Masteroppgave

NTNU
Norges teknisk-naturvitenskapelige universitet
Fakultet for naturvitenskap
Institutt for biologi

Marco Enoc Soto

Unraveling Regulated Cell Death in Plants

Challanges and Perspectives using Mass Spectrometry Imaging

Masteroppgave i Biology
Veileder: Dr. Daniela Sueldo
Mai 2023

NTNU
Kunnskap for en bedre verden

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2 Abstract

Norsk: Når klimaendringene akselererer og den globale temperaturen øker, utsettes planter for sterkere miljøbelastninger. Planter reagerer på stress ved å aktivere ulike typer fysiologiske endringer i rotmorfologi og molekylære responser, og en slik respons er programmert celledød. Programmert celledød er en kontrollert respons hvor skadede celler elimineres og fjernes. Dette er en viktig mekanisme, som gjør planter i stand til å takle miljøbelastninger. Det å forstå mekanismene som regulerer programmert celledød er derfor et viktig felt i plantebiologien. En sentral del av dette er å forstå de molekylære endringene som oppstår kort tid etter eksponering for stress. Dette har til nå vært vanskelig grunnet mangelen på metoder som tillater den spatiotemporale oppløsningen, men ny teknologi som MALDI-MSI gjør det nå mulig å foreta denne typen studier. MALDI-MSI er en molekylær avbildningsteknologi, som muliggjør identifisering og lokalisering av spesifikke molekyler i en prøve. Denne teknologien er tatt i bruk i det medisinske feltet, men er lite utprøvd på plantematerialet. Målet med denne oppgaven har derfor vært å undersøke de molekylære spatiotemporale endringene forbundet med programmert celledød ved å eksponere *arabidopsis thaliana*-røtter for lav pH, samt å fremme integreringen av massespektrometrisk avbildning for plantebaserte studier. Den første delen av oppgaven gikk ut på å undersøke effekten av ulike buffere og pH på celledød. Spirer av *arabidopsis* ble eksponert for ulike buffere (NaAc, MES og Pipes/$CaCl_2$) med pH 5.8 (normal) og 4.6 (lav) og eventuell celledød påvist ved hjelp av Evans Blue og SYTOX green. Eksponering for NaAc-buffere førte til celledød både ved pH 5.8 og 4.6. Ved pH 5.8 dannet plantene tilfeldige røtter, mens ved pH 4.6 ble de bleke. Dette indikerer at NaAc-buffer induserer celledød selv ved tilsynelatende normale pH-verdier så vel som ved lav pH. Dette var også tilfellet for MES-buffer, en buffer mye brukt i plantedyrking. Det kan dermed tyde på at MES-buffer ikke er en egnet buffer for planter, og ytterligere studier trengs for å kartlegge den fysiologiske effekten. Eksponering for Pipes/$CaCl_2$ derimot, gav ingen detekterbar celledød.

Målet med den andre delen av dette prosjektet var å etablere MALDI-MSI for plantestudier, inkludert preparering av prøver. Dette var et krevende arbeid, som leder til konklusjonen om at en omfattende metodeutvikling og testing er nødvendig for å oppnå reproduserbare resultater. Samlet sett understreker resultatene fra denne oppgaven behovet for studier som undersøker bufferinduserte effekter og forskjeller mellom dem, samt fremhever noen av utfordringene og potensielle løsninger for ytterligere å inkorporere MALDI-MSI for plantebaserte studier.

English: As climate change accelerates and global temperatures increase, plants are subjected to stronger environmental stressors. Plants respond to stress by activating different types of physiological changes in root morphology and molecular responses, one such response is regulated cell death. Regulated cell death is a controlled response which eliminates and removes damaged cells. It is an important preservation mechanism as without it plants would be unable to recover and overcome exposure to environmental stressors. Therefore, research investigating the mechanisms by which regulated cell death occurs in response to altered environmental conditions has become an important topic in the field of plant biology. One key part of this is understanding the molecular changes that occur shortly after stress exposure. This was previously difficult due to the lack of methodologies capable of reaching the necessary spatiotemporal resolution. With recent technological advances, equipment with such capabilities has been developed and has seen successful use in the medical field. One such methodology is MALDI-MSI, which allows the user to observe molecules present within a sample as well generating images illustrating the localization

of specific masses within the sample. However, it is necessary to develop protocols and overcome several challenges to successfully incorporate this technology for use in plant research. Therefore, the purpose of this thesis was to investigate the molecular spatiotemporal changes associated with regulated cell death upon exposure to low pH in *arabidopsis thaliana* roots, as well as furthering the integration of mass spectrometry imaging for plant based studies.

