission should be constant, eventually decreasing slowly with decreasing metabolic activity caused by the movement of the plant in the dark. However, despite this assumption, it can be seen from experiments that the spontaneous emission oscillates differently both in time for individual samples and between individual samples (Fig. 15).

It was found that the intensity of the number one control spontaneous UPE increased between the times of 20 minutes and 100 minutes. Subsequently, between the times of 100 minutes and 140 minutes, it decreased. Between 140 minutes and 160 minutes it increased slightly and then between 160 minutes and 180 minutes it decreased again. While the overall trend of control number one UPE was very slight decrease.

It was found that the intensity of control number two spontaneous UPE increased between times of 20 minutes and 40 minutes. Subsequently, between the times of 40 minutes and 160 minutes, it gradually decreased and, in the end, between the times of 160 minutes and 180 minutes, she remained at almost the same level of intensity. The overall trend in intensity for control number two UPE was a significant decrease.

It was found that the intensity of control number three spontaneous UPE periodically decreased and increased throughout the measurement period, starting with a decrease between times of 20 minutes and 40 minutes. While the overall trend in the intensity of control number three UPE was a very small decrease.

It was found that control number one and two started at similar UPE intensities, but during the experiments control one stayed at similar intensity levels, while the intensity of control two began to decrease significantly. Control number three started at a slightly lower intensity level, but during the experiment it stayed at this level approximately.

Spontaneous UPE was taken as a control in our experiments and, knowing that it oscillated in this way, it was subsequently subtracted from the injury data.

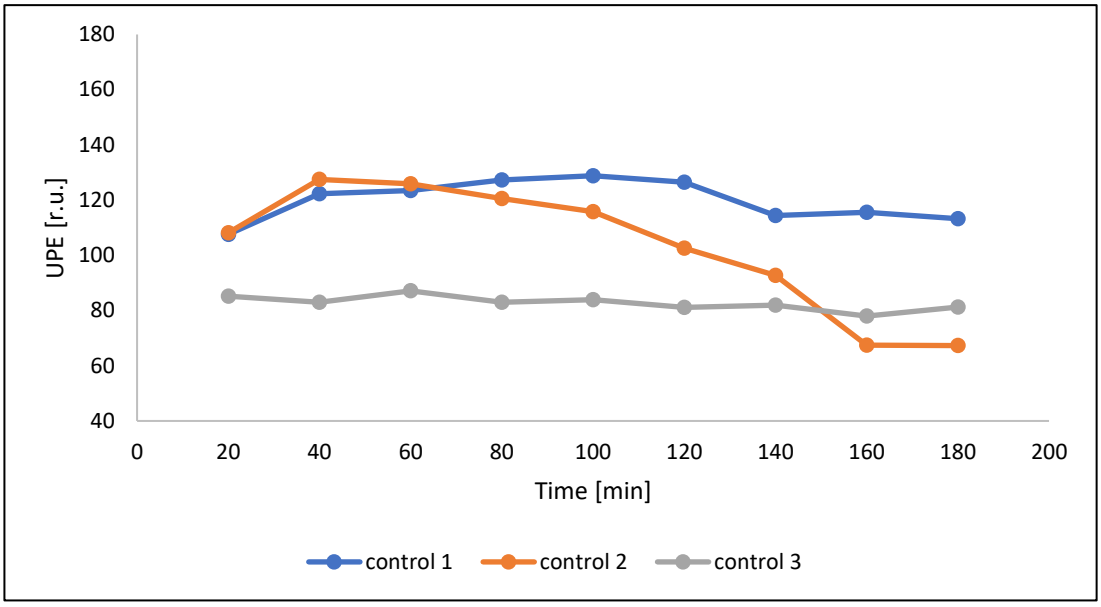

Figure 15. Courses of control UPE intensities from *H. vulgare* for experiments one (blue), experiment two (orange), and experiment three (Gray), showing individual increases or decreases of spontaneous UPE intensities for single experiments. The number one control showed a gradual slight decrease, the number two control showed a steep decrease, and the number three control showed a slight decrease. Control number three varies in different intensities of the UPE compared to controls number two and number three, with these intensities being a bit higher.

5. 3. 2 Comparison of spontaneous and induced UPE

The subject of this experiment was to determine the average values of the UPE from all injured leaves and from all controls to establish the relationship between control and injury and at the same time to prove that after a mechanical injury of the leaf there is an increase in the UPE.

It was found that after mechanical injury occurs in *H. vulgare* an increase in the UPE (Fig. 16). In addition, it was found that the course of the averaged graph corresponds to the course of the injury signal in individual experiments, when there was an increase in the UPE after damage and subsequently its decrease over time to a certain level of intensities. Over time, the intensity of UPE after injury decreases to below the intensity of control spontaneous UPE.

