Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biology

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Death signals during plant cell death.

Macroscopic study of cell death induced by low pH and eATP.

Master's thesis in Biotechnology
Supervisor: Daniela Sueldo
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Abstract

Research in cell death is currently a developing and very interesting topic. Cell death as the process of death in microscale is an important mechanism of control in all living organisms. Especially programmed cellular ‘suicide', which allows to remove damaged cells that are no longer needed, is fascinating. With every new publication, we gain knowledge about this process, in both animals and plants. However, cell death mechanisms are comparatively less well understood in plants. For optimal growth and development plants need a suitable pH of the soil for instance. Too low pH in the soil is a stress trigger, which can lead to cell death. Recently, extracellular ATP (eATP) has emerged as a signaling molecule in plants with several roles in development and stress response. Furthermore, eATP can be released during cell death but its role as a death signal in plants is not well understood yet. In this master thesis I aimed to answer the question whether low pH and exogenous ATP cause cell death in *Arabidopsis thaliana*. For this, I treated seedlings with adverse pH 4.6 in two different buffers, Sodium Acetate and MES buffer. I observed that low pH is a trigger for cell death and induced bleaching in cotyledons and primary leaves. Interestingly, bleaching was suppressed in the dark, suggesting these responses are light-dependent. Furthermore, to investigate if eATP can act as a ‘death molecule’ in plants, I treated seedlings with ATP for different timepoints. The results I obtained revealed that eATP induced cell death at shorter timepoints (1 hour).

For tiden er forskning på celledød et utviklende og svært interessant tema. Celledød som dødsprosessen i mikroskala er en viktig kontrollmekanisme i alle levende organismer. Spesielt programmert cellulært "selvmord", som gjør det mulig å fjerne skadede celler som ikke lenger er nødvendige, er fascinerende. Med hver nye utgivelse får vi kunnskap om denne prosessen, både hos dyr og planter. Imidlertid er celledøden mekanismer relativt mindre godt forstått i planter. For optimal vekst og utvikling trenger planter for eksempel en passende pH i jorda. For lav pH i jorda er en stress utløser, som kan føre til celledød. I det siste har ekstracellulær ATP (eATP) dukket opp som et signalmolekyl i planter med flere roller i utvikling og stressrespons. Dessuten kan eATP frigjøres under celledød, men dens rolle som døde signal i planter er ikke godt forstått ennå. I denne masteroppgaven hadde jeg et mål for å svare på spørsmålet om lav pH og eksogen ATP forårsaker celledød i *Arabidopsis thaliana*. For å gjøre det, behandlet jeg frøplanter med ugunstig pH 4,6 i to forskjellige buffere, Sodium Acetate og MES buffer. Jeg observerte at lav pH er en utløser for celledød og indusert bleking i cotyledons og primære blader. Forresten ble det bleking undertrykt i mørket, noe som tyder på at disse responsene er lysavhengige. Dessuten for å undersøke om eATP kan det fungere som et "døds molekyl" i planter, behandlet jeg frøplanter med ATP på forskjellige tidspunkter. Resultatene fikk jeg avslørt at eATP induserte celledød på kortere tidspunkt (1 time).

Acknowledgments

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1. Introduction

Constant balance between life and death is present in all life cycles. This regulation is crucial for multicellular organisms to remain in homeostatic equilibrium, allowing cells and organs to properly grow and function, in order to survive. Therefore, suitable mechanisms controlling life and death had to be introduced. Cell death is one of them.

In metazoans, several types of cell death have been described including apoptosis, necrosis, mitotic catastrophe, and autophagy (Okada; Mak, 2004). Senescence as a type of cell death is also mentioned in the context of cancer therapy (Ricci; Zong, 2006). Apoptosis and autophagy are active cell deaths pathways occurring in animals, while necrosis and mitotic catastrophe are passive processes (Stepien et al., 2007). Regulated Cell Death (RCD) is a physiological death in contrast to necrosis which is a pathological death occurring due to the mechanical injury of the tissue or the activity of strong stress factors such as exposure to toxic compounds or temperature changes (Wojciechowska, 2001). RCD, also referred to as "controlled suicide," is a key factor in controlling an organism's reactions, development and morphogenesis. Furthermore, RCD also participates in response to environmental conditions. Removal of dysfunctional or potentially harmful cells prevents their multiplication and spreading throughout the body (Wojciechowska, 2001). Despite variations in phenotypic patterns, specific components of the pathway, or its regulation, the occurrence of PCD is a universal event in the living world (Maruniewicz; Wojtaszek, 2007).

While animal cell death is constantly researched, there is comparatively limited information on cell death in plants. That makes it an interesting topic to research. There are still so many questions that need answers. For instance, what are the processes that lead to the decision that a particular cell should 'commit a controlled molecular suicide'? Can this process be stopped or reversed somehow? In my thesis I addressed the questions: What causes plant cells to die? How does it look macroscopically?

In both plants and animals, the regulation of cell death mechanisms is mostly controlled genetically (Wojciechowska, 2001). Numerous apoptosis-related genes have been discovered thus far that are also vital to cell proliferation, indicating a close connection between these two processes. A cell's entry into the cell death pathway is influenced by a wide range of regulatory elements. Physiological death-inhibitors and-activators have been identified in both plant (for instance, in *Nicotiana tabacum*) and animal organisms (Vacca et al., 2006). RCD in plants and animals may be characterized by the release of cytochrome c from mitochondria, activation of deoxyribonucleases (DNase), caspase-like proteases, and accumulation of reactive oxygen species (ROS). During this process, for example, DNA degradation with the formation of the so-called "DNA ladders" and changes in protein phosphorylation may also occur (Godlewski; Kobylinska 2016).

It is reported that adenosine triphosphate (ATP), a molecule that carries energy in the cells, is released during cell death and can act as a death signal when in the extracellular space (Martins et al., 2009). Extracellular ATP (eATP) serves a variety of purposes as a signaling molecule in addition to its function as an intracellular energy source. Animal cells have ATP receptors, known

as P2X and P2Y (Khakh; Burnstock 2010). Investigations based on sequence homology did not identify any homologous ATP receptor in plants, even though eATP can be perceived and is essential for plant development, growth, and stress responses. However, a decade ago, Choi et al. reported the identification of the first plant eATP receptor in *Arabidopsis*. This receptor – DORN1 (Does Not Respond To Nucleotides 1) possesses a high affinity for the ATP molecule and is necessary for the activation of the mitogen-activated protein kinase, the ATP-induced calcium response, and changes in gene expression. Choi et al. showed that the plant's reaction to physical injury was enhanced by DORN1's ectopic expression. It suggests DORN1 is crucial for eATP sensing and probably has a range of functions in plant stress tolerance (Choi et al. 2014). That generates an inquiry – does ATP itself cause any harm to the cell? In this thesis I will explore this question by treating the model plant *Arabidopsis thaliana* (Arabidopsis) to different concentrations of eATP (extracellular ATP). The results I achieved suggest the molecule is damaging cells in the shorter time point of 60 minutes.

Exposure to stress factors, such as low pH level, elevated temperature or toxic compounds may also cause cells to die. Focusing on pH level, for most of the plants the optimum pH ranges between pH 5.5 to pH 6.5 (Msimbira L.A; Smith D. L.; 2020). Therefore, lower, or higher pH level can be a stress factor for them. The optimum pH level for *Arabidopsis thaliana*, the species I work with, is 5.8. To address the effect of low pH in plant physiology and stress responses, I exposed Arabidopsis to low pH (4.6) for different time points. I discovered exposure to low pH caused bleaching of the tissue in some cases, and that this process is light dependent.

Abbreviations

ADP adenosine diphosphate

AMP adenosine monophosphate

APY1 apyrase 1

APY2 apyrase 2

ATP adenosine triphosphate

eATP extracellular ATP

iATP intracellular ATP

DNase deoxyribonuclease

DORN1 Does Not Respond To Nucleotides 1

EB Evans blue

ECM extracellular matrix

GSH glutathione

H+-ATPase ATP phosphohydrolase (H+-exporting)

HR hypersensitive response

MT melatonin

MQ mili-q water

PAs polyamines

PCD programmed cell death

PSI photosystem I

PSII photosystem II

RCD regulated cell death

REF ultraviolet light

ROS reactive oxygen species

SAMDC S-adenosylmethionine decarboxylase

TB trypan blue

V-H+-PPase Vacuolar H^+-pyrophosphatase

2. Background

2.1 Cell Death in plants

The term “apoptosis” is not the correct name for process of cell death that occurs in plants. Apoptosis is regarded as the active type of death that needs ATP and recognized because of morphological signs, which include cell shrinkage, chromatin marginalization and condensation, DNA fragmentation, preservation of cell membrane integrity until late stages of the process. Finally, apoptosis involves the formation of apoptotic bodies that are then ingested by nearby cells or specialized phagocytic cells. As a result, absence of apoptotic cell death has been proposed for organisms whose cells are surrounded by a rigid cell wall that prevent cell fragmentation, such as plants and fungi (Maruniewicz; Wojtaszek, 2007), (Minina et al. 2021). Cells may randomly necrotize under conditions of acute stress, which is basically an uncontrolled collapse of biological function. Contrarily, regulated cell death (RCD) is a form of cellular suicide that is genetically programmed and integrated into the multicellular organism's physiology (Galluzi et al. 2015). For instance, programmed cell death (PCD), a subtype of RCD intimately associated with proper development, inhibits cell proliferation by removing senescent cells and is crucial to morphogenesis by removing surplus cells and generating new structures (Minina et al. 2021). RCDs also have a role in stress reactions, preventing infections into tissues, and reducing the harm caused by abiotic causes (Minina et al. 2021) (Huysmans et al. 2017), (Coll et al. 2011), (Jorgensen et al. 2017).

Even though some of the alterations seen during apoptosis in animal species are also seen in plants, these changes are referred to as RCD rather than being the same as apoptosis. Plants do not exhibit heterophagy elimination of dead cells, which is a characteristic of apoptosis in animals. Plant cells do not possess lysosomes, instead they have vacuoles which house acidic hydrolases (lytic vacuoles). One key feature that differentiates the process of cell death in animals and plants is the fact that plants have a cell wall, and it appears that plant cells are eliminated through autolysis and autophagy. This attribute procures the leaking of dying cell’s content to the extracellular environment, where it is being recycled later during further stages of cell death. Plants also have plastids, and chloroplasts participate in RCD (Godlewski; Kobylinska, 2016). In the course of apoptosis in animals on the other hand, content of the dying cell is being absorbed by macrophages or neighboring cells thereby preventing the inflammatory reaction (Wojciechowska, 2001).

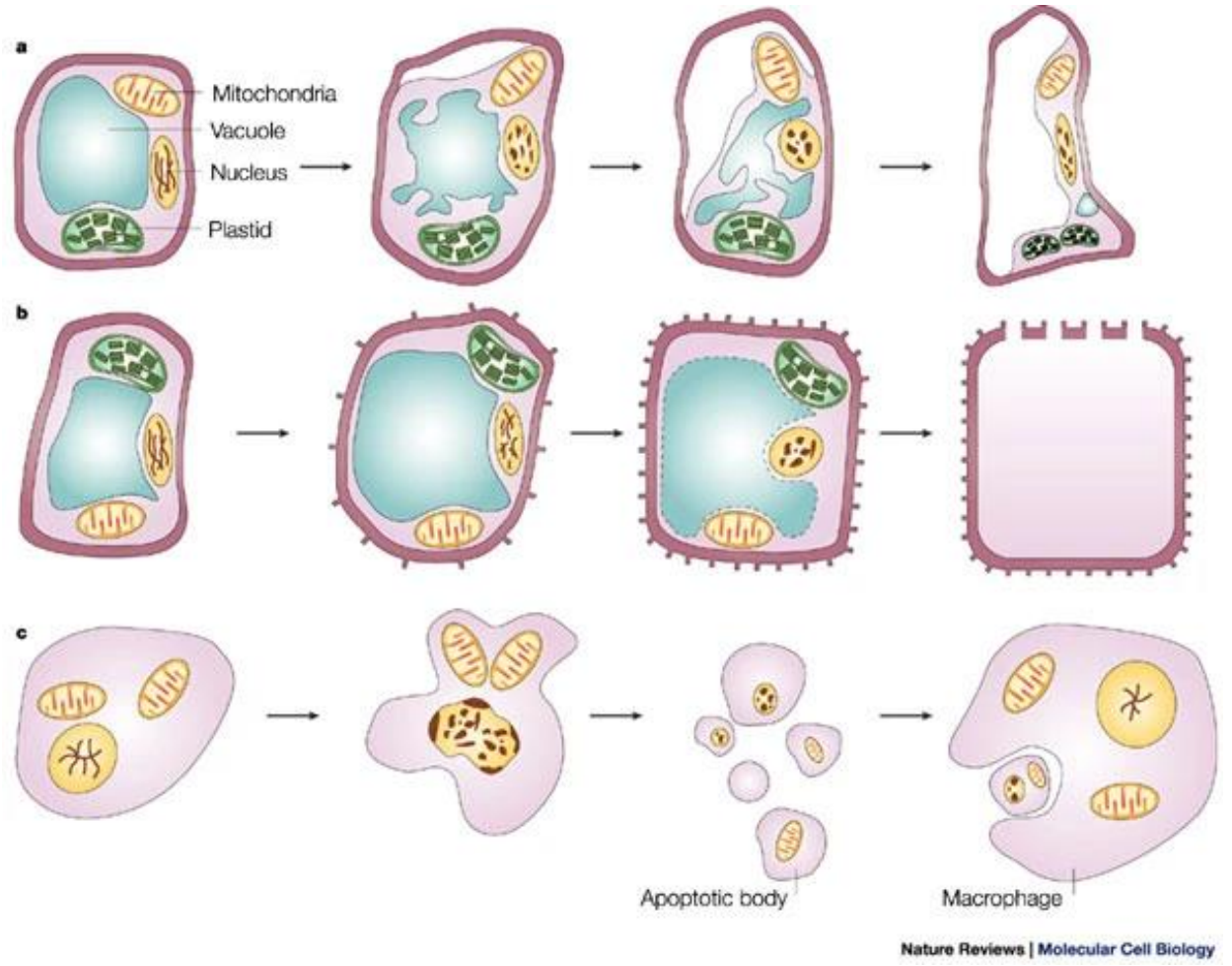

Figure 1. Regulated cell death in plants versus animal apoptosis – the morphological comparison.

a. Cell death induced by pathogen recognition (hypersensitive response): Before the apparent breakdown of the vacuole, which takes place during the last stages of cell death, chromatin condensation and DNA breakage into 50-kb fragments are seen. Late organelle breakdown and blebbing of the vacuole and plasma membranes are also observable. The plasma membrane splits from the cell wall and collapses at the end of this cell-death process, allowing the contents of the dead cell to flow into the apoplast. The nuclei of dying cells are seen in the illustration as irregular, brown lumps, which represent fragmented nuclear DNA.
b. Developmental cell death (tracheary elements differentiation): Vacuole enlargement and rupture coincide with the thickening and remodeling of the cell wall during the differentiation of tracheary elements. Nuclear DNA fragmentation, which takes place at the end of the cell death process before the final autolysis of the cell, occurs right before the final collapse of the vacuole. On differentiating tracheary components, short stubbles signify reticulated secondary cell walls. Terminally differentiated tracheary elements' broken cell walls show signs of spatially localized holes.
c. In animal cells, chromatin condensation and fragmentation signal the start of apoptosis. The development of apoptotic bodies from the repackaging of the cell's contents and their eventual engulfment by neighboring cells or macrophages occur next to plasma-membrane ruffling (Adapted from Lam 2004).