In an attempt to induce a localised cell death response, *arabidopsis* seedlings were exposed to various buffers (NaAc, MES and Pipes/$CaCl_2$) at two pH conditions (Normal: 5.8 and Low: 4.6). Exposure to NaAc buffers led to Evans Blue and SYTOX green cell death patterns indicative of primary root death. This was further confirmed with the observation that seedlings also developed adventitious roots at pH: 5.8. Seedlings at pH: 4.6 exhibited bleaching and complete cell death, indicating NaAc buffer had induced toxicity effects at seemingly "normal" pH values as well as in low pH conditions. Interestingly, in MES buffers which are extensively used in plant cultivation, cell death was observed at both normal and low pH values as well as various tested concentrations and time points. These results suggest MES buffer may not be a suitable control for experiments and further research investigating its effects on plant physiology should be conducted. Contrastingly, Pipes/$CaCl_2$ treated seedlings exhibited no discernable cell death patterns and were seemingly unaffected by buffer exposure.

The second part of this project was focused on furthering the integration of mass spectrometry imaging technology for plant studies. For this, sample preparation and cryosectioning steps were conducted before mass spectrometry analyses. Difficulties encountered in the aforementioned steps led to the conclusion that extensive method development and testing is required in order to obtain reproducible results. Taken together, the results generated from this thesis emphasise the need for studies investigating buffer induced effects and differences between them, as well as highlighting some of the challenges and potential solutions to further incorporate MALDI-MSI for plant based studies.

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3 Introduction

Plants are complex multicellular organisms that are subjugated to both biotic and abiotic stresses on a regular basis. Like animals, they have evolved specialised techniques to help cope with and overcome stressors. However, unlike animals, plants lack an adaptive immune system. One strategy plants use to cope with stressors is induction of controlled suicide in damaged and exposed cells. This controlled process is known as regulated cell death (RCD).

RCD is a fundamental cellular process that enables an organism to cope and overcome stressors in a controlled manner by localising cell death to the affected area and thereby preventing further damage to surrounding tissue. Two types of RCD have been described in plants – developmental programmed cell death (dPCD) and pathogen triggered cell death (pPCD), which correspond to different functions [1]. For example, dPCD participates in growth, reproduction, and senescence whereas pPCD mainly serves in responding to plant-pathogen interactions as well as other abiotic stimuli such as wounding [1]. Mechanical wounding can be defined as the physical damage resulting from the opening of plant tissue, and can also induce RCD in plants. Wounding leads to tissue exposure towards the external environment, which can have detrimental effects on plant survival as it can facilitate pathogen infection [2]. Importantly, signalling molecules and pathways activated by different modes of RCD differ in shape and intensity, and research concerning these responses is an ongoing effort.

Some molecules that have been identified in the regulation of cell death include: calcium, iron, reactive oxygen species (ROS), reactive nitrogen species (RNS), as well as several phytohormones such as salicylic acid (SA), jasmonic acid (JA) and abscisic acid (ABA), amongst others [3, 4]. Additionally, it is known these molecules are not working independently from one another and exhibit crosstalk at several steps of the signal transduction process. For example, calcium is a well known signalling molecule in eukaryotes. In plants, it serves roles ranging from long distance signalling to conferring tolerance to abiotic stressors [5, 6] Similarly, iron is an essential micronutrient involved in the production of various enzymatic cofactors [7]. However, its involvement has recently been linked to a novel category of plant cell death, ferroptosis [8]. These examples illustrate how signalling molecules serve multiple roles and are not exclusive to responding to a specific stressor/stimulus. Even though there is extensive literature reviewing the numerous roles these molecules serve in stress modulation, information regarding the spatiotemporal distribution of these molecules upon RCD is currently lacking. This blackbox of information can aid in identifying some of the key players involved in RCD, as well as unveiling some of the physiological mechanisms underlying this fundamental process.