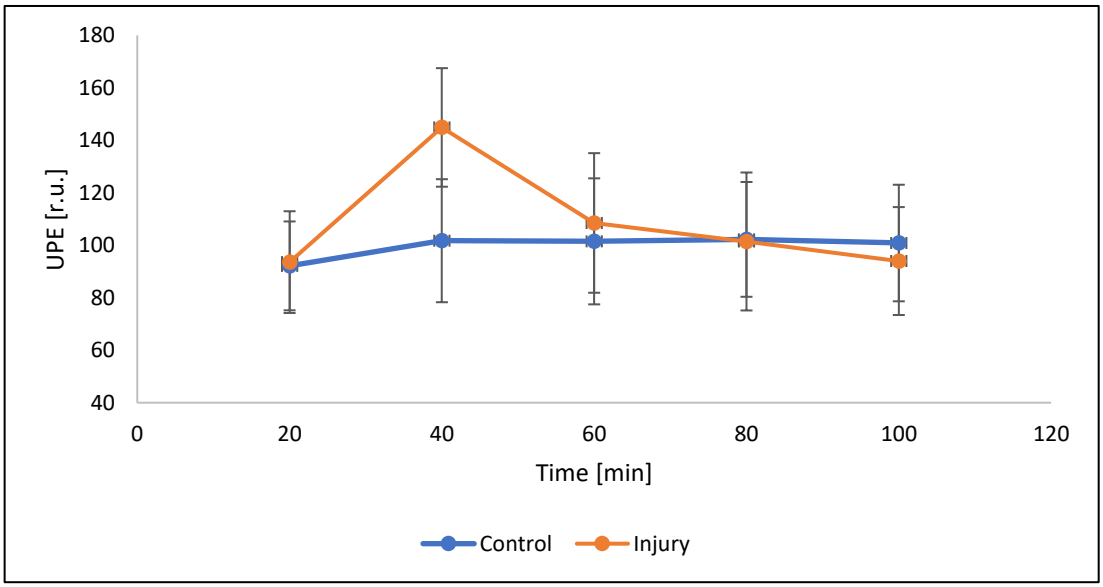

Figure 16. Course of the average UPE intensity for injuries (orange) of all *H. vulgare* leaves with error sections in the form of a standard deviation. Next, the graph shows the progress of the average intensity of the control UPE (blue) again with error segments in the form of standard deviations. The average intensity of the UPE after the injury increased sharply, subsequently also decreased, and over time reaches intensities comparable to control. Subsequently, it reaches intensities lower than those of control.

5. 3. 3 First, second and third leaf UPE comparison

In this experiment, the increase in the UPE between the first, second and third injured leaves on one *H. vulgare* plant was compared in one experiment over five frames of 20 minutes, with the first frame recording the leaf in its uninjured state, after capturing the first frame, the leaf was mechanically injured, and the UPE of the site of this injury was captured in the next frame, that is, at 40 minutes. Further, the progress of the injured leaf UPE was monitored on 3 additional images, that is, at a time of 60, 80 and 100 minutes.

It was found that the differences in the intensity of induced UPE are very small, nevertheless, the highest increase in the intensity of induced UPE occurred with the first injured leaf, the second highest increase in induced UPE occurred with the third injured leaf, and the lowest increase in UPE occurred after the injury of the second leaf (Fig. 17).