Developmental programmed cell death (PCD) in plants is one of the most significant processes that contributes to the development of plant tissues and the subsequent maintenance of their homeostasis. Consequently, it is a mechanism required at various stages of ontogenesis (Zagorska-

Marek 2007). Depending on the differences in their structure, the necessary rate of viability inhibition, and the ultimate resting place of the dead cells, the course of changes occurring in the area of dying cells also varies. In developmental PCD, cells that have served a purpose for a predetermined amount of time are eliminated, such as suspensor cells during embryo development and aleurone cells in granulomas. Unneeded cells such as root caps and stamen buds in same-sex flowers, protoplasts of wood cells that are undergoing vessel differentiation, as well as aerenchyma produced during aging and organ shedding. (e.g., leaves, shoots, excess flowers) are also removed. In both plants and animals, the commitment of cells toward developmental PCD is determined by endogenous factors, whose actions can be affected by external factors, such as environment. This follows a morphogenetic plan. PCD in plants is controlled by phytohormones like ethylene, abscisic acid, jasmonates, polyamines, salicylic acid, as well as by nitric oxide (NO) and higher concentrations of cytokinins (Godlewski, Kobylinska 2016).

2.2 Types of cell death

Developmental PCD

One distinctive process for developmental PCD in plants is the occurrence of the cell digesting its own components. Because of autophagy, only the protoplast of the dying cell is subject to degradation processes during PCD, excluding nearby cells (Figure 1). The vacuole, containing an assortment of enzymes that break down cellular components, plays a crucial part in the autophagy process (Wojciechowska, 2001). Vacuoles are multipurpose structures, and a cell can contain several types of them. The type of proteins that reside inside vacuoles is one factor that influences how they differentiate in function (Muntz 2007). The vacuolar fluid contains proteins such as cysteine proteases, serine proteases, endonucleases (RNAses, DNAses), acid phosphatases, and lipases, among others. These are the same enzymes that are crucial for the apoptosis of cells in animals. Then the vacuole's volume significantly increases until it takes up nearly the entire protoplast (Fukuda 2000). The series of events that result in the lysis of the complete protoplast only happens when the tonoplast is broken. This implies that the cell continues to function physiologically until the vacuolar contents are released, a crucial PCD phase. (Obara et al. 2001).

After being "equipped" with the necessary enzymes, the vacuole goes through additional changes linked to the autophagy process. There may be various routes taken by this procedure. Autophagy is a katabolic process, and it can be divided into microautophagy and macroautophagy. Microautophagy consists of a tiny amount of the cytoplasm being taken up by the lysosome membrane (in animals) or the vacuole (in plants) and it is then divided into vesicles (autophagic bodies) and transported into these organelles where it is digested (Godlewski, Kobylinska 2016). Macroautophagy has more stages: a larger part of the cytoplasm, filled with organelles, is surrounded by the membranes of a vesicle likely originating from the ER, and cup-shaped pre-autophagic structures are created. The autophagosome, a vesicle encircled by two membranes, is created when the membranes enclosing this region of cytoplasm fuse. In animals, the autophagosome fuses with the lysosome, and the contents are digested in the resulting autolysosomes. In plants, the autophagosome's outer membrane fuses with the tonoplast, and the autophagic body's contents are released into the lytic vacuole and digested there. The most

prevalent form of autophagy and the one that is most frequently induced when under stress is macroautophagy (Van Doorn W. G., Woltering E. J., 2002), (Yang et al., 2005).

Apart from micro- and macroautophagy, megaautophagy may be crucial to developmental PCD in plants. Hydrolytic enzymes are heavily synthesized in the initial stages of this autophagy form and then found in the vacuolar sap. The tonoplast then loses its selective permeability, its continuity is broken, and hydrolytic enzymes are released from the vacuole to begin the process of degrading cell components. This results in a substantial increase in the volume of the vacuole (Wojciechowska 2001). The cell may engage in at least two distinct kinds of autophagy during PCD. There have also been instances where megaautophagy succeeded simultaneous micro- and macroautophagy.

Pathogen-induced RCD

Plants do not possess an adaptive immune system. In their situation, a pathogenic microorganism is frequently fought by a different line of defense. Hypersensitive response (HR) is an essential element in this case (Greenberg, 2005). HR involves the protoplasts of infected and nearby cells dying rapidly, which helps to stop the infection from spreading throughout the plant tissue (Figure 1) (Zagorska-Marek 2007). Cytoplasm shrinking is another trait that sets HR apart from other RCD pathways. Sometimes pathogen-infected cells will create cell fragments that resemble the apoptotic bodies developed during animal apoptosis. NO and Reactive oxygen intermediates (ROI) may cause the death of nearby cells (REF). HR is typically accompanied by an increase in the augmentation of the vacuoles and may call for the release of vacuolar processing enzymes (VPEs) after tonoplast rupture (Godlewski, Kobylinska 2016).

2.3 eATP – its role and link to cell death

Adenosine 5′-triphosphate (ATP) is a molecule used by cells as ‘energy currency’. It provides energy that is needed in different biochemical reactions. Despite its intracellular nature, numerous inquiries have shown that animal, plant, and microbial cells release this chemical into the extracellular matrix from the cytosol from stressed or dying cells (Boyum and Guidotti, 1997; Parish and Weibel, 1980; Thomas *et al*., 2000). Extracellular ATP (eATP) in animal cells is necessary in physiological processes such as immunological responses or growth and death of the cell, whereas plant cells require it as a signaling molecule in terms of plant’s growth, development, and response to environment (Feng, 2014).

Furthermore, it is important to mention, that some research indicates that it has a special role in the regulation of plant cell death. ATP synthase has not been identified at the cell surface or plasma membrane of plant cells; it suggests that intracellular ATP (iATP) may be a significant source of plant eATP. Additionally, certain external factors, such as pathogen infection, hypertonic stress and injury, can raise the level of eATP (Cao et al., 2014; Choi et al., 2014b; Sheppard, 2014; Tanaka et al., 2010a; 2014). Plant cells have apoplastic nucleotidases and apyrases, which hydrolyze eATP, just like animal cells do. Altering the amount of plant eATP can have an impact on cell development, viability, biotic and abiotic stress responses, thigmotropism, and

gravitropism. It demonstrates that eATP has significant physiological functions in the plant (Cao et al., 2014; Choi et al., 2014b; Sheppard, 2014; Tanaka et al., 2010a; 2014). Because eATP cannot readily diffuse across the plasma membrane, it is thought that the effects of eATP on various plant physiological processes depend on a membrane-associated receptor protein(s) (Feng, 2014).

In animal cells, eATP causes an increase in cytosolic Ca levels by activating its receptor proteins in the plasma membrane, which serves as an early signaling step for the eATP-mediated physiological activities (Dichmann et al., 2000). Additionally, exogenous ATP treatment can cause a particular rise in cytosolic Ca in plant cells as well (Demidchik et al., 2009; Möhlmann, 2014; Tanaka et al., 2010b) In plant cells, eATP is released at levels as high as nanomolar into the surrounding media (Sun et al., 2012b; Tanaka et al., 2010a, 2014).

Furthermore, extracellular and intracellular ROS are produced more frequently by plant cells in response to eATP (Clark et al., 2010; Demidchik et al., 2009; Kim et al., 2006; Lim et al., 2014; Möhlmann, 2014; Song et al., 2006). Exogenous ATP treatment of *Populus euphratica* cell cultures has also been shown to produce mitochondrial ROS (mtROS) (Sun et al., 2012a).

In addition to cytosolic Ca and ROS, exogenous ATP stimulation causes an increase in NO (Clark et al., 2010; Foresi et al., 2007; Möhlmann, 2014; Reichler et al., 2009; Salmi et al., 2013; Wu and Wu, 2008). Additionally, exogenous ATP has been shown to trigger the transcription of the genes encoding the enzymes in the biosynthetic pathways for ethylene $[Ca^{2+}]_{cyt}$jasmonic acid, two plant hormones involved in stress responses (Song et al., 2006). Several studies have demonstrated that the increase in cytosolic Ca $[Ca^{2+}]_{cyt}$eATP-induced production of NO and ROS (including extracellular, cytosolic, and mitochondrial ROS) (Demidchik et al., 2009; Möhlmann, 2014; Sueldo et al., 2010; Sun et al., 2012a; Wu and Wu, 2008).

2.3.1 eATP and cell death

Chivasa et al. (2005, 2009, 2010) demonstrated that the removal of eATP caused cell death in both cell cultures and whole plants (*Arabidopsis thaliana; Nicotiana tabacum; Zea mays; Phaseolus vulgaris*) by employing various enzymes that can consume or hydrolyze eATP. Further evidence that cell death is a distinct response to the depletion of ATP rather than to the accumulation of ADP or AMP is provided by the fact that neither AMP nor ADP, the products of ATP hydrolysis, can cause cell death (Chivasa et al., 2005). However, later studies in eATP in plants show that stable eATP level is essential for plant cell viability, because radical changes in increase or decrease may cause cell death (Figure 2) (Feng, 2014).

Since adjacent cells in multicellular organisms share eATP, changes in eATP release from cells undergoing specific environmental stimuli, such as pathogen infection or injury from herbivore attack, may have an immediate impact on the fate of nearby cells. Therefore, this opens the question of whether eATP released from a dying cell could act as a death molecule for other cells.

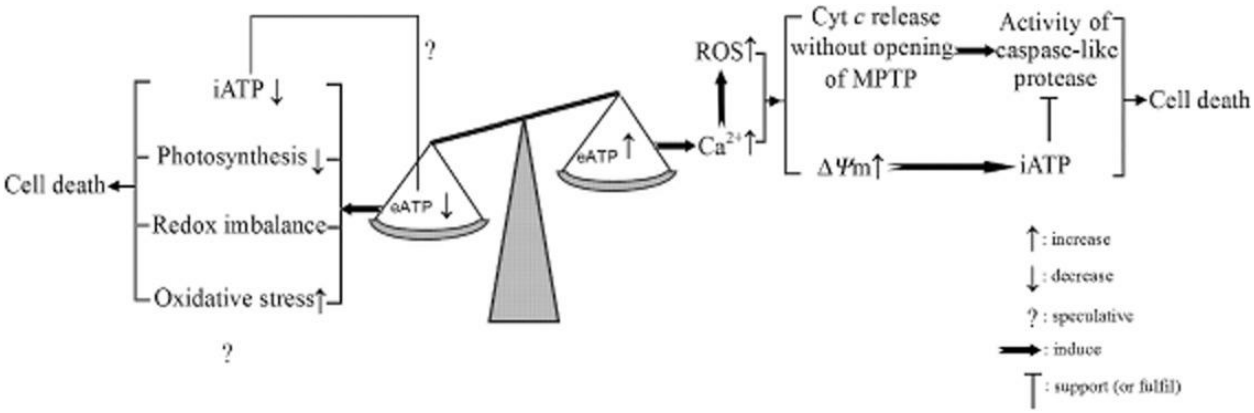

Figure 2. Extracellular adenosine 5′-triphosphate (eATP) levels have an impact on cell death. Both an increase or decrease of eATP can cause cell death. Model for the probable signaling pathways or physiological events mediated by eATP may help explain how cell death is induced. Cyt c stands for cytochrome c, iATP stands for intracellular ATP, whereas MPTP and ROS stand for mitochondrial permeability transition pores and reactive oxygen species, respectively (Adapted from Feng 2014).

2.4 Low pH stress

Hydrogen is one of the chemicals elements that are essential for all life forms. On Earth, from the atmosphere to the planet's deep core, hydrogen is abundant and generally exists as chemical compounds like water or in solutions in the protonated state (H+). All organisms depend on protons for their survival as these create gradients across cellular membranes, that power the intracellular or cell-to-cell movement of nutrients and other chemicals required for cellular function. However, an overabundance of protons in the surrounding environment can be detrimental and interfere with normal cell growth and function. In addition to causing nutritional issues, acidity is a particular abiotic stress that lowers the availability of phosphorus (P), calcium (Ca), ferrum (Fe), and magnesium (Mg) (Poschenrieder et al., 1995). Plant survival and metabolic activities are impacted by it.

Being sessile, plants are unable to migrate away from an acidic environment. Acid soils are those that have a surface layer pH between 5.0 and 5.5 and are known to restrict plant growth (Edmeades et al., 1995). For proper operation, plant cells must keep the cytoplasmic pH in the range of 7.0-7.5 (Marschner, 1991). The active function of proton pumps allows for the outflow of protons into the vacuole or the apoplastic space, which regulates the proton concentrations in the cell cytoplasm (Felle, 1998; Netting, 2002; Gao et al., 2004). Three separate proton pump types are used: two H+-ATPases, one each for the plasma membrane and the vacuole, and a V-pyrophosphatase, or V-H+-PPase, with varying activity but comparable roles. Most plants can survive in situations with a relatively low pH, but if the surrounding environment is very acidic, the proximity of root cells to soil solutions can make it difficult for plants to regulate their cellular pH. Strong acidic environments can have a significant impact on the structure and operation of root cells in plants (Foy, 1984; George et al., 2012).

Ca2+ may help to lessen the effects of low-pH stress (Moore, 1974; Kinraide, 1998, 2003; Rangel et al., 2005; Watanabe and Okada, 2005). Using mutant *Arabidopsis* plants that were sensitive to acidity stress, Kobayashi et al. (2013) showed an ameliorative impact of external Ca2+ and suggested that the reaction may be caused by Ca2+ stabilizing the cell wall. Strong acidity stress brought on by a high proton concentration eventually causes PM H+-ATPase activity in root cells and other enzymes across the entire plant to gradually shut down, stopping plant growth and creating standstill (Dyhr-Jensen and Brix, 1996).

Exogenous low pH stress results in cell death of root cells, restricting root growth and crop yield (Graças et al. 2020). Low pH is thought to cause the root development to slow down as a proactive plant reaction to reduce cell death (Graças et al. 2016). Different studies have shown restricted root development upon low pH dicot crops like *Solanum lycopersicum*, monocots like *Triticum aestivum*, or the model plant *Arabidopsis thaliana* (Graças et al. 2016), (Kinraide et al. 1992), (Koyama et al. 2001). Observed cell death was for instance visible in growing root hair cells, lateral root tip and primary root tip cells in *Arabidopsis* (Kobayashi et al. 2013), (Graças et al. 2020). The results obtained Koyama et al. suggest that calcium plays a significant role in the pectic polysaccharide network, which is the principal target of proton toxicity in plant roots, because the simultaneous delivery of calcium during treatment reduced the low-pH damage (Koyama et al. 2001). Despite the knowledge available on the effect of low pH on roots, there is comparatively little understanding of its impact on leaves.

2.5 Photooxidative stress and damage

Plants use active oxygen species as part of their alarm-signaling mechanisms. These alter gene expression and metabolism so that the plant can react to harmful environmental factors such as - invasive species, pathogens and ultraviolet light (REF). During these moments, the antioxidative defense system's capability is frequently strengthened, but if the reaction is insufficient, radical production will outpace scavenging, which will eventually disrupt metabolism.

High light causes oxidative damage mostly when it works in concert with other stressors like pollution or chilly conditions. The photooxidative processes can be altered by environmental stress in a number of ways, from direct involvement in the production of light-induced free radicals to the suppression of metabolism that causes previously optimal light levels to become excessive. In precisely these circumstances, the ability to produce active oxygen species may be greater than the capacity of the antioxidative defense systems to scavenge them (Foyer, 1994).

ROS function as signaling molecules but can also oxidize lipids in cells, causing direct harm. Sunlight energy is captured by plant leaves to power CO_2 fixation during photosynthesis. When the equilibrium between energy absorption and consumption is upset, leaves become exposed to photooxidative stress. By producing distinct ROS at various spatial levels of the cell, excess excitation energy in the photosystems (PSI and PSII) inhibits photosynthesis (Apel and Hirt, 2004; Asada, 2006; Van Breusegem and Dat, 2006). When the CO2 availability is reduced, and high light intensifies superoxide radicals (O2.) are produced at PSI. In combination with Hydrogen peroxide (H2O2) produced by superoxide dismutation, this contributes to the dissipation of extra

energy (Asada, 2006). Reduced CO2 availability influences the first step of CO2 fixation by causing the Rubisco carboxylase-oxygenase enzyme to switch from carboxylation to oxygenation, a process known as photorespiration.