Consequently, this need for higher spatiotemporal resolution has driven the development of new technologies or modifications of existing techniques for alternate purposes. An example of such technology is Matrix Assisted Laser Desorption/Ionization Mass Spectrometry Imaging (MALDI-MSI). MALDI-MSI is a spectrometry technique that enables detection of a wide range of biomolecules such as proteins, metabolites, lipids, as well as sugar and various macromolecules in situ via laser desorption/ionisation of the tissue sample. Its ease of use, coupled with information output make it an attractive technology for use in medical, and clinical studies, where the generation of proteomic profiles can aid in disease diagnosis [9]. In plant biology it has been used for studying protein imaging, in situ analysis of endogenous molecules such as lipids, metabolites, ginsenosides, amino acids, flavonoids and organic acids amongst other applications. [10, 11, 12]. To date, MALDI-MSI has mainly been used for metabolic profiling in stems and leaf tissue areas of various

plant species under normal growth conditions [13]. Research further incorporating MALDI-MSI for studies concerning molecular responses to abiotic stressors, and more specifically cell death, is a scarce and an ongoing effort. These topics are of incredible interest as MALDI-MSI can yield information on spatiotemporal regulation of many molecules, and thus help us further understand the enigmatic processes surrounding cell death.

My thesis will mainly focus on furthering the incorporation of MALDI-MSI to plant based studies in the context of RCD. This work is being conducted as part of the "Cell Death and Communication" group at the Norwegian University of Science and Technology (NTNU). The Deathwall lab consists of PI's Daniela Sueldo and Thorsten Hamann as well as various members ranging from post-Doctoral researchers to master students. Interests include cell death signalling triggered by environmental stressors, cell wall integrity, as well as cell wall composition and structure amongst others.

Cell Death Type	Morphology	Features	Supporting Literature
Vacuolar	• Decreased cytoplasmic volume • Increased lytic vacuole volume • Tonoplast rupture • Digested cellular components • Cell corpse remaining	• Release of vacuolar hydrolases • Protoplast clearing • Two types of Vacuolar-mediated cell death (non-destructive and destructive)	72,73,74
Necrotic	• Undigested organelles • Mitochondrial swelling • Protoplast shrinkage	• Mitochondrial disfunction • Shift in Ca2+,ROS and RNS levels	72,75
Hypersensitive Response (HR)	• Localized response at site of infection and surrounding cells • Shared morphology from vacuolar and necrotic cell death	• Rapid response to biotic stressors • Accumulation of ROS • Sudden concentration increase of Ca2+	72,76,77,78
Ferroptosis	• Cytoplasmic retraction • Mitochondrial shrinkage • Increased mitochondrial density	• Iron-dependent form of cell death • Accumulation of lipid ROS • GSH depletion • Observed under specific heat stress conditions	8,27,80
Apoptosis-like (AL-PCD)	• Cell shrinkage • Protoplast-cell wall separation	• Nuclear fragmentation and condensation • Calcium influx • Mitochondrial Membrane Permeabilization (MMP)	81,82,83

Figure 1: Known cell death mechanisms in plants and associated features.

4 Background

Unlike animals, plant cells are immobile and lack an adaptive immune system. However, they have developed specialised responses to constantly changing environmental conditions such as: temperature, salinity/osmotic, and pH as well as biotic stressors. This section will briefly review some of the stressors utilised to trigger RCD in this thesis project and associated responses.

4.1 Salt Stress

One of the major factors affecting crop cultivation worldwide is salinity stress. Salt stress is defined as an accumulation of abnormal levels of sodium contents within soil. Soil salinification occurs via "primary salinization" and "secondary salinization". In "primary salinization", water evaporation

exceeds water precipitation levels, leading to the accumulation of sodium ions in the upper soil layers [14]. Contrastingly, in "secondary salinization", salinization stems from a more anthropogenic source via use of modified irrigation systems and use of growth enhancing agents [14]. Salt stress has been shown to lead to arrested growth and eventual death in plants. Indicators of salinity stress include reduced growth, reduced biomass, and fruit number and size [15]. Consequently, plants have developed various mechanisms to overcome salinity stress depending on the concentration and location of the sodium ions with respect to the plant.