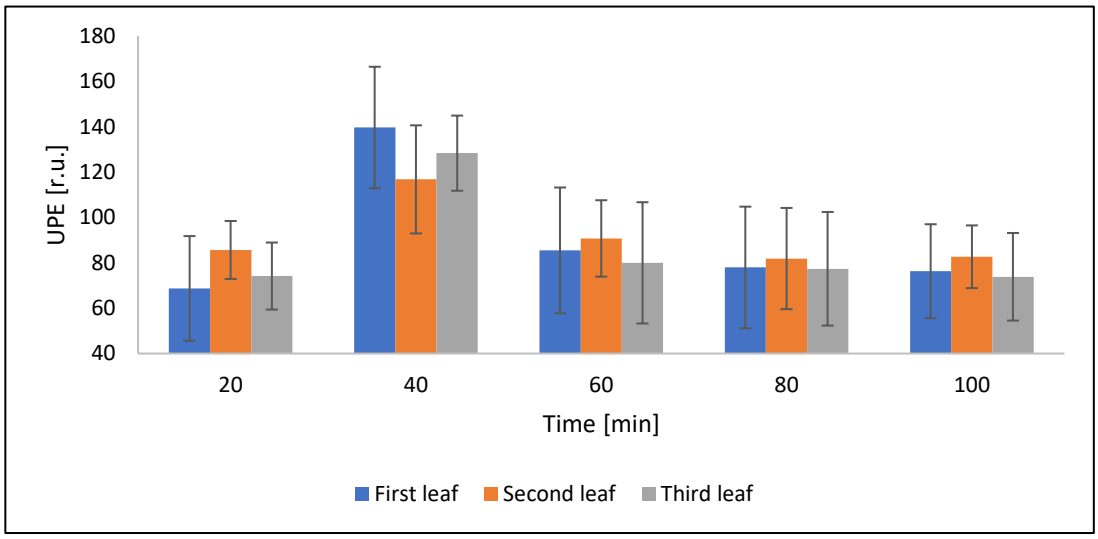

Figure 17., Changes in UPE in injured *H. vulgare* leaves number one (blue), two (orange) and three (gray). With leaf number one providing the highest intensity of UPE, leaf number two providing the lowest UPE intensity and leaf number three providing the higher than leaf number two and lower than leaf number one intensity. The subsequent course of the UPE for leaf number one involves a steep decrease to the medium level of intensities out of the three injured leaves. The subsequent UPE progression for leaf number two involves also a decrease to the levels of the highest intensities out of all three leaves. The course of the UPE for leaf number three includes a decrease, but in this case to the lowest level of intensities out of all the injured leaves.

5. 3. 4 Kinetics of UPE from injured leaf

Imaging of the UPE from plant *H. vulgare* was performed over the time period of 180 min, while accumulation time of each frame was 20 min (Fig. 19). In image one, which was captured at a time of 20 min, only spontaneous leaf emission was observed and white pixels, caused by cosmic radiation, or bad pixel readout from the chip, these white pixels appear in every image. After capturing image one, a mechanical injury to the leaf one was performed, resulting in the increase in UPE in Fig. 19, 40 min, followed by slow decrease over the next 40 min (Fig. 19, 60 – 80 min). Figure captured at 100 min reveals a very small increase in the UPE from the injured leaf, possibly a result of systemic reaction to the plant injury. Further images (Fig. 19, 120 – 140 min) show a slow decrease in the UPE from injured leaf. The next image (Fig. 19, 160 min) shows an increase in UPE, which may have also been a result of a systemic reaction. The last image (Fig. 19, 180 min) shows a final decrease in the UPE, while the injury is still visible even 180 min after the cut. The average intensity of UPE at the place of injury is plotted in graph (Fig. 18).

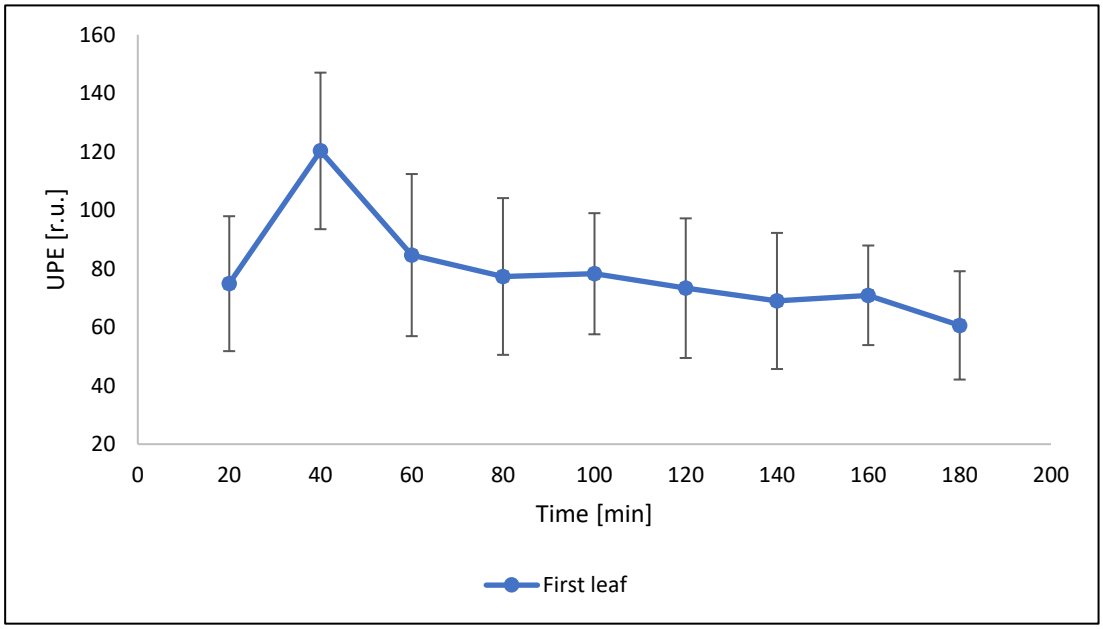

Figure 18. The course of UPE in the injured *H. vulgare* leaf with reaching a maximum in 40 minutes, that is, after the injury and with a subsequent decrease until 80 min. Subsequently, with a small increase at 100 minutes with a subsequent decrease in UPE to 140 minutes. Further with the increase at 120 minutes due to a probably systematic reaction. Further, a decrease in UPE can be observed.