Increased cellular ROS levels cause stress signaling and RCD signal transduction events when antioxidant mechanisms are overloaded (Mittler et al., 2004; Van Breusegem and Dat, 2006). On the other hand, excessive ROS accumulation harms lipids, proteins, pigments, and nucleic acids, which either causes or contributes to cell death (Halliwell and Gutteridge, 2007).

As it was mentioned previously, despite sharing similarities in the process of RCD with animals, plants' cells are distinct due to the existence of chloroplasts, a conspicuous vacuole, and the cell wall, all of which have an impact on RCD responses (Hatsugai et al., 2006; Samuilov et al., 2003). According to current understanding, chloroplasts may participate as a global communications system in many plant PCD responses by controlling reactive oxygen species (ROS), because they are an important ROS source. Many plant RCD responses, including those brought on by developmental, abiotic, and biotic stimuli, require light. Accordingly, one of the earliest and most notable cellular changes before leaf senescence is chloroplast breakdown (Chen and Dickman, 2004). Through their involvement in the production of salicylic acid, a recognized hormone and signaling molecule in plant defense responses as well as a mediator of PCD, chloroplasts have also been connected to pathogen induced PCD responses. The redox environment modification and ROS regulation might turn out to be important in plant PCD.

Experiments I conducted in my thesis indeed suggest a relationship between RCD and chloroplast breakdown upon stress by low pH.

2.6 Preliminary data

The experiments in my thesis were designed based on the preliminary data from the host research group which were conducted to evaluate a possible role of eATP as a death molecule. Initial experiments suggested *Arabidopsis* seedlings bleached after exposure to 1mM ATP for different timepoints, but not in the control condition. Furthermore, trypan blue staining confirmed induction of cell death for both timepoints (1h and 24h) of exposure to eATP. However, when these experiments were further replicated, it became clear that the bleaching and cell death observed were due to a combination of low pH (pH 4.6) and 1mM ATP (Figure 3). Therefore, this sparked the question of whether low pH and eATP can also act as inducers of cell death individually, leading to the research objectives of my MSc Thesis.

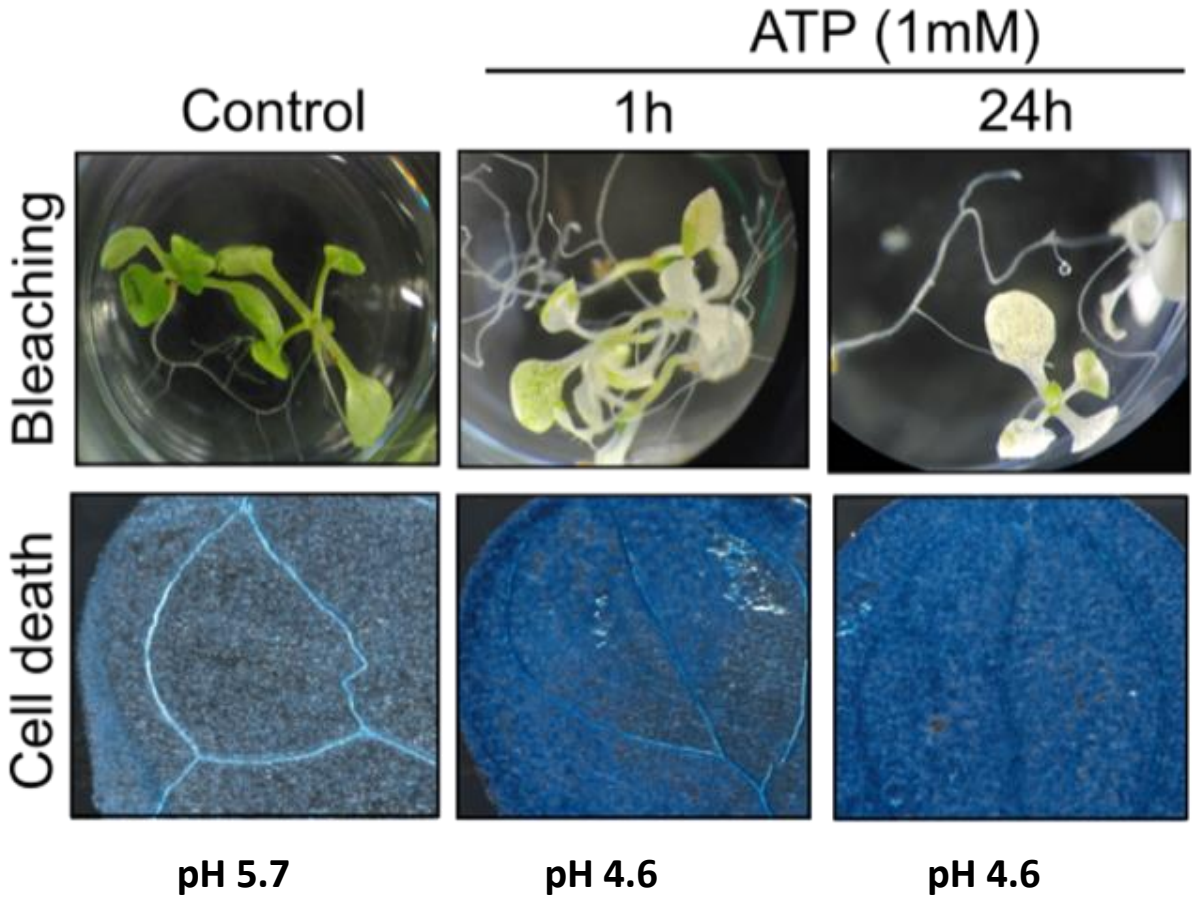

Figure 3. Exposure to eATP and low pH (4.6) lead to bleaching and cell death in leaves of Arabidopsis seedlings.

6-days old Arabidopsis seedlings were exposed to 1mM ATP in low pH (4.6) for 1h or overnight (24h). Seedlings were then washed with MQ water and stained with Trypan Blue to determine cell death. White leaves indicate bleaching, and blue indicates cell death. Control seedslings were exposed to phosphate buffer pH 5.7. Preliminary data Ana Dominguez-Ferreras (Warwick University); Lucia Gimenez-Lopez (NTNU).

2.7 General and specific objectives

The general objective of my MSc thesis is to understand the process of regulated cell death in plants and what triggers it. It is known that plants, like every living organism, need specific conditions to grow and develop. They have several mechanisms that help them remain in homeostasis. If there is a need for it, particular cells are being removed and it is a controlled event as a response to the stressor.

The specific objectives were as follows:

1- Characterize the bleaching and cell death induced in *Arabidopsis* leaves by exposure to pH 4.6

2- Evaluate the effect of extracellular ATP as a 'death molecule' in *Arabidopsis* leaves

3. Materials and methods

3.1 Plant material

Arabidopsis thaliana wild type seeds Col were available in the host research group.

3.2 Plant growth conditions

3.2.1 Tissue culture on plate (solid media)

Non-sterilized seeds, stored in a dark environment, were surface sterilized in 1 ml 70% ethanol, 1 ml 50% bleach and washed 4 times with 1 ml sterile Mili-Q (MQ) water. Sterilized seeds were spread on a plate with ½ Murashige and Skoog (MS) sucrose solid medium at pH 5.8, wrapped in aluminum foil and incubated in the fridge for 2 days. Seeds were then transferred to a growth chamber under standard conditions (150 µmol light intensity for 16 h at 22°C followed by 8 h dark at 18 °C, 50 % relative humidity) for 7 days. In this project, the standard growth conditions were used for all tissue cultures.

3.3 Buffer solutions

Sodium Acetate (NaAc) buffer

Two 1M NaAc buffers were available in the host research group: pH 5.8 and pH 4.6. For my experiments I needed 50 ml 50 mM NaAc buffer pH 4.6 and the same amount of pH 5.8. 2,5 ml of the stock solution was transferred to an empty flask and diluted with 47,5 ml sterile MQ water. Then I checked the pH and adjusted it if needed with HCl or NaOH. For some experiments I also needed 50 ml 50 mM NaAc buffer pH 5.0, so I would pipette out 2,5 ml of the base buffer 5.8, add 47,5 ml sterile MQ water and follow the same steps with checking the pH to adjust it to the correct range.

MES buffer and ATP

1M MES buffer was available in the host research group at pH 5.7. For my experiments I needed 2 x 50 ml 50 mM MES buffer, so I prepared these solutions by pipetting out 2,5 ml of the base buffers into an empty flask and then adding 47,5 ml sterile MQ water to it. I checked the pH and if needed I would adjust it to the correct range. Then to one of the solutions, I would add 25,359 mg of ATP (which was stored in the freezer) for a final concentration of 1mM. pH of the ATP solution was checked and corrected back to 5.7 if needed.

3.4 Arabidopsis treatments

Arabidopsis seedlings were exposed to low pH stress (Sodium Acetate buffer; MES buffer) or to extracellular ATP (eATP). All treatments described below were performed with 7 days old

seedlings, and incubations were done for different timepoints, as indicated for each experiment. In the following sections, the procedure for each experiment is described.

3.4.1 Low pH and ATP treatment in Erlenmeyer flask

3 solutions of 50 mM Sodium Acetate buffer (NaAc) were prepared: 40 ml pH 5.8 as a control; 40 ml pH 4.6 and 40 ml pH 4.6 with 1 mM ATP added. Two time points were set for the treatment: 60 minutes and 16 hours. Fresh plant material was divided into 6 Erlenmeyer flasks: 10 seedlings per 1 flask.

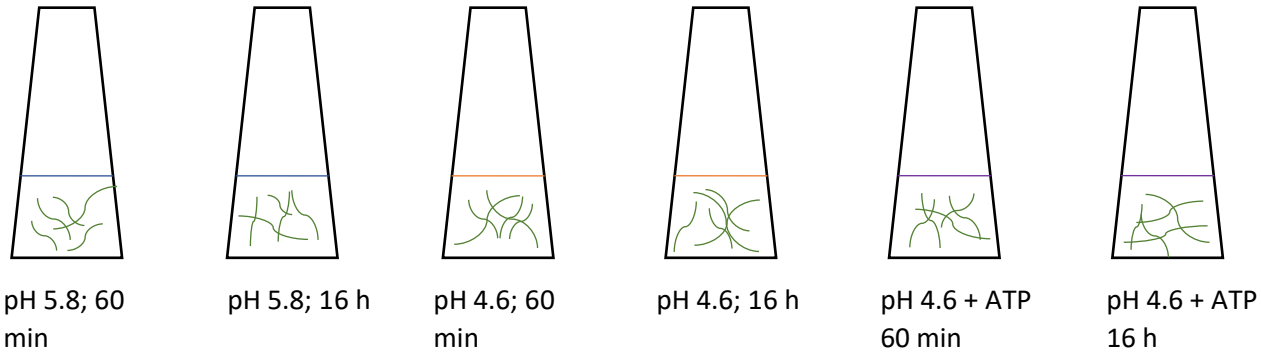

Figure 4. Experimental design to test low pH stress and eATP in *Arabidopsis* seedlings. Different treatments were carried out, as described in the figure. Two time points were used in this treatment: 60 minutes and 16 hours.

Flasks were put in the growth chamber under standard growth conditions; and left shaking for 60 minutes and overnight (16 hours), respectively. After the time of treatment passed, seedlings were washed 3 times with sterile MQ water and put back to the growth chamber in the control NaAc buffer of pH 5.8 till the next day. Pictures were taken on the next day with a Zeiss-AXIO Zoom.V16 Microscope, fluorescence stereo zoom microscope.

3.4.2 Low pH treatment in 6-well plate

50 mM NaAc buffer were prepared: 50 ml of pH 5.8; 50 ml of pH 5.0 and 50 ml of pH 4.6. Two time points were set for the treatment: 60 minutes and 16 hours. Fresh plant material was transferred to the wells containing 4 ml of each buffer. 10 seedlings were used per well.

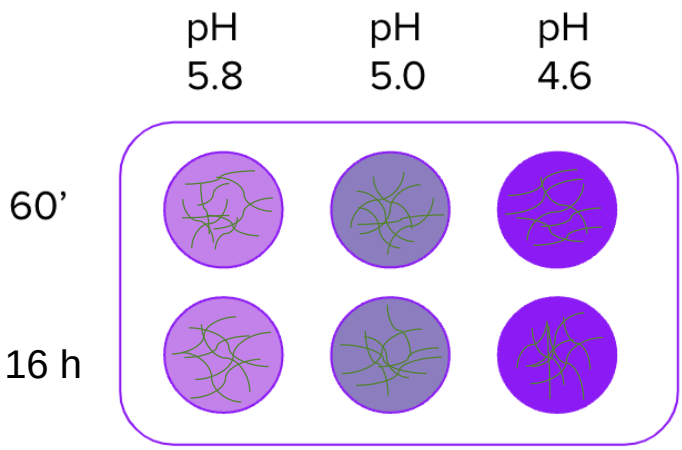

Figure 5. Experimental design to test low pH treatment. Three different pH were tested solutions of 50 mM NaAc buffer: pH 5.8 (control); pH 5.0 and pH 4.6. Two time points were used in this treatment: 60 minutes and 16 hours.

The plate was put in the growth chamber under standard conditions and left for 60 minutes and overnight (16 hours), respectively. Afterwards seedlings were washed 3 times with sterile MQ water and put back to the growth chamber in the control NaAc buffer of pH 5.8. Pictures were taken on the next day with a Zeiss-AXIO Zoom.V16 Microscope, fluorescence stereo zoom microscope.

3.4.3 ATP treatment in 6-well plate

50 mM MES (2-(N-morpholino) ethane-sulfonic acid) were prepared: 50 ml of pH 5.7; and 50 ml of pH 5.7 with 1 mM ATP added. Two time points were set for the treatment: 60 minutes and 16 hours. Fresh plant material was divided into a 4-well plate: 10 seedlings per 1 well.

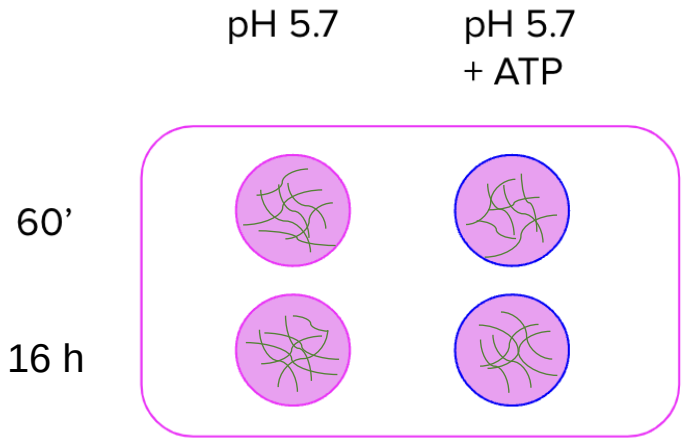

Figure 6. Experimental design to test 1 mM ATP treatment. Two solutions of MES buffer at pH 5.7 were prepared. 1 mM ATP was added to one of the solutions. Two time points were used in this treatment: 60 minutes and 16 hours.

The plate was put in the growth chamber under standard conditions and left for 60 minutes and 16 hours, respectively. Afterwards seedlings were washed 3 times with sterile MQ water and put back to the growth chamber in the control MES buffer of pH 5.7. Pictures were taken on the next day with a Zeiss-AXIO Zoom.V16 Microscope, fluorescence stereo zoom microscope.