While salinization refers to the method of salt accumulation, the location where sodium is located within the plant is important when differentiating between a short term stress response and long term tolerance. Plant growth under saline conditions can be separated into two phases 1) osmotic phase: defined as a short term exposure, characterised by sodium ions located outside the root and 2) ionic phase: defined by infiltration of sodium within root and tissue matter, caused by prolonged exposure to saline conditions [16]. Depending on the time of exposure and location of salt within the plant, salt detoxification responses may differ. During short term exposure, stomatal closure, increases in leaf temperature, as well as arrested growth of both roots and shoot have been observed [16]. Contrastingly, long term exposure leads to sodium accumulation within plant tissue and is often accompanied by decreased photosynthetic activity, reduced leaf growth and accelerated leaf senescence [16]. Moreover, the aforementioned responses are generalisations and are not to be assumed for all types of plants as there exist salt tolerant species of plants, halophytes, and more sensitive ones, glycophytes. Halophytes are known to tolerate higher salinity levels compared to glycophytes. This is made possible via unique mechanisms such as use of salt-secreting glands, toxic ion compartmentalization via specialised transporters, as well as effective use of ROS detoxification pathways [17]. Similarly, some halophytes thrive on increased levels of salinity, to the extent that their distribution can be severely limited by insufficient sodium content in the soil. Contrastingly, glycophytes lack some of mechanisms utilised by halophytes thereby leading to the development of alternate methods to aid when exposed to conditions of increased salinity.

Just like there are different responses to salinity based on strength and time of exposure, there are also various mechanisms plants employ in response to salinity stress. According to Roy et al. (2014), there are three main classes of mechanisms which confer salinity tolerance in plants: tissue tolerance, ion exclusion and osmotic tolerance [18]. Tissue tolerance occurs during the ionic phase of salt exposure. A unique feature of tissue tolerance is the compartmentalization of ions into vacuoles. This ion compartmentalization strategy effectively prevents and limits further sodium accumulation induced cellular damage [18]. Furthermore, translocation of ions is made possible via the use of ion transporters, proton pumps and the synthesis of solutes to aid in transportation coordination [18]. Another category of mechanisms that confers salinity tolerance is ion exclusion. As the name suggests, the process involves the expulsion of Na+ and Cl- ions from plant tissue and mainly occurs within roots [18]. This process can occur via various mechanisms such as ion efflux and vacuolar assisted ion compartmentalization [18]. This strategy consisting of a series of interactions occurring within the xylem, apoplast and epidermis, allow plants to withstand moderate levels of salinity stress [18].

Moreover, the strategies described above are employed when the salinity stress is mainly in the ionic phase and correspond to an extended period of salinity exposure. Contrastingly, a short-term response also exists, and given its rapid nature, it is heavily dependent on efficient sensing and signalling cascades triggered by osmotic differences. Therefore, the third kind of strategy

is known as osmotic tolerance, and is defined as an immediate response to an osmotic stress with the goal of minimising the deleterious effects of salinity stress on growth [18]. Minimization of damage is stringent upon effective sensing mechanisms as well as accelerated long-distance signalling. Given the speed and short time in which these responses occur, it makes information acquisition increasingly difficult, and thereby limits our current understanding surrounding short term signalling and sensing modalities in plants. However, research strongly suggests there exists direct involvement of roots in response to abiotic stimuli [5, 19, 20].

Because of the root's fundamental role in nutrient absorption, it is hypothesised that the effects of ion dysregulation are primarily sensed in roots, and can lead to direct responses affecting cells along the primary root end in plants. Moreover, it has been shown that upon exposure to NaCl, salt-sensitive plants exhibit adaptive responses via RCD induction, targeting cells located at the primary root end [21, 22]. In *arabidopsis thaliana*, seedlings exposed to NaCl at concentrations of 0.16M and 0.2M displayed DNA fragmentation localised within the primary root cells in wild type and sos1 defective variants [21]. Further supporting these results, various studies conducted in barley, wheat, and rice suggest a direct link exists between primary root cells, salinity stress and PCD induction [23, 24, 25]. Salinity induced RCD was shown to alter root morphologies, growth behaviour as well as triggering cell death at the primary root end [23, 24, 25].

4.2 Temperature

When discussing abiotic factors affecting plant growth and development worldwide, temperature is amongst the largest and most influential. Like in many animal species, temperature alone can limit the distribution of a large quantity of plant species, and can greatly affect productivity, photosynthetic capabilities, crop yields, and maturation time amongst other variables. Moreover, with radical temperature fluctuations becoming more common, research investigating the effects of heat stress on plant physiology has become a hot topic in the research community.