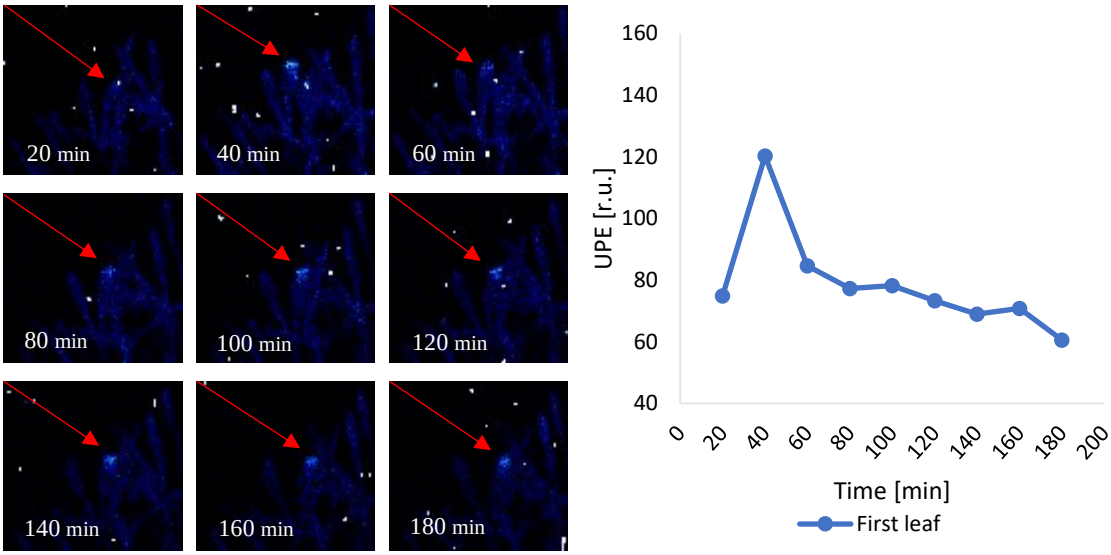

Figure 19. UPE from *H. vulgare* leaf, that was mechanically injured (The site of injury marked by red arrow). Only spontaneous UPE is visible at 20 min and white pixels caused by cosmic radiation, or bad pixel readout. At 40 min a massive increase in UPE is visible, induced by mechanical injury, also shows that the plant moved a bit while cutting. Followed by rapid decay at 60 – 80 min. At 100 min shows a slight increase in UPE, with its gradual decline until 140 min. At 160 min shows a slight increase in the UPE, followed by its final decrease at 180 min.

5. 4 Comparison of results among *A. thaliana, N. rustica* and *H. vulgare*

The subject of this comparison was to illustrate the differences in the courses of UPE from injured first leaves in *A. thaliana*, *N. rustica* and *H. vulgare*. The data from leaf injury from individual experiments, over a period of 100 minutes, i.e., from five images at 20-minute intervals were compared. For the mutual comparison of the data, the normalization of the individual UPE values was carried out, from which the control values were subtracted, that is, the spontaneous UPE from the uninjured leaf. Standard deviations were calculated from at least three experiments.

In fact, all three plant species reacted similarly in the experiments, but the differences in the course and intensity of UPE are their biggest differences. Prior to injury in all three plant species there was only spontaneous UPE, which was the most intense in *A. thaliana*, in *N. rustica* the spontaneous UPE was very low. In *H. vulgare*, spontaneous UPE was very low but higher than in *N. rustica*. After injury, there was an increase in UPE in all three species, which was most intense in *A, thaliana*. For *N. rustica*, the increase in UPE was also observable, but very small. In *H. vulgare*, there was also an intensive increase in UPE, as in *A. thaliana*, but it was slightly lower in comparison with it. At 60 minutes of measurement, i.e., 20 minutes after injury, a decrease in UPE was observed in all of three species. In *A. thaliana*, there was a decrease, which, however, was still much more intense than the spontaneous UPE. For *N. rustica*, the UPE was reduced below the control values, i.e., again to negative values. In *H. vulgare*, the UPE was reduced to values slightly higher than the spontaneous UPE. At 80 minutes, i.e., 40 minutes after the injury, all three species experienced a re-reduction in UPE. In *A. thaliana*, there was only a very small decrease compared to the previous value, and still the UPE remained above the level of spontaneous UPE. For *N. rustica*, the intensity of UPE was reduced again to more negative values. In *H. vulgare*, the intensity of UPE was reduced to levels comparable to spontaneous UPE. At 100 minutes, all three species experienced a re-reduction in UPE again, which was similar to the previous point. In *A. thaliana*, there was a small decrease in UPE, which, however, was still much higher than the spontaneous UPE. For *N. rustica*, the UPE was again reduced to more negative values, and for *H. vulgare*, the UPE was reduced to negative values (Fig. 20).