3.4.4 Low pH treatment with aluminum foil

50 mM NaAc buffer were prepared: 50 ml of pH 5.8; 50 ml of pH 5.0 and 50 ml of pH 4.6. Two time points were set for the treatment: 60 minutes and 16 hours. Fresh plant material was divided into 2 x 6-well plate: 10 seedlings per 1 well and transported there with tweezers. One of the plates was covered with aluminum foil to prevent light exposure.

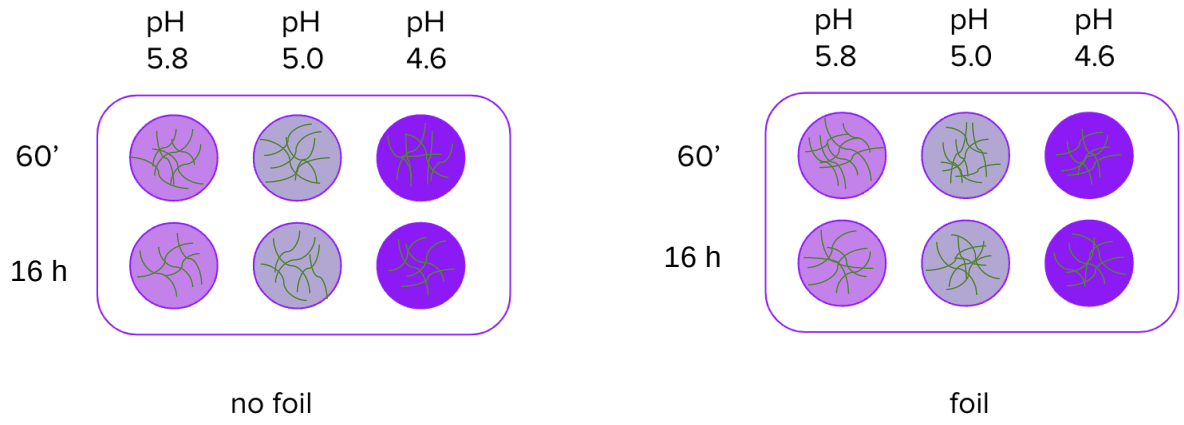

Figure 7. Experimental design to test low pH treatment. Three solutions of 50 mM NaAc buffer: pH 5.8 (control); pH 5.0 and pH 4.6. Two time points were used in this treatment: 60 minutes and 16 hours. One of the plates was covered with aluminum foil to prevent it from light exposure.

The plates were put in the growth chamber under standard conditions and left for the time of treatment for 60 minutes and 16 hours respectively. After the time of treatment passed, seedlings were washed 3 times with sterile MQ water and put back to the growth chamber in the control NaAc buffer of pH 5.8. Pictures were taken on the next day with a Zeiss-AXIO Zoom.V16 Microscope, fluorescence stereo zoom microscope.

3.4.5 MES buffer low pH treatment with aluminum foil

50 mM MES were prepared: 50 ml of pH 5.7; 50 ml of pH 5.0 and 50 ml of pH 4.6. Two time points were set for the treatment: 60 minutes and 16 hours. Fresh plant material was divided into 2 x 6-well plate: 10 seedlings per 1 well and transported there with tweezers. One of the plates was covered with aluminum foil to prevent it from light exposure.

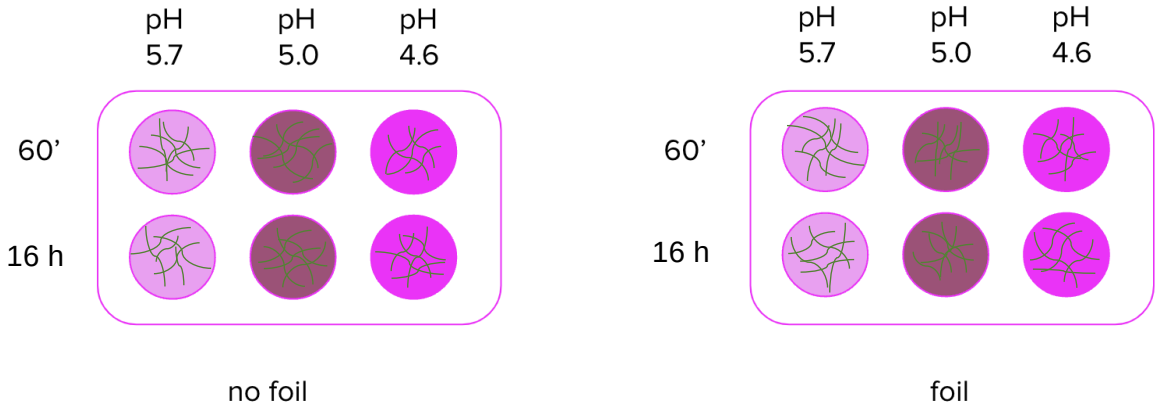

Figure 8. Experimental design to test low pH treatment. Three solutions of 50 mM MES buffer: pH 5.7 (control); pH 5.0 and pH 4.6. Two time points were used in this treatment: 60 minutes and 16 hours. One of the plates was covered with aluminum foil to prevent it from light exposure.

The plates were put in the growth chamber under standard conditions and left for the time of treatment for 60 minutes and 16 hours respectively. After the time of treatment passed, seedlings were washed 3 times with sterile MQ water and put back to the growth chamber in the control MES buffer of pH 5.7. Pictures were taken on the next day with a Zeiss-AXIO Zoom.V16 Microscope, fluorescence stereo zoom microscope.

3.4.6 ATP treatment in different time points

50 mM MES were prepared: 50 ml of pH 5.7; and 50 ml of pH 5.7 with 1 mM ATP added. Three time points were set for the treatment: 30 minutes, 60 minutes, and 16 hours. Fresh plant material was divided into 6-well plates: 10 seedlings per 1 well and transported there with tweezers.

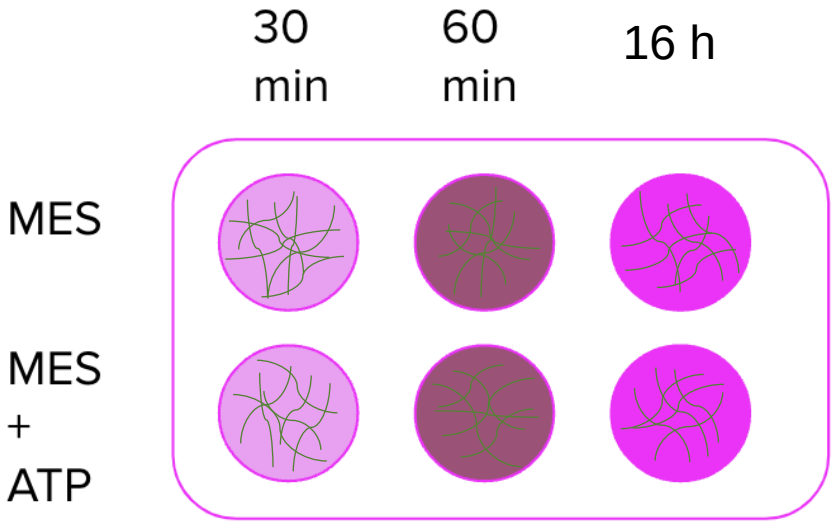

Figure 9. Experimental design to test 1 mM ATP treatment. Two solutions of MES buffer at pH 5.7 were prepared. 1 mM ATP was added to one of the solutions. Three time points were used in this treatment: 30 minutes, 60 minutes and 16 hours.

The plates were put in the growth chamber under standard conditions and left for the time of treatment for 30 minutes, 60 minutes and 16 hours respectively. After the time of treatment passed, seedlings were washed 3 times with sterile MQ water and put back to the growth chamber in the control MES buffer of pH 5.7. Pictures were taken on the next day with a Zeiss-AXIO Zoom.V16 Microscope, fluorescence stereo zoom microscope.

3.4.7 ATP treatment in different time points

50 mM MES were prepared: 50 ml of pH 5.7; and 50 ml of pH 5.7 with 1 mM ATP added. Three time points were set for the treatment: 15 minutes, 30 minutes, and 60 minutes. Fresh plant material was divided into 6-well plates: 10 seedlings per 1 well and transported there with tweezers.

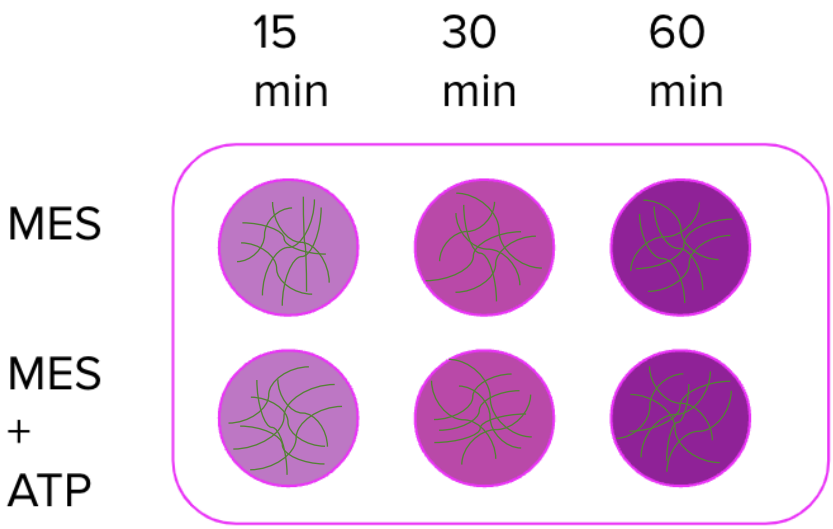

Figure 10. Experimental design to test 1 mM ATP treatment. Two solutions of MES buffer at pH 5.7 were prepared. 1 mM ATP was added to one of the solutions. Three time points were used in this treatment: 15 minutes, 30 minutes and 60 minutes.

The plates were put in the growth chamber under standard conditions and left for the time of treatment for 15 minutes, 30 minutes and 60 minutes respectively. After the time of treatment passed, seedlings were washed 3 times with sterile MQ water and put back to the growth chamber in the control MES buffer of pH 5.7. Pictures were taken on the next day with a Zeiss-AXIO Zoom.V16 Microscope, fluorescence stereo zoom microscope.

3.4.8 Low pH treatment in different time points

50 mM NaAc buffer were prepared: 50 ml of pH 5.8; 50 ml of pH 5.0 and 50 ml of pH 4.6. Three time points were set for the treatment: 15 minutes, 30 minutes, and 60 minutes. Fresh plant material was divided into 2 x 6-well plate: 10 seedlings per 1 well and transported there with tweezers. One of the plates was covered with aluminum foil to prevent it from light exposure.

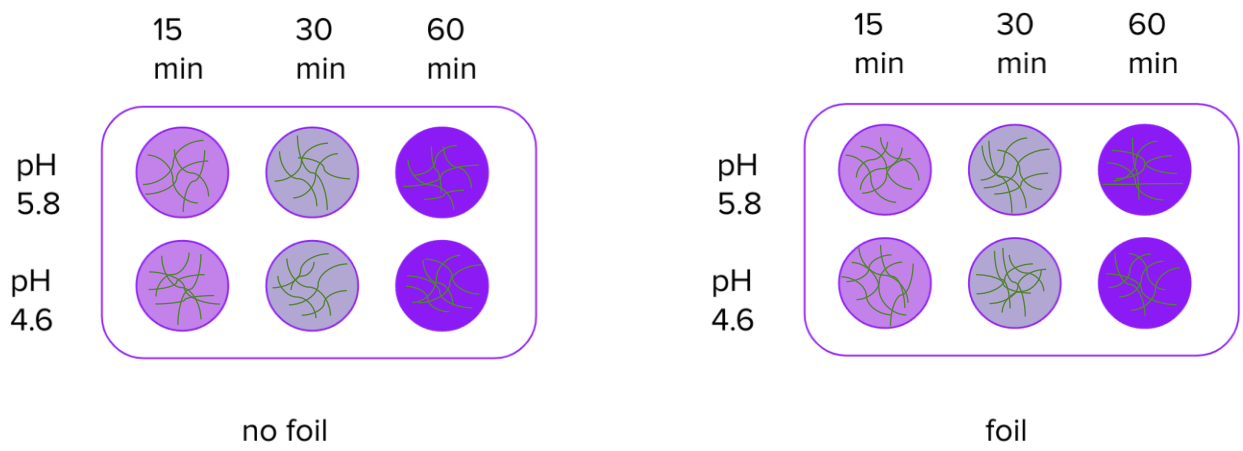

Figure 11. Experimental design to test low pH treatment. Two solutions of 50 mM NaAc buffer: pH 5.8 (control) and pH 4.6. Three time points were used in this treatment: 15 minutes, 30 minutes and 60 minutes. One of the plates was covered with aluminum foil to prevent it from light exposure.

The plates were put in the growth chamber under standard conditions and left for the time of treatment for 15 minutes, 30 minutes and 60 minutes respectively. After the time of treatment passed, seedlings were washed 3 times with sterile MQ water and put back to the growth chamber in the control NaAc buffer of pH 5.8. Pictures were taken on the next day with a Zeiss-AXIO Zoom.V16 Microscope, fluorescence stereo zoom microscope.

3.5 Cell Death Staining

3.5.1 Evans Blue solution

Evans Blue staining

Collected plant tissues were transferred to 2 ml Eppendorf tubes and 2 ml of Evan's blue solution was added to each tube. The tubes were shaked in an orbital shaker at 50 oscillations/min per 20 minutes in order for the tissue to have contact with the dye. Leaves and roots were washed 3 times to unbound dye washed from the surface. They were later observed under the transmitted light microscope. Photographs of them were taken under the brightfield microscope. A detailed protocol and recipe are provided in the supplementary data.

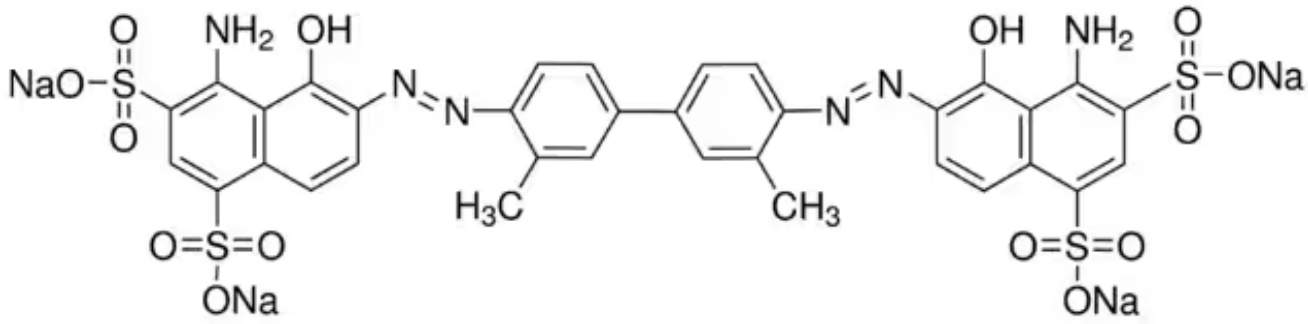

Figure 12. Evan's blue chemical formula (Saiah et al. 2008)

3.5.2 Trypan Blue solution

Trypan blue staining

Collected plant tissues were transferred to 2 ml Eppendorf tubes with Safe lock and 1 ml of Trypan blue solution was added to each tube. There were 30 tubes in total – 5 plants for each treatment. Negative (MS medium, room temperature for 10 min) and positive (MS medium, 55°C for 10 min) controls were appended. They were left in the heatblock for 1 minute at 99°C and then left to cool down for an hour. After that, trypan blue solution was pipetted out of the tubes and the clearing solution (Chloral Hydrate) was pipetted in and left for 4 hours on laboratory rocker. The clearing was repeated 2 times and then the tubes were filled with 1 ml of 50% glycerol solution. Photographs were taken under the brightfield microscope.