Heat stress is defined as exposure to temperatures above an optimal range. Temperature increases can lead to the disruption of homeostasis and altering of various processes at multiple biological levels. Heat stress has been shown to disrupt photosynthetic efficiency, CO2 uptake, photophosphorylation, membrane fluidity, and electron transport amongst other photosynthesis associated processes [22]. However, despite the deleterious effects imposed by heat stress, some plants have evolved adaptations to aid with heat tolerance. Like salinity specialists, there also exists three categories of plants that exhibit varying degrees of thermal tolerance. Categories are: 1) psychrophiles, these are plants that typically inhabit environments with temperatures of 0-10 C°, 2) Mesophiles, which thrive in temperatures of 10-30 C°, 3) Thermophiles, which are adapted to temperatures of 30-65 C°[26]. As described above, plants can inhabit a wide range of environments with an even wider range of temperatures. However, plants are still prone to drastic temperature fluctuations that trigger the activation of defensive mechanisms to prevent further damage.

Similar to salinity stress, exposure time plays an important role in determining the strategies implemented to overcome heat stress. Plants frequently subjected to heat stress are likely to develop long term adaptations to limit the damage heat stress imposes, these mechanisms can be described as tolerance mechanisms. Contrastingly, if the exposure is brief, developing long term tolerance mechanisms may be cost inefficient, leading to the development of avoidance strategies. However, plants are not bound to either of these categories, and can exhibit mixed responses

consisting of characteristics shared by both tolerance and avoidance strategies in order to maximise their chance of survival.

Avoidance strategies include: stomatal closure, production of superficial wax on leaf surface, leaf rolling, leaf orientation, lipid membrane compositional changes, early maturation, and leaf transpiration [26]. Noteworthy, the short term physiological mechanisms described above are similar to the ones utilised by plants during drought conditions. Contrastingly, tolerance mechanisms are defined as mechanisms that allow for the maximisation of growth and production while experiencing constant heat stress. Mechanisms that confer heat tolerance include: increased levels of antioxidants, expression of heat stress proteins such as Heat Shock Proteins (HSPs), ion transporters, as well as signalling cascades that lead to transcriptional changes and upregulation of heat tolerance associated genes [26].

Temperature increments lead to a wide variety of stress responses in both animals and plants. A common response of temperature surges is defective/inefficient protein synthesis, disruption of homeostasis leads to a unfolded protein response (UPR) in an attempt to mitigate the harmful effects caused by misfolded proteins. It has been shown that misfolded proteins trigger the upregulation of Heat Shock Proteins (HSPs) that dispose of the faulty proteins. Moreover, Heat Shock (HS) has been shown to trigger increases in Reactive Oxygen Species (ROS). HSPs aid in short term temperature tolerance by assisting with the refolding and assembly of damaged proteins [22]. Furthermore, increases in antioxidants are often observed in response to elevated ROS levels [22].

Heat shock induced cell death Ferroptosis is a recently described iron-dependent form of cell death in plants. In both plants and animals, ferroptosis is dependent on intracellular iron for the formation and subsequent accumulation of oxidised lipids known as lipid ROS. While fairly common in animals, ferroptosis has only been shown to develop under specific conditions of heat stress in plants. For example, *arabidopsis thaliana* seedlings exposed to 55 C°for 10 minutes developed an RCD response located in various types of root cells, most notably root hairs [27]. Additionally, when seedlings were pretreated with ferroptosis inhibitors prior to heat exposure, it significantly impacted the extent of cell death, further hinting at iron's role in responding to heat stress [27]. However, if heat exposure is increased in either duration or temperature, a form of non regulated cell death mechanism closely resembling necrosis occurs [27]. Further research has to be conducted in order to fully comprehend the mechanisms at play and identify if they remain conserved across various plant species, and the extent to which ferroptosis might participate in other stress responses in plants.

4.3 pH

Another major stressor plants are commonly exposed to is pH, soil acidification via extensive use of ammonia rich fertilisers and acid rain are leading causes of soil acidification. Contrastingly, climatic events such as weathering and flooding lead to soil alkalinization. These pH disturbances trigger various responses in plants in an effort to mitigate the deleterious effects brought on by the sudden change in pH. Moreover, responses vary by exposure time and plants can exhibit a combination of short term tolerance and long term adaptation responses. Similarly, pH disturbances can affect environmental factors such as trace element solubility, microbial community composition, soil enzyme activity, biodegradation, and soil nitrification processes [28]. Because of the wide range of effects on plant-growth associated factors, pH is often used as a general indicator of overall health

in plants.