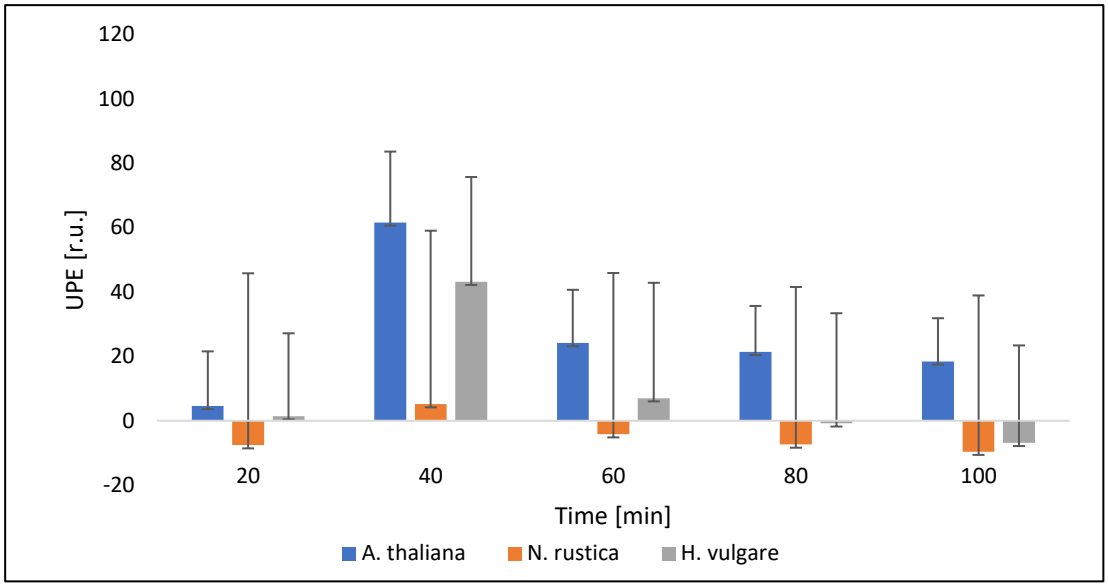

Figure 20. The comparison in the courses of the increases or decreases of the signal in A. thaliana (blue), N. rustica (orange) and H. vulgare (gray), when in 20 min. the measured values of the UPE represent spontaneous emission, at 40 min, an increase in UPE after injury to the leaf, and at 60-, 80- and 100-min individual decreases in UPE intensity over time.

5. 5 Binning

Binning, or combining adjacent pixels on the sensor so that they behave as a single pixel. Binning is a feature that allows a virtual increase in pixel size, which can be used in CCD sensors for several reasons; it reduces the number of imaging elements, hence the resolution, but increases the dynamic range of the associated pixels, speeds up the reading of information and reduces read noise. Pixels can be binned in multiples of 1x1, 2x2, 3x3, 4x4, 8x8, etc., and in the horizontal direction, as well as in the vertical direction, with more modern types of cameras there is also the possibility of an asymmetric binning.

In the experiment, different types of binning were tested, namely 1x1, 8x8 and 16x16 on images with exposures of 600 seconds (Fig. 21)., 1200 (Fig. 22) and 1800 seconds (Fig. 23), and it was found that images with 8x8 binning are the most acceptable in terms of image noise reduction in proportion to the resolution. Images with 1x1 binning were of very poor quality and did not provide acceptable information. The same was true for the 16x16 binned images, which had too much resolution distortion and too much white pixels. It was also found that the ideal exposure time is 1200 seconds

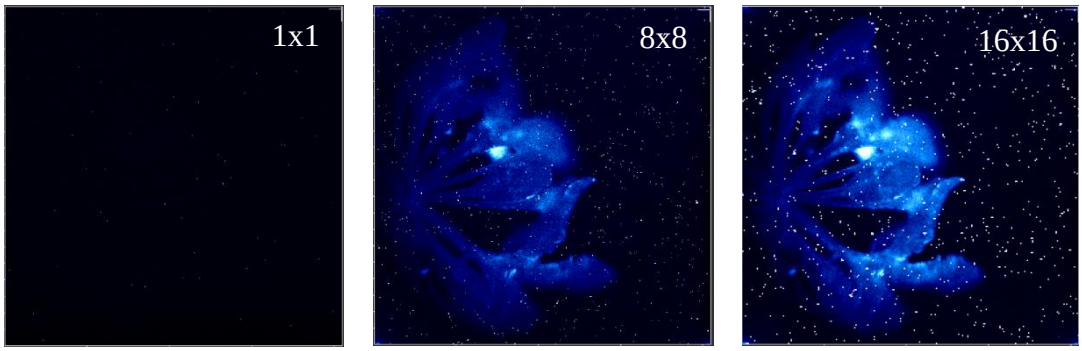

Figure 21. Images with 600 seconds exposure. Figure 1x1 (binning) shows almost zero information. Figure 8x8 (binning) shows nice detail of *A. thaliana* leaf. Figure 16x16 (binning) shows too much resolution distortion.