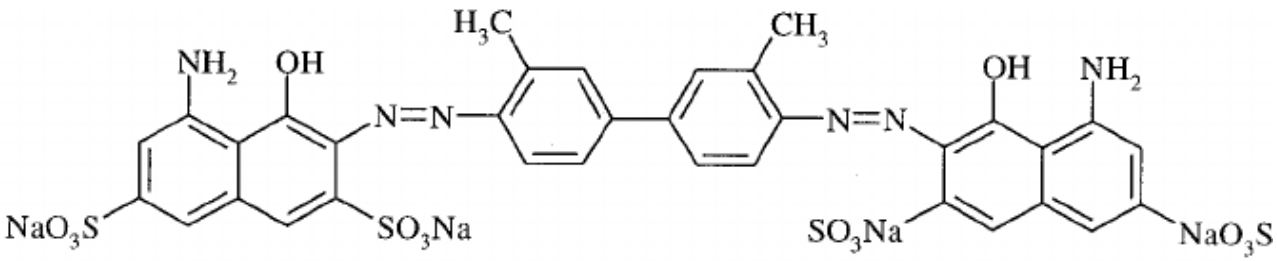

Figure 13. Trypan blue chemical formula (Sarma et al. 2000).

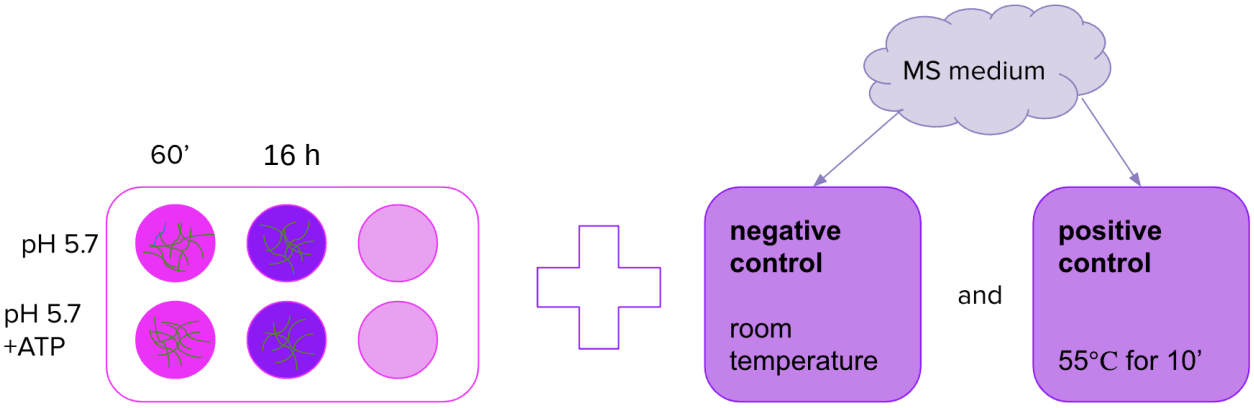

Figure 14. Experimental design to test 1 mM ATP treatment. Two solutions of MES buffer at pH 5.7 were prepared. Two time points were used in this treatment: 60 minutes and 16 hours. Two controls were appended: negative (MS medium, room temperature for 10 min) and positive (MS medium, 55°C for 10 min) control. There were 30 tubes prepared for Trypan blue staining – 5 plants for each treatment.

4. Results

Preliminary results obtained by the Cell Death and Communication Group suggested that a combined treatment of 1mM ATP and pH of 4.6 induced bleaching and cell death in *Arabidopsis* seedlings (Figure 3 in Background). In order to determine whether this effect is due to a combined stress or induced by eATP or low pH individually, I treated *Arabidopsis* plants with low pH and eATP independently. The results for low pH stress and eATP are presented in the following sections.

4.1 Low pH induces light-dependent bleaching in *Arabidopsis*

In the beginning, the treatments were performed in Erlenmeyer flasks. However due to the mechanical damage done to the tissue by moving the seedlings constantly, I decided to switch to the multi-well plates.

To evaluate the effect of low pH on *Arabidopsis*, 7-days old seedlings were treated with two different buffers: Sodium acetate (NaAc) 50mM and MES 50mM. For both buffers control was designated as pH 5.8 and low pH corresponded to pH 5.0 and pH 4.6. Plants were exposed to the treatments for 1 hour or 16 hours in the presence of light, washed with MQ water and afterwards remained in the growth chamber in control buffer (pH 5.8). Results were evaluated the next day. 10 seedlings were used per treatment, and the experiment was repeated 5 times with similar results. Results of one replicate are shown in Figure 15 and the one replicate is shown in Supplementary data (Figure 24)

Seedlings treated with NaAc buffer were bleached at pH of 5.0 and 4.6, even for short exposure times (1 hour) (Figure 15). Furthermore, a gradient of bleaching was visible – the lower the pH, the more bleached the cotelydons and primary leaves were. This indicates that the effect of low pH is dose-dependent within the tested acidity levels. Therefore, the impact on chlorophyll stability is likely fast and irreversible. I also observed that when the plates were left outside of the growth chamber, even control seedlings bleached after a few days. This suggests that chlorophyll degradation occurs also during aging and can be accelerated upon exposure to low pH in the presence of light.

I then investigated whether the bleaching observed upon low pH treatment is light-dependent. To test this, two 6-well plates were prepared with NaAc buffer, identical as previously, but one of the plates was covered with tin foil during incubation in the growth chamber. This way, the tin foil prevented the samples from light exposure. The results showed that indeed, the bleaching process was blocked when the plants were covered with foil, which suggests photosynthesis-related ROS (Figure 15).

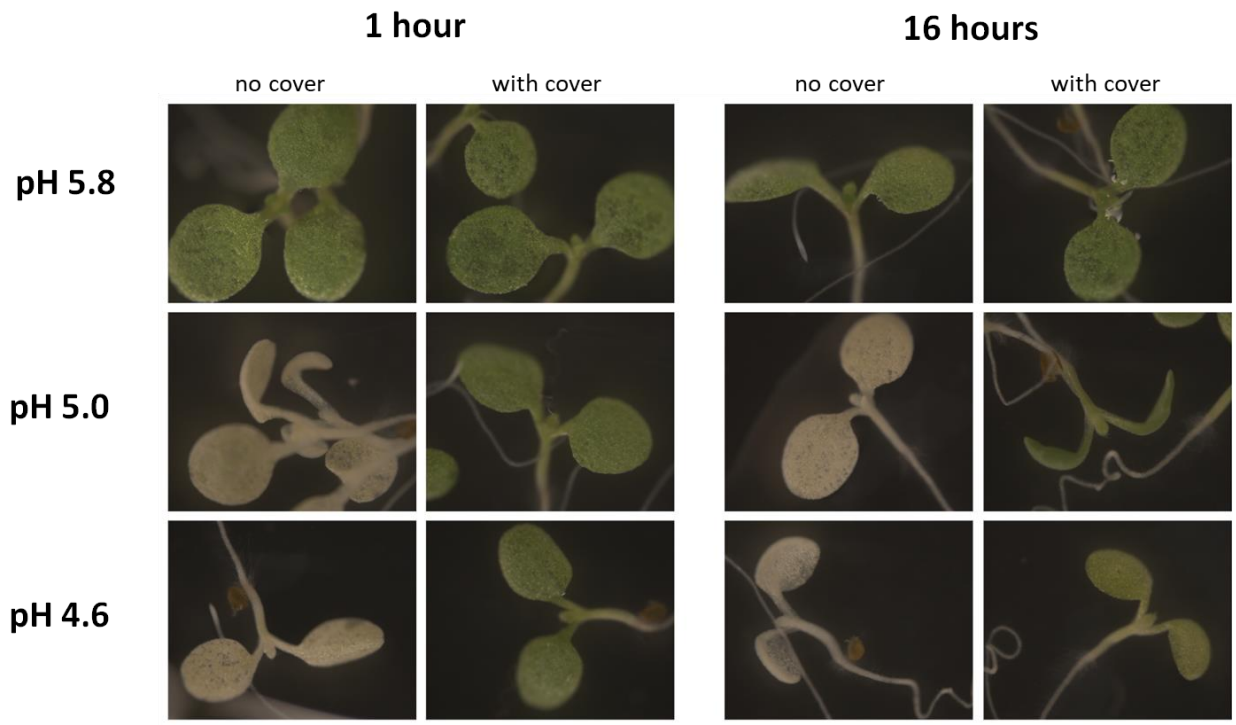

Figure 15. Low pH (4.6) induces light-dependent bleaching in *Arabidopsis* seedlings

7-days old *Arabidopsis* seedlings were exposed to three solutions of 50 mM NaAc buffer: pH 5.8 (control); pH 5.0 and pH 4.6. Two time points were used in this treatment: 60 minutes and 16 hours. One of the plates was covered with aluminum foil to prevent it from light exposure. In the case of treatment without aluminum foil cover bleaching is visible at pH 5.0 and 4.6, in both time points.

After confirming the reproducibility of the results (see Supp. Fig. 24), I decided to shorten the time of exposure to low pH to 15 and 30 minutes to identify the shortest exposure time that leads to bleaching. In this case, 1 hour treatment was kept as a control. The results obtained show that even in the 15 minutes of exposure to pH 4.6 the damage was already visible. Furthermore, bleaching was suppressed in the absence of light for all timepoints.

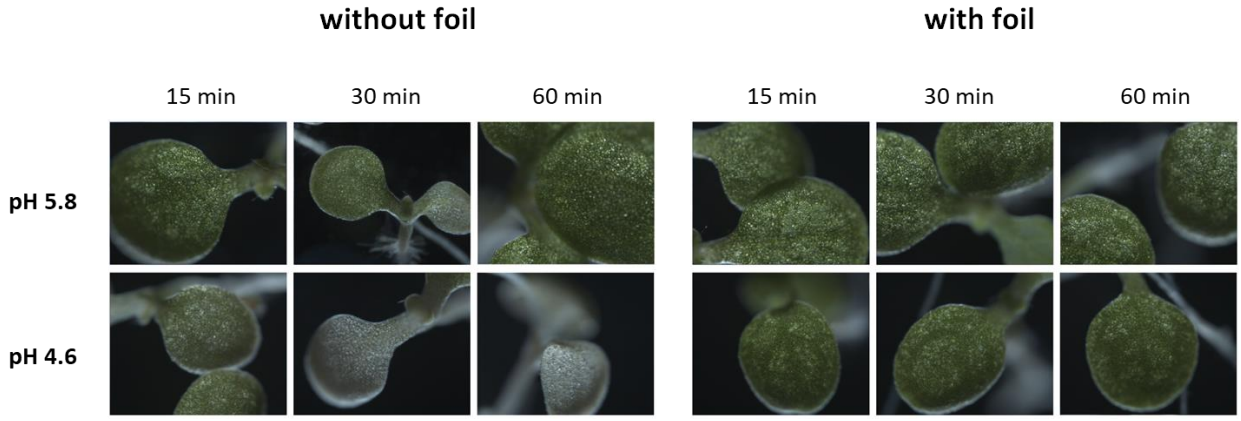

Figure 16. Short timepoints (15 minutes) at pH 4.6 induce light-dependent bleaching in *Arabidopsis* seedlings.

7-days old *Arabidopsis* seedlings were exposed to 50 mM NaAc buffer: pH 5.8 (control) and 50mM pH 4.6 for three different timepoints (15, 30 and 60 minutes). One of the plates was covered with aluminum foil to prevent it from light exposure. In the case of treatment without aluminum foil cover bleaching is visible at 4.6, in all time points.

4.2 Low pH-induced bleaching is not observed with MES buffer

I next wanted to test whether the bleaching observed upon low pH exposure is a general response to any buffer solution. Therefore, I exposed *Arabidopsis* seedlings to pH 4.6 in a MES buffer. Interestingly, in this case, seedlings remained green at all tested pH and timepoints, and no gradient was observed as for the NaAc treatment (Figure 17). This suggests that the bleaching observed with NaAc at pH 4.6 and 5.0 cannot be attributed only to the low pH itself. Furthermore, the absence of light had no impact either.

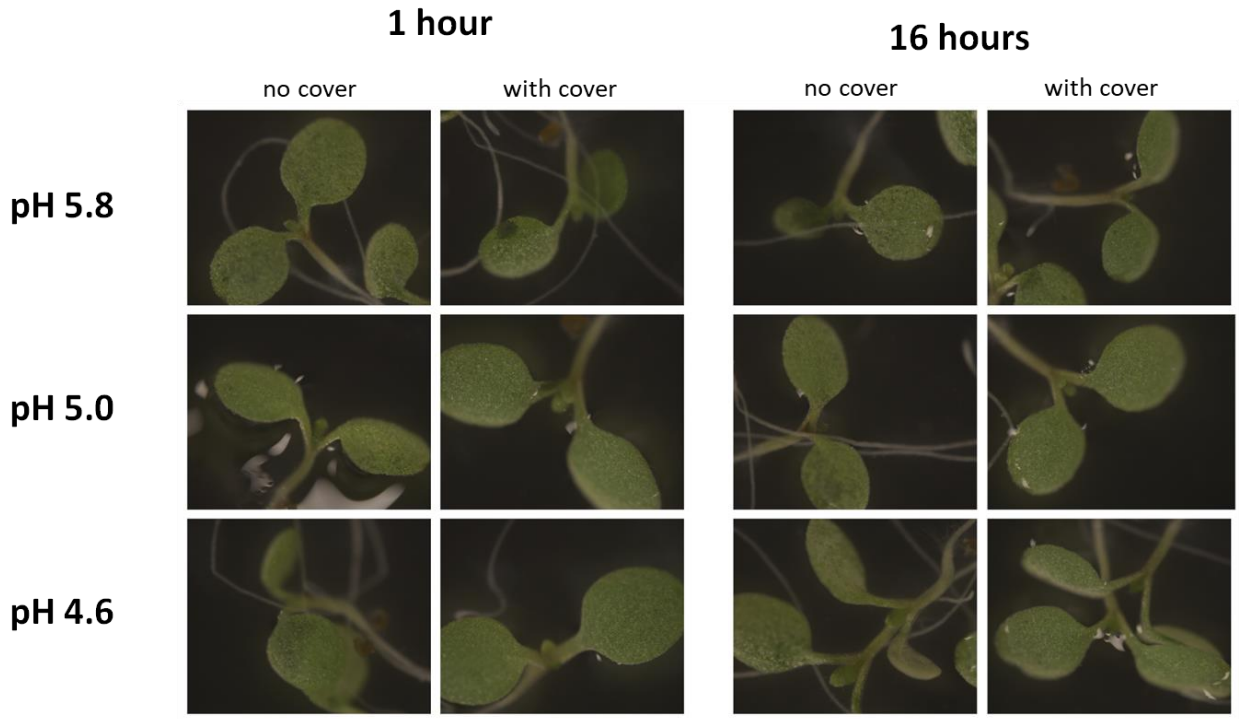

Figure 17. Low pH (4.6) in MES buffer does not induce light-dependent bleaching in *Arabidopsis* seedlings

7-days old *Arabidopsis* seedlings were exposed to three solutions of 50 mM MES buffer: pH 5.8 (control); pH 5.0 and pH 4.6. Two time points were used in this treatment: 60 minutes and 16 hours. One of the plates was covered with aluminum foil to prevent it from light exposure.

In the experiments described above, plants are washed with MQ water after exposure to pH 4.6 or 5.8. This change from 50 mM buffer to MQ could induce an osmotic shock and partly be responsible for the observed phenotype. To address this, I decided to wash the seedlings with 50 mM NaAc pH 5.8 (the control buffer). As seen in Figure 18 this had no impact on the results. Therefore, I can conclude that the bleaching response was not connected with the osmotic shock and is indeed linked to the low pH plants are exposed to.

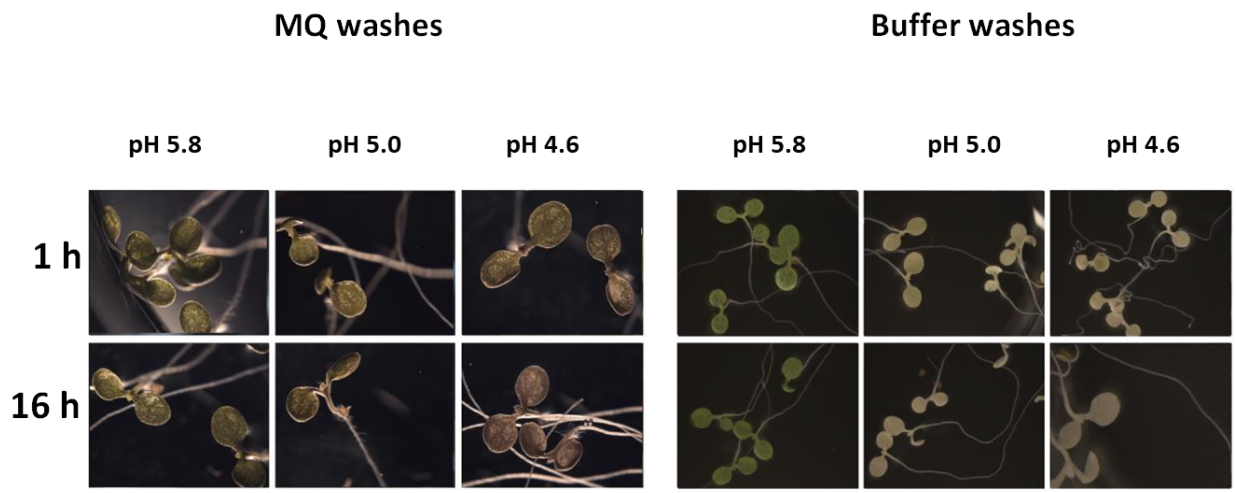

Figure 18. Osmotic show does not induce bleaching in *Arabidopsis* seedlings

7-days old *Arabidopsis* seedlings were exposed to three solutions of 50 mM NaAc buffer: pH 5.8 (control); pH 5.0 and pH 4.6 and then washed with 50mM NaAc pH 5.8. Two time points were used in this treatment: 60 minutes and 16 hours.

4.3 Low pH induces cell death in *Arabidopsis* seedlings

Evans Blue Staining

Exposure to pH 4.6 in a NaAc buffer leads to bleaching of *Arabidopsis* seedlings. I then wanted to assess whether this is linked to the activation of cell death. Evans blue (T-1824) dye is characterized by its high-water solubility and ability to stain dead or damaged cells as it does not enter live cells. I used EB to evaluate if the bleaching observed with low pH in NaAc buffer is associated with cell death. Plants were treated as described above (section 4.1 from the results) and stained with EB to evaluate cell death. I only observed staining in the roots, and no staining was observed in the cotyledons, suggesting a problem with the staining (Figure19). Due to unsatisfying results obtained with this dye, I decided to switch to a similar dye – Trypan blue.

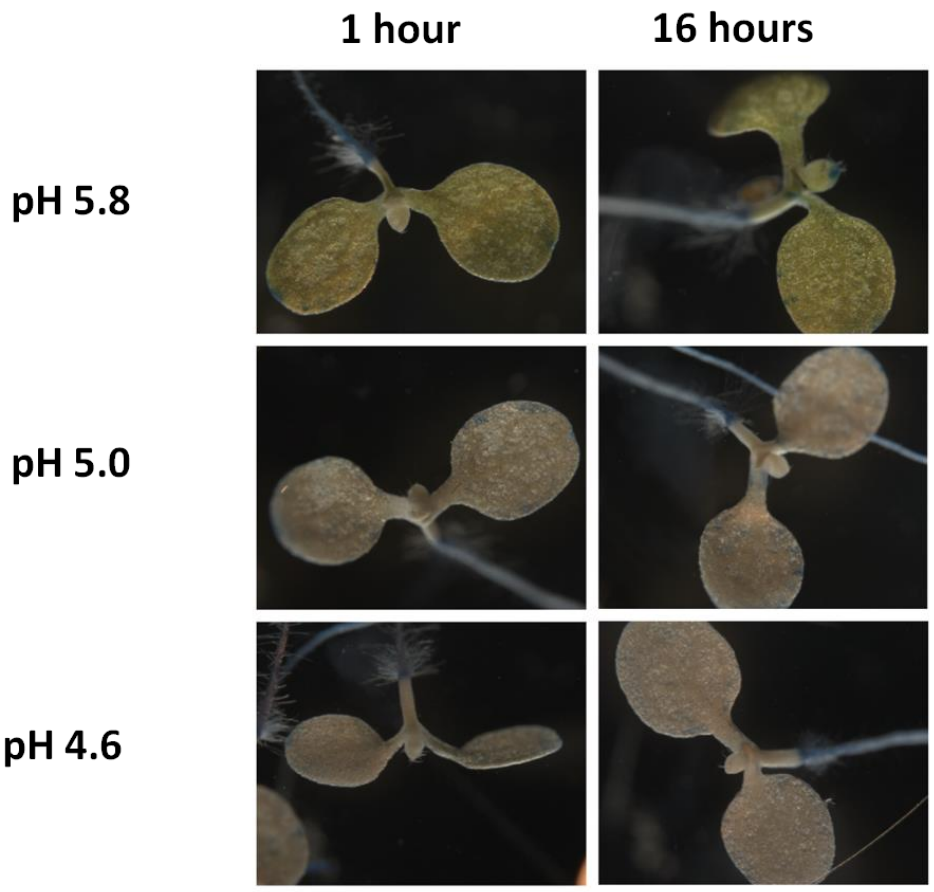

Figure 19. Evans Blue staining of *Arabidopsis* seedlings exposed to low pH (4.6)

7-days old *Arabidopsis* seedlings were exposed to three solutions of 50 mM NaAc buffer: pH 5.8 (control) and pH 4.6. Two time points were used in this treatment: 1 and 16 hours. After, seedlings were stained with Evans Blue to evaluate cell death.

Trypan blue staining

Trypan blue selectively dyes dead tissues or cells blue. In a dead cell, trypan blue passes through the cell membrane and enters the cytoplasm. Live cells have an intact cell membrane and therefore trypan blue cannot penetrate the cell membrane of live cells and enter the cytoplasm (Hunger et al. 2005).

To establish a Trypan Blue staining protocol in the lab, I used heat shock as a positive control for cell death. *Arabidopsis* seedlings exposed to 55°C for 10 minutes undergo RCD, which can be detected by staining with Sytox Green, a dye that penetrates dead cells and stains DNA (Distefano et al. 2017) and with Trypan Blue (Figure 20). After exposure to heat shock, the seedlings were stained blue in both the cotyledons and primary root, indicating that cell death indeed occurred in the tissue (Figure 20, controls).

To evaluate cell death upon exposure to low pH, I treated *Arabidopsis* seedlings with NaAc buffer pH 5.8 and 4.6 as described before and then stained them with Trypan Blue. After the staining,

seedlings exposed to low pH were blue, indicating that NaAc pH 4.6 induces both bleaching and cell death. Overall, my results indicate that exposure to a pH of 4.6 in NaAc buffer induces cell death and light-dependent bleaching of cotyledons in *Arabidopsis* seedlings.

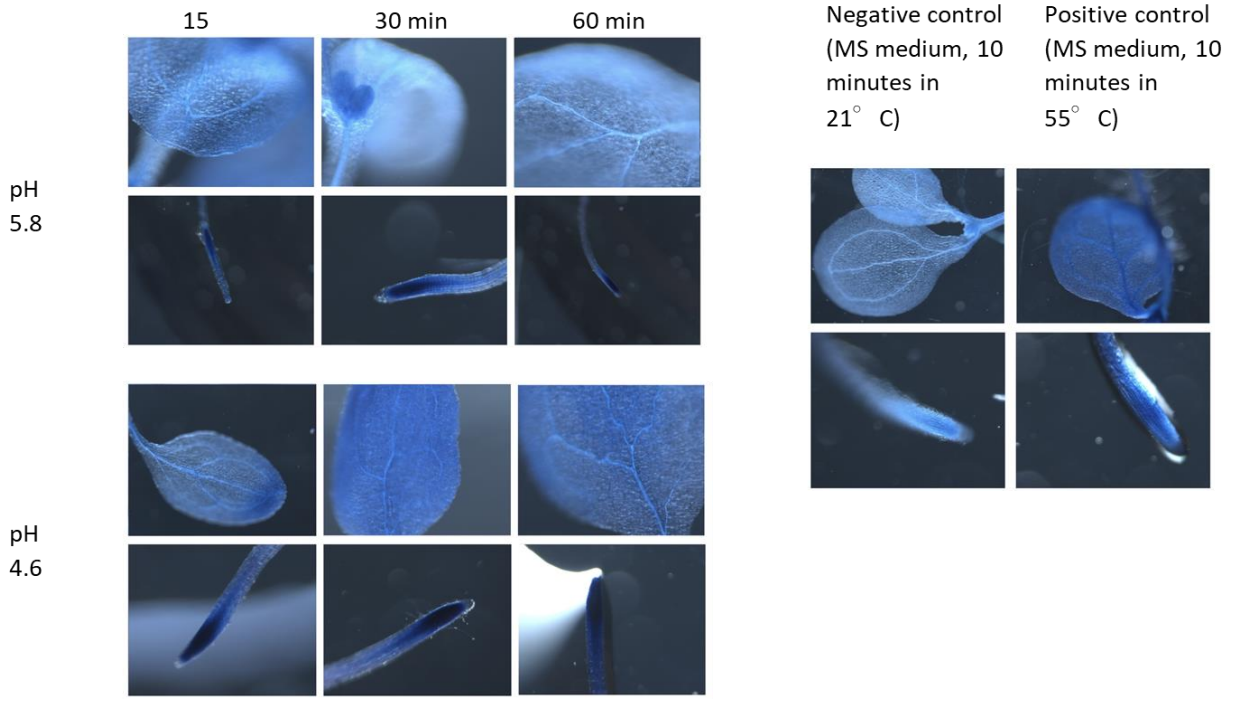

Figure 20. Low pH (4.6) induces cell death in *Arabidopsis* seedlings

7-days old *Arabidopsis* seedlings were exposed to three solutions of 50 mM NaAc buffer pH 5.8 (control) and pH 4.6. for 15, 30 and 60 minutes. As a positive control for cell death, seedlings were treated for 10 minutes at 55C to induce heat shock. Afterwards, seedlings were stained with Trypan Blue, Dark blue indicates cell death, lighter blue (as in control at room temperature) represents background.

4.4 eATP as a death molecule in *Arabidopsis*

My original aim was to evaluate whether low pH and eATP can induce bleaching and cell death individually. As shown before, exposure to low pH indeed induces cell death in Arabidopsis (Figure 20). Therefore, I next tested whether eATP has a similar effect in Arabidopsis seedlings. Seedlings were exposed to 1mM ATP in a MES buffer at 5.7. Control seedlings were exposed to MES buffer pH 5.7 only. As seen on Figure 21 treatment with eATP did not cause bleaching in *Arabidopsis* seedlings at any timepoint tested. This suggests that the bleaching reported in the preliminary data (Figure 3 of Background) was probably due to the low pH rather than due to the eATP treatment.

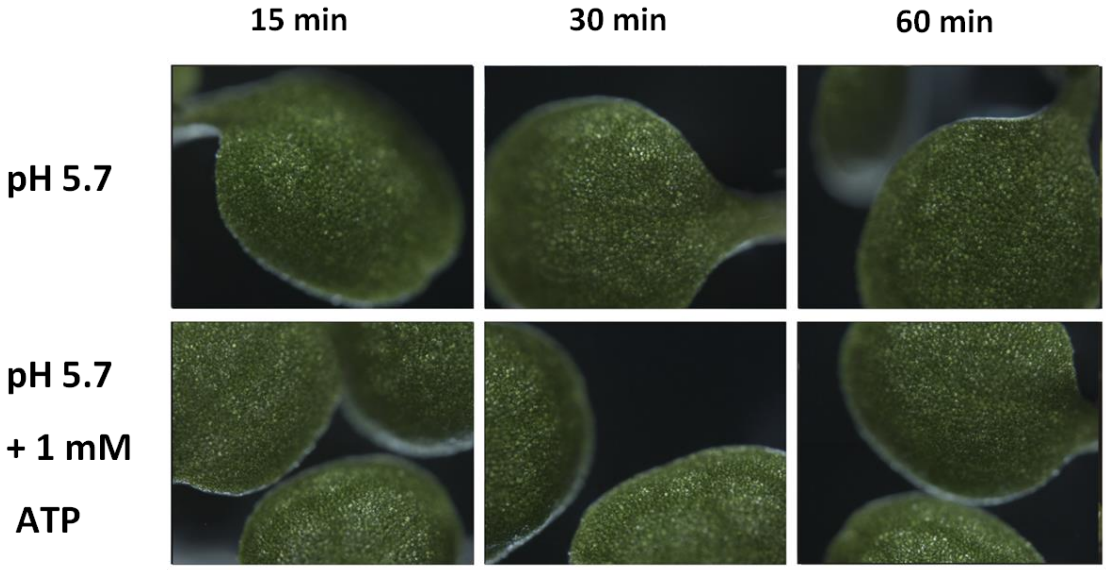

Figure 21 eATP as a death molecule in *Arabidopsis*

7-days old *Arabidopsis* seedlings were exposed to two solutions of 50 mM MES buffer: pH 5.7 (control) and pH 5.7 with addition of 1 mM ATP. Two time points were used in this treatment: 1 and 16 hours.

Next, I tested if eATP can induce cell death. For this, I exposed *Arabidopsis* seedlings to eATP as explained before and then stained them with Trypan Blue. Interestingly, I observed that exposure to 1mM ATP leads to localized cell death at short times of incubation (1 hour) but not at prolonged exposure (16 hours) (Figure 22). This result was repeated 5 times and one repetition is presented in Supplementary Figure 27. These results indicate that ATP induces cell death, but in a shorter version of treatment (1 h).