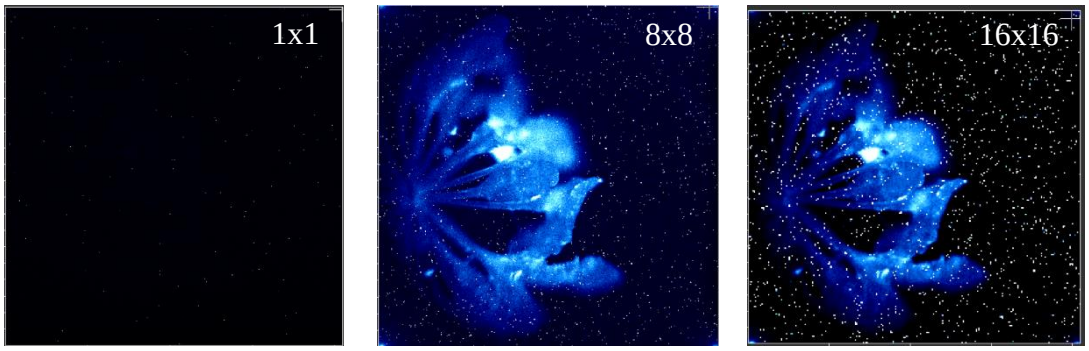

Figure 22. Images with 1200 seconds exposure. Figure 1x1 (binning) shows almost zero information. Figure 8x8 (binning) shows nice detail of *A. thaliana* leaf. Figure 16x16 (binning) shows too much resolution distortion.

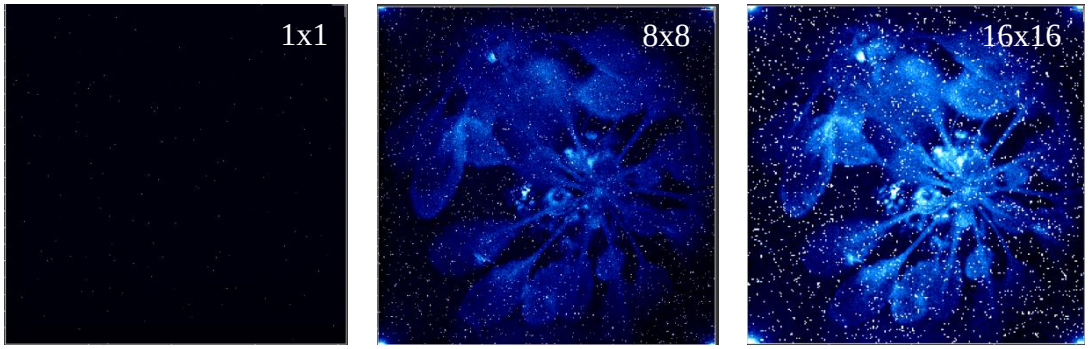

Figure 23. Images with 1800 seconds exposure and also a different *A. thaliana* plant. Figure 1x1 (binning) shows almost zero information. Figure 8x8 (binning) shows decent resolution of *A. thaliana*. Figure 16x16 (binning) shows too much resolution distortion

Images taken with 8x8 binning provide an ideal compromise between resolution and image quality.

5. 6 ROI analysis using Andor Solis vs. ImageJ

The ROI function, i.e., region of interest, allows to create a region of precisely defined dimensions on the image in which the signal analysis is performed. The ROI function can calculate various parameters such as median, total counts, maximum, etc. Another important feature in the experiment is that it can create these regions at the same locations in other frames of the series, thus allowing us to analyze the decrease of the injury signal, or compare the intensity of the injury signal against the spontaneous emission signal of the leaf and/or the background.

In this experiment, three images of *A. thaliana* were captured at 20-minute intervals. The first image at 20 minutes recorded only spontaneous emission from an intact leaf, at 40 minutes the UPE of the injured leaf was captured, and at 60 Minutes an UPE intensity decrease from the wound was recorded.

The images were further analyzed in Andor Solis, using the Analysis Region Of Interest function (Fig. 24)

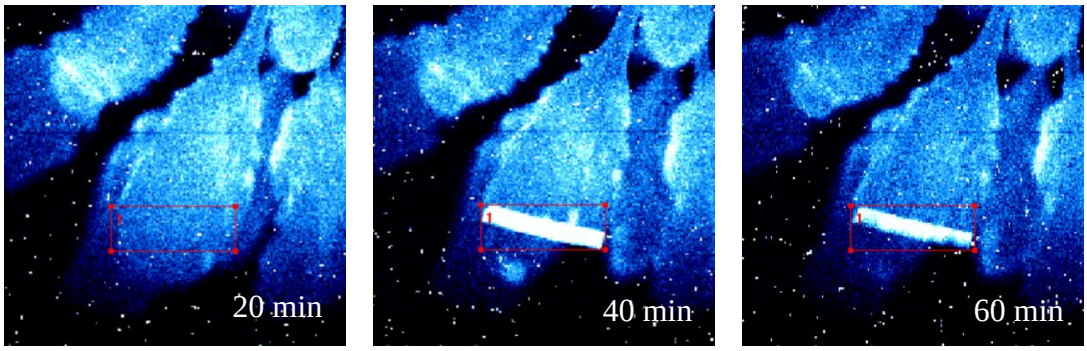

Figure 24. Signal analysis (Red rectangle) from *A. thaliana* using Andor Solis and Analysis Region of Interest function, from left showing analysis of the intact – uninjured leaf at 20 minutes, in the middle showing injured leaf at 40 minutes and on right showing signal development after injury at 60 minutes.