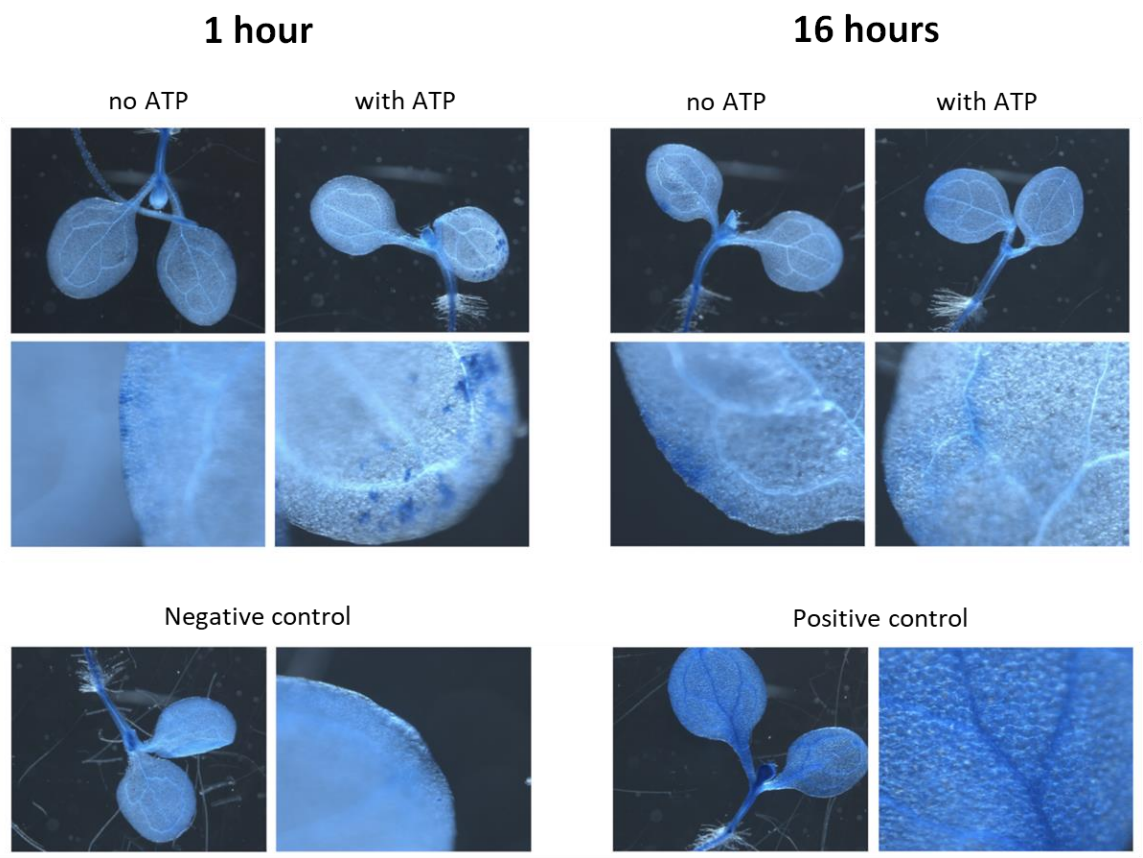

Figure 22. eATP as a death molecule in *Arabidopsis*

7-days old *Arabidopsis* seedlings were exposed to two solutions of 50 mM MES buffer pH 5.7 (control) and pH 5.7 with addition of 1 mM ATP. Two time points were used in this treatment: 1 and 16 hours. As a positive control for cell death, seedlings were treated for 10 minutes at 55C to induce heat shock. Afterwards, seedlings were stained with Trypan Blue, Dark blue indicates cell death, lighter blue (as in control at room temperature) represents background.

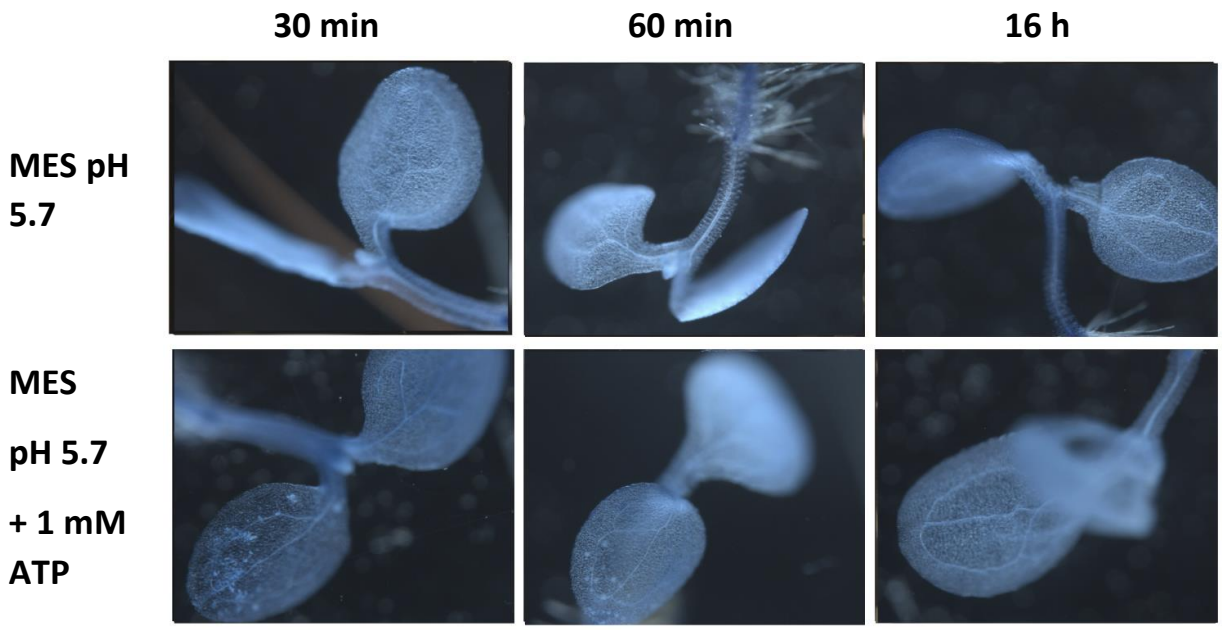

Figure 23. ATP as a death molecule in *Arabidopsis*

7-days old *Arabidopsis* seedlings were exposed to two solutions of 50 mM MES buffer pH 5.7 (control) and pH 5.7 with addition of 1 mM ATP. Two time points were used in this treatment: 30 minutes, 60 minutes and 16 hours. As a positive control for cell death, seedlings were treated for 10 minutes at 55C to induce heat shock. Afterwards, seedlings were stained with Trypan Blue, Dark blue indicates cell death, lighter blue (as in control at room temperature) represents background.

5. Discussion

The main aim of this thesis was to gain knowledge on the process of regulated cell death in *Arabidopsis* and its triggers. *Arabidopsis* seedlings were treated with low pH and eATP separately to address the effect of exposure to low pH (pH 4.6), as well as the potential role of eATP as a cell death inducing molecule in plants.

The bleaching response might be a sign of visible senescence, what Weaver et al. described in their report as leaf yellowing. They believed that senescence brought on by stress has many biological characteristics with senescence brought on by aging (Weaver et al. 1998). Wi et al. examined the types of responses to the different stresses (salt stress, cold stress, acidic stress, and abscisic acid treatment) in wild-type and transgenic plants with sense SAMDC (S-adenosylmethionine decarboxylase) cDNA. SAMDC is a crucial enzyme in polyamines (PAs) biosynthesis. PAs are found in all forms of life and are involved in many different cellular physiological functions. Conclusion of this research was that transgenic plants had a bigger tolerance to abiotic stresses than wild-type plants, which suggests the possibility of PAs' role in the plant's response to the trigger (Wi et al. 2006). Further investigation of bleaching in case of this thesis could contain using transgenic *Arabidopsis thaliana* seedlings with sense SAMDC cDNA in comparison to the wild-type ones to test suggested higher tolerance to stress.

Buffer and pH-dependent bleaching in *Arabidopsis*

To evaluate the impact of low pH stress in cell death induction in *Arabidopsis*, I exposed seedlings to two different buffers: NaAc and MES. Outcome from treatment with NaAc pH 4.6 shows that even a short period of time (15 minutes) to this pH results in bleaching of the cotyledons and primary leaves in a pH-dependent manner (Figure 16). Koyama et al. reported that low pH reduces cell viability in roots (Koyama et al. 2001) and pH 5 has also been associated with chlorophyll degradation in *Sargassum*, a macroalgae species (Wahyuningtyas et al. 2019).

Further experiments revealed that bleaching is light dependent (Fig 15,16), as it was suppressed when the *Arabidopsis* seedlings were exposed to low pH in the absence of light. This suggests photooxidative damage might be involved.

Low pH treatment with MES buffer however, showed no sign of bleaching at any given pH level as seedlings remained green. Therefore, bleaching is caused not only by specific buffer's low pH impact but rather by its combination with the high light and suggests photosynthesis related ROS. To test this hypothesis, I could treat the seedlings with a molecule that protects them from photooxidative stress, such as melatonin, glutathione or vitamin E. Melatonin (MT) for instance a plant hormone that has great capabilities to enhance plant performance under various abiotic stressors. MT could be promising in terms of protection from high light due to its improvement of physiological and molecular procedures (Hassan et al. 2022). Glutathione (GSH) contributes to cellular defense against oxidative aggression, redox control of protein thiols, and maintenance of redox homeostasis, all of which are essential for the efficient operation of cellular functions (Circu and Aw, 2012). Therefore, it could potentially intensify plant defense from high light.

My research adds new information about low pH in context of cell death. This discovery is relevant considering acid soils, since a decrease of yield crop is observed in these circumstances. About 30% of the world's ice-free terrain is covered in acid soil (Uexküll; Mutert 1995). Scientists are trying to develop new ways for agriculture and agroforestry on acidic soils to gain suitable amounts of harvest without extensive damage for the plants (Uexküll; Mutert 1995). Knowing now that combination of low pH and high intensity light, new sowing protocols could be launched which would say how to protect the plants from the cell death response in these adverse conditions.

eATP as a death-inducing molecule in *Arabidopsis*

I found that short exposure to eATP can induce localized cell death in *Arabidopsis* (Figure 22), but not in longer timepoints.

To determine whether eATP can induce cell death *Arabidopsis* seedlings were incubated in 1mM ATP for 1hr or 16hr, and then stained with TB to evaluate cell death. I observed that eATP can induce cell death at shorter timepoints of incubation. These results are in line with what has been previously described for *Populus euphratica,*-where incubation with eATP lead to cell death. (Sun et al., 2012). However, this research does not mention the exact time of the treatment and therefore should be explored further.

Extracellular nucleotides are hydrolyzed by apyrases (NTPDases), which prevents their buildup in the extracellular matrix (ECM) (Clark; Roux 2011). Depending on the tissue and the surrounding circumstances, these ectonucleotidases' relative significance in regulating eATP may vary. *Arabidopsis* has two apyrases (APY1 and APY2), and the locations where the plant is actively developing and producing ATP are where they are most expressed (Wu et al. 2007). Light that quickly increases guard cell growth and ATP release also quickly increases APY1 and APY2 expression (Clark et al. 2011). These and other findings point to a close connection between rising eATP and rising APY1 and APY2 expression (Clark; Roux 2011). These findings suggest higher activity of apyrases during longer exogenous ATP and could explain my discovery that this molecule seems to damage the plant tissue, but in a shorter version of treatment (1 hour).

Furthermore, Chivasa et al. described in their report that plant cell viability must be maintained by extracellular ATP. Both cell cultures and entire plants died when eATP was eliminated by the enzymes glucose-hexokinase and apyrase, which are cell-impermeable traps (Chivasa et al. 2005). They suggested that plants' natural death pathway is inhibited by extracellular ATP, while several pathogen-induced cell death processes involve the depletion of extracellular ATP (Chivasa et al. 2005).

Research limitations

Every research has its limitations including this Master Thesis. The main limitation in this case was time. The question is extensive and since it is experimental research, everything had to be double checked to make sure the way of thinking and designing experiments was correct. Having more time, I could do extra treatments such as protecting the seedlings from photooxidative stress using melatonin, for instance. Strong aspect of experiments performed for this thesis is the fact that I have been working with a species that is a model organism. *Arabidopsis thaliana* is a well examined plant, which was very helpful considering researching a topic that we have so limited information about.

Future research

Future direction of this research would contain protection from photooxidative stress with melatonin, glutathione, or vitamin E. Wahyuningtyas et al. observed reduced content of chlorophyll-α in *Sargassum sp. thallus* after treating it with pH 5.0 and noticing bleaching (Wahyuningtyas et al.2019) so it would be beneficial to also measure the chlorophyll content in *Arabidopsis* leaves after NaAc buffer treatment. Common method of measuring cell death is measuring electrolyte leakage which can proxy the degree of cell death (Hatsugai and Katagiri, 2018). In the case of experiments for this thesis, measurement the electrolyte leakage would be specifically for eATP. Testing Ca spiking upon cell death could supply more information about activity of eATP and Ca2+ level rise caused by it. Another thing worth testing considering the relationship between eATP and Ca2+ is glutamate receptor KO mutants. Glutamate receptor (GLR)-like channels, which are calcium permeable channels involved in stomatal control, wound signaling, and root and pollen tube formation, are plant counterparts of glutamate receptors found in vertebrate synapses (Alfieri et al. 2019). What would happen if these receptors were genetically modified?

All in all, working on this Master Thesis was quite an exciting experience. I have gained a lot of knowledge and I hope my work will be continued which will lead to new discoveries in plant cell death topic.

6. Supplementary data

Evans blue staining solution:

Dissolve 0,25 g of Evans blue dye in 100 ml of 0,1 M Ca solution at pH 5.6 and mix well until it is dissolved. Evans blue solution should be prepared freshly each time.

M Ca of pH 5.6

To prepare 0,1 M Ca, dissolve 1,10 g of Ca (Molecular Weight = 110,989) in distilled water (1 L) and adjust pH to 5.6 using HCl. Ca solution can be stored for a month in RT.

Procedure:

1) Transfer the collected leaf and root tissue to 2 ml Eppendorf tubes and add 2 ml of Evans blue solution to each tube. Note: Adjust the volume to ensure that tissues are immersed in the solution.
2) Shake the tubes in an orbital shaker at 50 oscillations/min for 20 minutes. Note: Shaking is just to ensure that all of the tissue is in contact with the Evans blue dye solution.
3) Wash the roots and leaves thoroughly with distilled water thrice or until unbound dye washes out from the surface.
4) Observe the stained leaf or root under the normal, transmitted light microscope. Examine the leaves or roots and take photographs of images under a brightfield microscope. Furthermore, the Evans blue stained images can be quantified using ImageJ software to assess the viability of the cells.

Trypan Blue staining protocol

Trypan Blue solution:

- 10 ml water
- 10 ml lactic acid
- 10 ml glycerol
- 10 g phenol
- 10 mg trypan blue powder [Takemoto, D. (2005)]

Dilute trypan blue solution 1:1 in EtOH.

Clearing solution (Chloral hydrate): Dilute chloral hydrate crystals in MQ-water. It is better to prepare it right before the staining experiment and to have a fresh batch for every new staining.

Initial chloral hydrate concentration: 2,5g/ml

1 ml (or 0,5 ml) of Chloral hydrate x ___ number of tubes x 3 washes = volume needed of chloral hydrate for the experiment.

*Whole bottle of Chloral hydrate is 500 g and since a great quantity of it is needed for the washes it is perfectly fine to use 0,5 ml per tube per wash. It uses less product and there is no difference in washes.

Staining procedure:

1) Prepare 1 eppendorf tube for each well with seedlings. Use 2 ml eppendorf with Safelock.
2) Stain seedlings with 1 ml trypan blue, leave in heatblock at 99°C for 1 minute.
3) Left to cool at room temperature for about an hour. All the seedlings look completely blue at this point.
4) Pipette the trypan blue and dispose at a special container (carcinogenic chemical).
5) Pipette 1 ml clearing solution and leave for 4-5h, if there is laboratory rocker available, put it inside the chemical hood and leave the tubes with the solution rocking at slow speed (in a tube rack).
6) Pipette and dispose the chloral hydrate from the seedlings after ~4h. Repeat clearing at least 2 times more.
7) After the last time clearing, prepare a solution of 50% glycerol (in MQ water) and pipette 1 ml into each tube

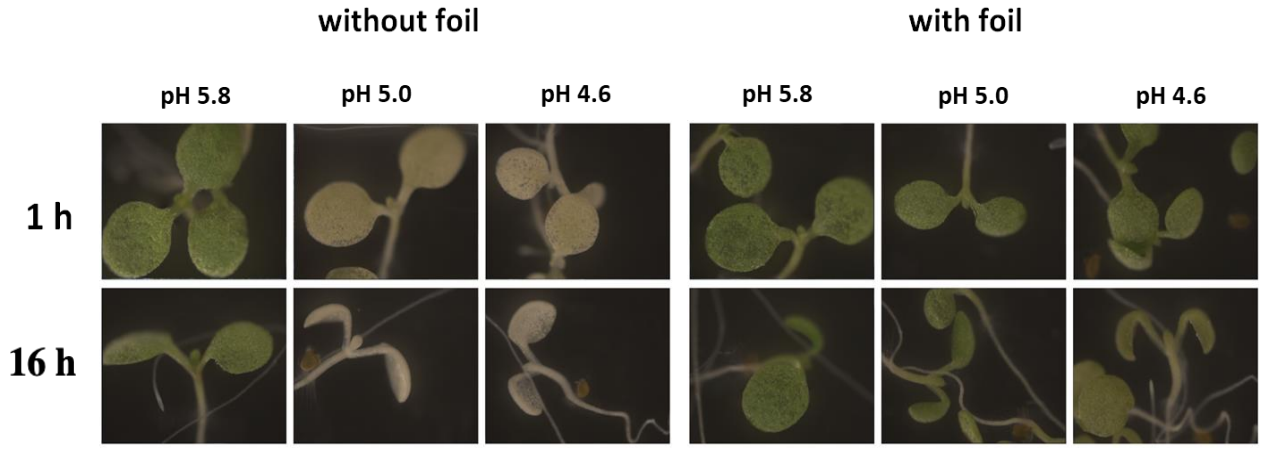

Figure 24. Low pH (4.6) induces light-dependent bleaching in *Arabidopsis* seedlings

7-days old *Arabidopsis* seedlings were exposed to three solutions of 50 mM NaAc buffer: pH 5.8 (control); pH 5.0 and pH 4.6. Two time points were used in this treatment: 60 minutes and 16 hours. One of the plates was covered with aluminum foil to prevent it from light exposure. In the case of treatment without aluminum foil cover bleaching is visible at pH 5.0 and 4.6, in both time points.

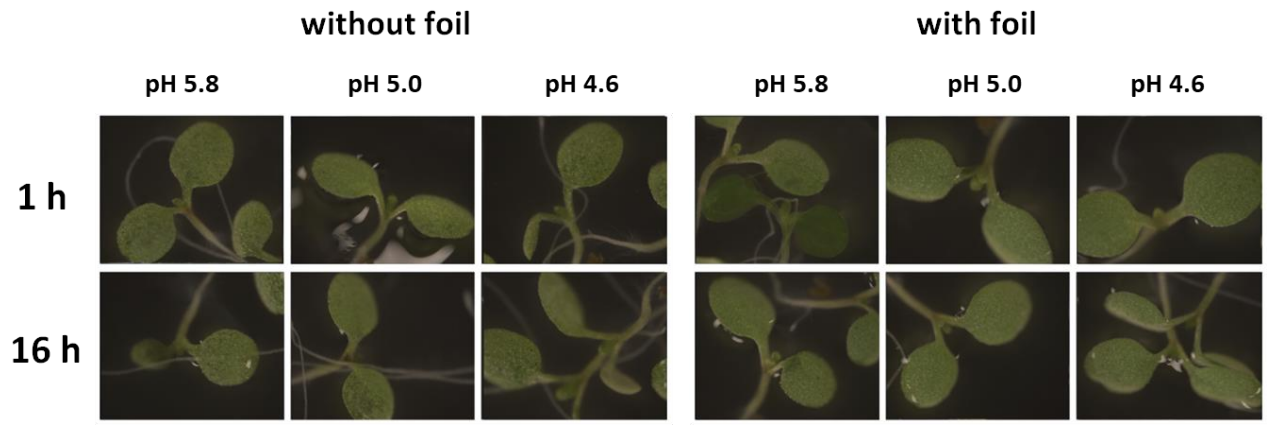

Figure 25. Low pH (4.6) in MES buffer does not induce light-dependent bleaching in *Arabidopsis* seedlings

7-days old *Arabidopsis* seedlings were exposed to three solutions of 50 mM MES buffer: pH 5.8 (control); pH 5.0 and pH 4.6. Two time points were used in this treatment: 60 minutes and 16 hours. One of the plates was covered with aluminum foil to prevent it from light exposure.

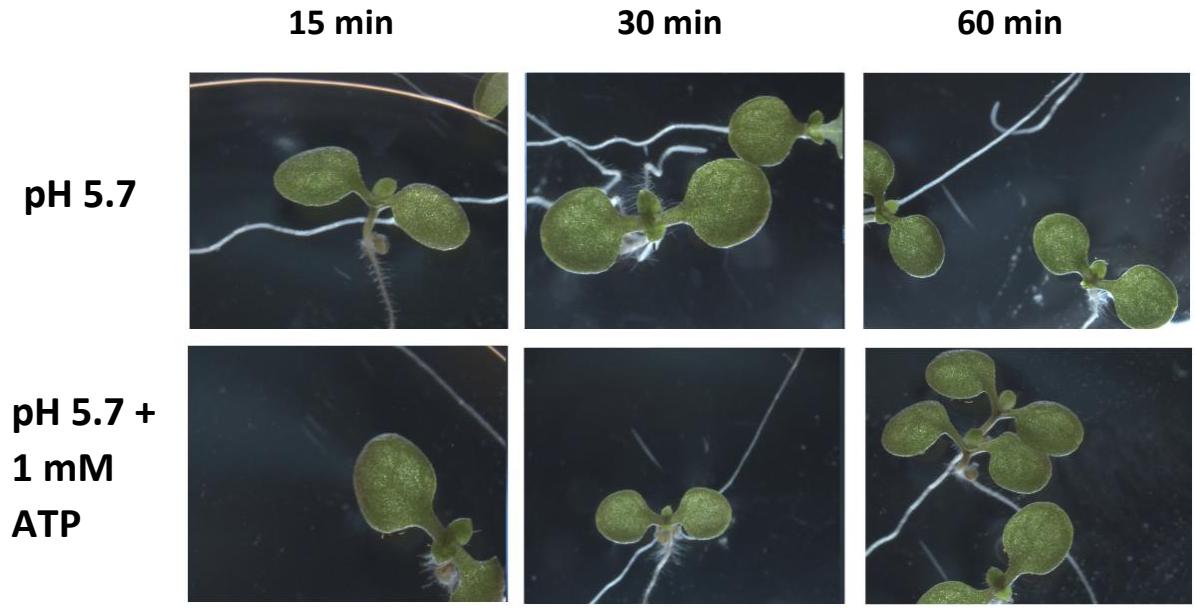

Figure 26. eATP as a death molecule in *Arabidopsis*

7-days old *Arabidopsis* seedlings were exposed to two solutions of 50 mM MES buffer: pH 5.7 (control) and pH 5.7 with addition of 1 mM ATP. Three time points were used in this treatment: 15 minutes, 30 minutes and 60 minutes.

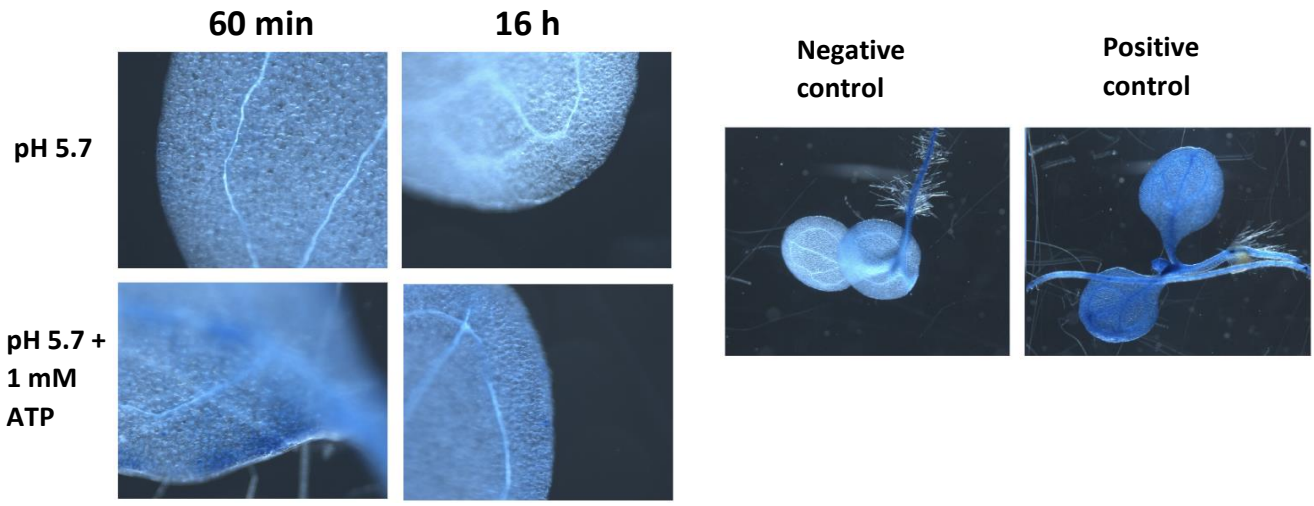

Figure 27. Replicate: ATP as a death molecule in *Arabidopsis*

7-days old *Arabidopsis* seedlings were exposed to two solutions of 50 mM MES buffer pH 5.7 (control) and pH 5.7 with addition of 1 mM ATP. Two time points were used in this treatment: 1 and 16 hours. As a positive control for cell death, seedlings were treated for 10 minutes at 55C to induce heat shock. Afterwards, seedlings were stained with Trypan Blue, Dark blue indicates cell death, lighter blue (as in control at room temperature) represents background.

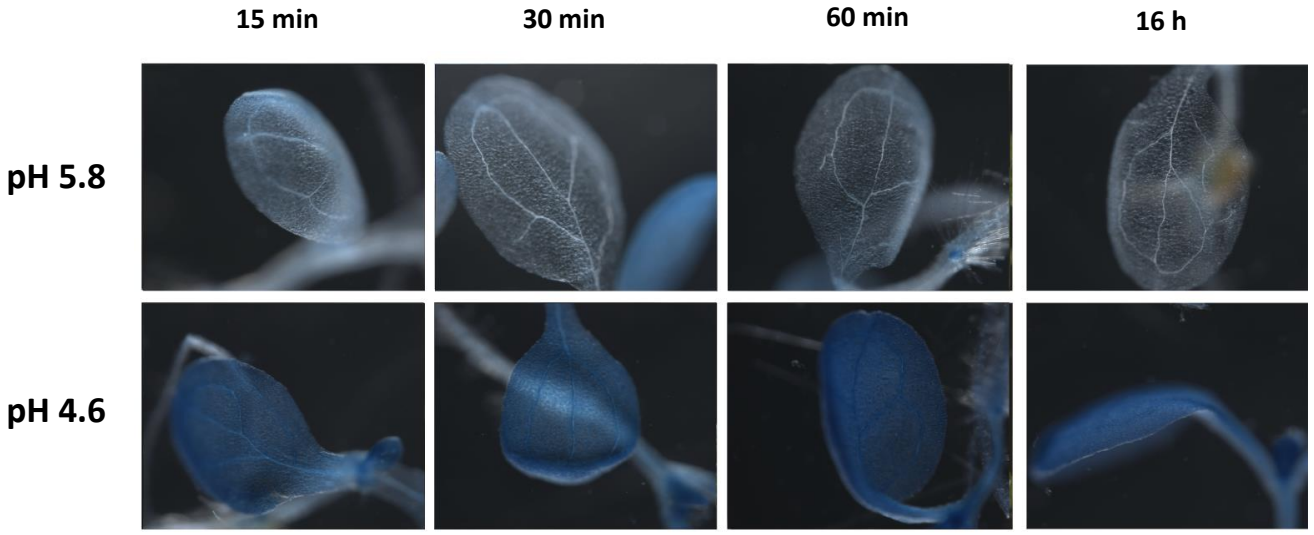

Figure 28. Replicate: Low pH (4.6) induces cell death in *Arabidopsis* seedlings

7-days old *Arabidopsis* seedlings were exposed to three solutions of 50 mM NaAc buffer pH 5.8 (control) and pH 4.6. for 15, 30 and 60 minutes. As a positive control for cell death, seedlings were treated for 10 minutes at 55C to induce heat shock. Afterwards, seedlings were stained with Trypan Blue, Dark blue indicates cell death, lighter blue (as in control at room temperature) represents background.

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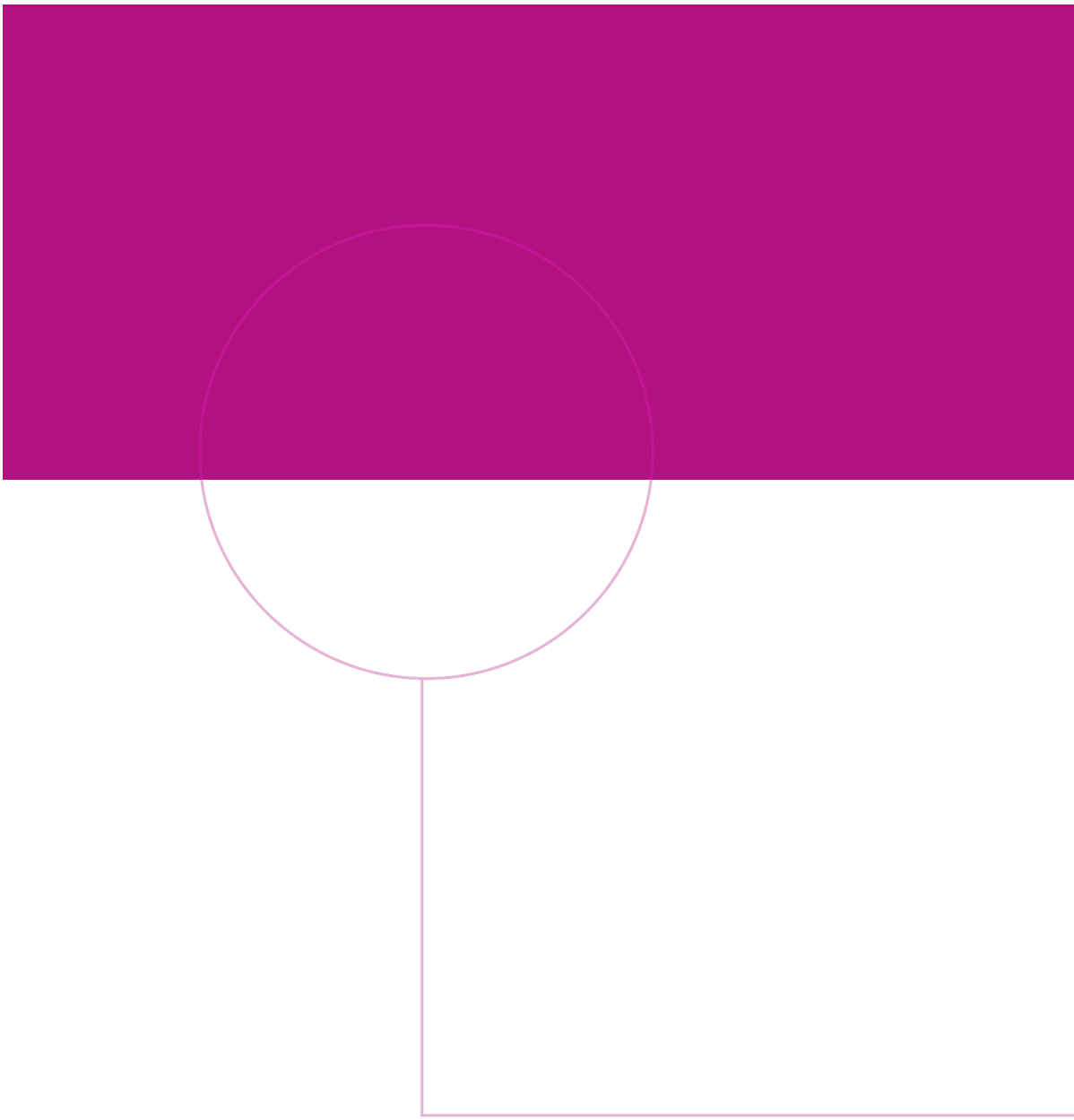

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