ImageJ is a free software that is widely used in science in the analysis of images. It has a large number of functions, however, for the purposes of this experiment, the most suitable option is to create an area in which analysis is subsequently possible. Thus, the same series of images was analyzed using this software (Fig. 25)

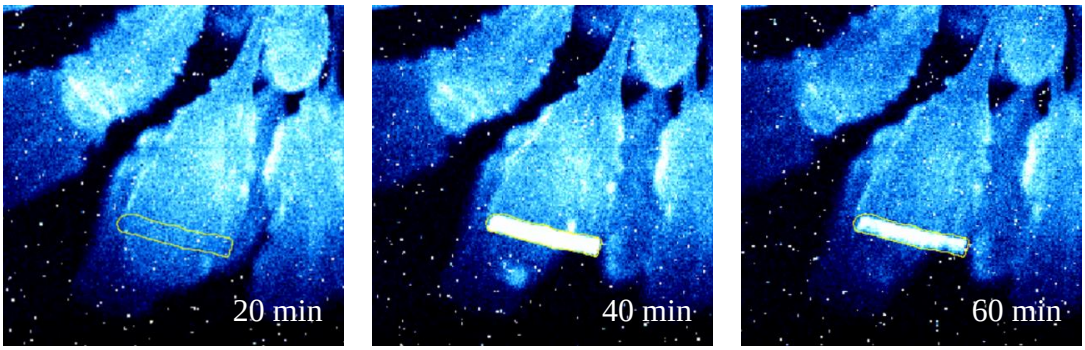

Figure 25. Signal analysis (Yellow marking) from A. thaliana using ImageJ, from left showing analysis of the intact – uninjured leaf at 20 minutes, in the middle showing injured leaf at 40 minutes and on right showing signal development after injury at 60 minutes.

Further, after exporting the data and normalizing it, it was found that the ROI function in Andor Solis provides a much lower signal compared to the data that was obtained from ImageJ software. At the same time, the data from Andor Solis are much less accurate and do not provide the necessary sensitive resolution of intensities, as is possible with the analysis in ImageJ (Fig. 26). The data provided by the ROI function shows that the intensity of UPE after the leaf injury increased very little, and their subsequent decrease to a similar level as with an undamaged leaf. While the data from ImageJ indicate a massive increase in the intensity of UPE after injury, i.e., captured in 40 minutes, and its gradual decrease, but far from the levels of the intact leaf.

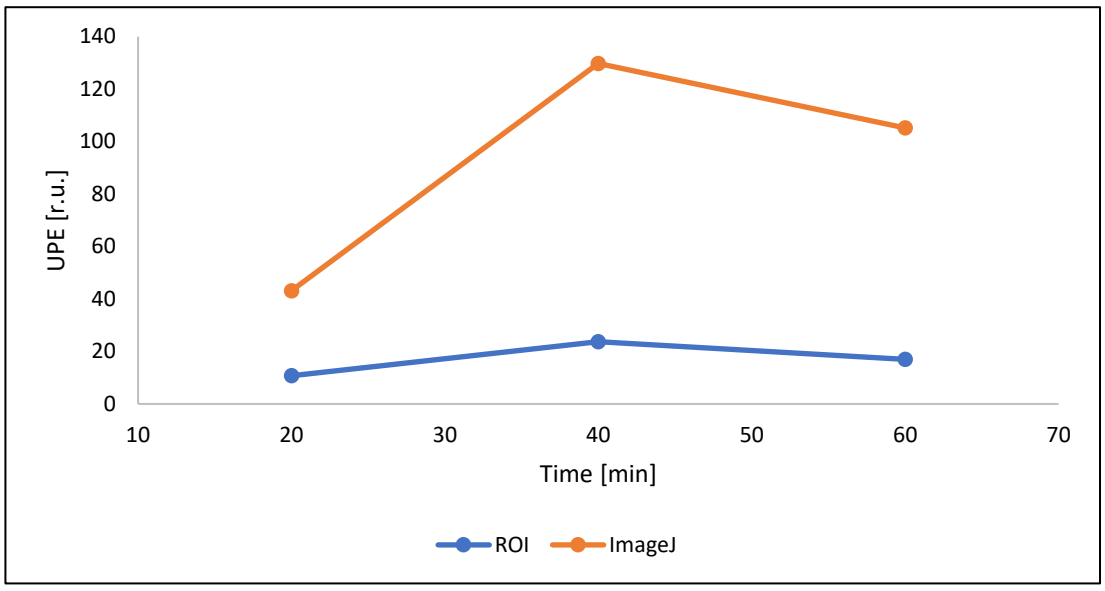

Figure 26. The difference between image analysis using ROI in Andor Solis, and data analysis in ImageJ software. With ROI analysis (Blue), only a small increase after damage (40 min) and a subsequent small decrease (60 min) can be observed. In contrast to the data analyzed with ImageJ (Orange), a massive increase in the intensity of UPE and its subsequent gradual decrease (60 min) can be observed.

6 Conclusion and discussion

We measured spontaneous and induced UPE from three different plant species in this work. Based on previously published studies, we expected slow decrease in spontaneous UPE during the measurements (Pónya & Somfalvi-Tóth, 2022), which, however, was not confirmed (Chapters 5. 1. 1, 5. 2. 1 and 5. 3. 1).

In *A. thaliana*, the intensity of spontaneous UPE oscillated between experiments (Fig. 5). It could not be assumed that individual spontaneous UPE decreased over time, this statement was confirmed only in one experiment (Fig. 5, control 3), in the other two experiments there was an oscillation and an overall increase in spontaneous UPE (Fig. 5, control 1, 2). The decrease was also not confirmed for *N. rustica*, as spontaneous UPE was at different intensity levels in the experiments (Fig. 10). Again, in one of the experiments, it was found that spontaneous emission decreased over time (Fig. 10, control 2), but in the other two experiments this was not confirmed (Fig. 10, control 1, 3). In *H. vulgare*, it was found by analogy that spontaneous UPE does not decrease in time (Fig. 15), on the contrary, it oscillates differently, with a slight increase in two experiments (Fig. 15, control 1, 3). In the third experiment, a slight increase and subsequent decrease in time to lower values of the intensity of UPE was noted (Fig. 15, control 2).

The consequence of the finding that the spontaneous UPE oscillates over time and does not decrease according to the assumptions was that for the comparison of the UPE course in plant species, the measurement results had to be normalized by subtracting the values of spontaneous UPE from the values of the induced UPE (Fig. 20).

After subsequent normalization of the data, the induced UPE patterns among plant species were compared. It was found that the injury increases the intensity of UPE at the site of the injury on the leaf in *A. thaliana*, which corresponds to the literature (Prasad et al., 2020), as well as in *N. rustica* and *H. vulgare*. With *A. thaliana* having the most intense increase and *N. rustica* having the least intense increase (Chapter 5. 4). Furthermore, the kinetics of UPE, was also different for different plant species. Induced UPE in *A. thaliana* remained increased over the level of spontaneous UPE throughout the whole experiment, while induced UPE in *N. rustica* decreased back to the level of spontaneous UPE at 60 min. and in case of H. vulgare at 80 min.

(Chapter 5. 4, *A. thaliana*: Chapter 5. 1. 4, *N. rustica*: Chapter 5. 2. 4, *H. vulgare*: Chapter 5. 3. 4). Those data suggest different kinetics in ROS formation upon mechanical injury in *A. thaliana* compared to *N. rustica* and *H. vulgare*.

The systemic response of plants to mechanical injury on leaf has been previously described (Jakšová et al., 2021). The transmission of signal occurs within seconds through electrical signals from injured leaves into adjacent leaves resulting in the formation of stress hormones and jasmonic acid. In this work, we were focusing the whole plants with CCD camera in order to examine possible effect of the systemic response on UPR induced by mechanical injury. We expected to see gradual decay of induced UPE signal between first, second and third injured leaf in *A. thaliana* and *N. rustica*, where we injured the leaves within one plant in comparison to *H. vulgare*, where each leaf represents separated plant. Comparison of induced UPE from all three plant species did not reveal any differences (*A. thaliana*: Chapter 5. 1. 3, *N. rustica*: Chapter 5. 2. 3, *H. vulgare*: Chapter 5. 3. 3) suggesting that there was no effect of systemic response on UPE induced by mechanical injury. The gene expression of stress genes should occur less intensively between 0 and 120 minutes after exposure to stress, but can also occur in the time range of 0 to 240 minutes (Bendjilali et al., 2017). The time period (40 and 80 min.) chosen by us to monitor the dependence of the UPE response on the injuries of the second and third leaves may not have been long enough in order for the plant to accumulate enough antioxidants and stress proteins in order to affect the intensity of UPE.

From the results obtained, we conclude that the UPE measurement method can be applied to the study of all three plant species with certain limitations. In *A. thaliana* and *H. vulgare*, the UPE study can be applied without problems, but in *N. rustica*, the UPE study encounters the problem of a weak response after injury. Another complication with *N. rustica* is the size of the plant itself, as it carries a problem with proper focusing of the CCD camera. In our work, the whole plant was focused, so we could observe the differences between individual leaves, but for the next study of *N. rustica* using UPE, we would suggest focusing individual leaves. For *A. thaliana* and *N. rustica*, the method can be applied without any changes.

Furthermore, we suggest to extend the exposure time for *N. rustica* resulting in a better signal-to-noise ratio and to more distinct differences between spontaneous and induced UPE, while losing time resolution of the kinetics of induced UPE. In case the

kinetic of induced UPE is the priority, we suggest to use photon multiplier tube (PMT) (Rác et al., 2015) for the study rather than CCD camera.

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