4.3.1 Low pH

In circumstances of low pH it has been shown that plants exhibit limited root growth, altered root morphology, fresh weight, height, and chlorophyll contents amongst other photosynthetic parameters [29, 30]. As previously mentioned, one of the major effects of soil pH disruption is element solubility. Under low pH conditions, trace elements are soluble but not absorbable, leading to nutritional deficits and altered metal solubility in soil [28]. Similarly, microbial composition is also greatly impacted at low pH's. Additionally, at low pH levels, soil respiration decreases and favours fungal activity over bacterial [28]. This change alone can be detrimental to plant survival as microbes have a major impact on plant survival due to their pH-adjusting and nutrient acquisition capabilities [31].

Furthermore, research in *arabidopsis thaliana* investigating the effects of low pH (4.6-4.8 approximately) show that exposure ranging from 30-240 minutes can lead to irreversible damage in the form of localised cell death along the primary root end [32, 33]. Similarly, primary and lateral roots ceased growth and displayed cell death along the meristematic and elongation zones of the primary root [33]. Interestingly, application of MgCl, $CaCl_2$ and SrCl reduced low pH induced damages in *arabidopsis thaliana* seedlings [32]. Taken together, these results suggest that sensing and possibly stress-associated signal transduction capabilities are harboured in cells localised along the primary root end.

4.3.2 High pH

In conditions of elevated pH levels, plants exhibit similar responses as in conditions of low pH stress. Some of these symptoms include reduced growth and photosynthetic productivity, increased electrolyte leakage, as well as altered root morphology [34]. According to Turner et al. (2020), Rhododendrons (*Rhododendron spp.*) exposed to heightened pH conditions (pH: 6.5) exhibited altered root morphology compared to controls. This change manifested as decreased growth and an increase of short branched roots [34].

5 Sensing

Just as important as the responses elicited to the stressors labelled above, is the ability to sense and coordinate a controlled response. It is well known that plants have developed a wide array of mechanisms that enable them to respond to specific stimuli in such a manner as to minimise and localise damage. This section will discuss some of the major pathways involved in the aforementioned stressors as well as associated molecules and current knowledge on their spatiotemporal distribution.

5.1 Salt Stress

One of the main mechanisms triggered in conditions of heightened salinity is the Salt Overly Sensitive (SOS) pathway. The SOS pathway currently consists of five genes termed "SOS" identified in *arabidopsis thaliana*, who's activity is highly regulated by changes in salinity levels [35, 36, 37]. Interestingly, SOS pathway components have been identified in other plant species, indicating conservation amongst species [38, 39, 40, 41]. Over 40 SOS mutant lines have been identified to date, which are differentiated by their unique sensitivity to external sodium and lithium. The SOS pathway has as a goal to reduce the detrimental effects of salinity exposure and confer tolerance. The SOS pathway is triggered by detection of elevated levels of intracellular sodium, and is followed by increases of cytosolic Ca_2+. Increases of cytosolic Ca_2+ lead to the binding of SOS3 that then activates a protein kinase (SOS2). Furthermore, binding of the SOS3-2 complex leads to increased expression of SOS1, an antiporter that transports cytosolic Na+ into the extracellular space, thereby maintaining osmotic and ion balance, preventing cellular damage [37].

Even though the genes involved in the SOS pathway have been identified since the 90s, the exact mechanism by which Na+ ions are detected has elucidated researchers until recently. A group of sodium sensing cells located in the differentiation zone have been shown to form a "sodium sensing niche" [19]. This group of cells have not only been found to respond to initial Ca^{2+} signals, but they additionally propagate the stimulus upstream in a wave-like form [19]. Moreover, it was shown that the amplitude and speed of this signal directly correlates to Na+ concentrations [19]. This result would indicate that plants are not only capable of sensing shifting Na+ concentrations, but also capable of producing tailored responses to various Na+ levels.

5.2 Temperature

Temperature increments lead to a wide variety of stress responses observable in both animals and plants. Therefore, effective sensing is necessary in order to maximise chances of survival upon heat stress. One of the main strategies plants use to detect changes in temperature is via use of thermosensors, these are capable of sensing warm and cold temperatures. These biological sensors can be divided into four categories: DNA, RNA, protein-protein, and protein-membrane [42]. However only three have been described in plants: RNA and protein thermosensors as well as a protein-metabolite based sensor [43].

At warm temperatures, plants have been shown to activate a developmental, morphology-altering program known as thermomorphogenesis [43]. This type of response can be identified by the elongation of the hypocotyl and petiole [43]. Moreover, another mechanism plants employ to detect heat stress is via use of phytochromes. These photoreceptors work by using crosstalk in light and temperature signalling pathways to regulate thermomorphogenensis [44]. In addition to various photoreceptors, RNA thermosensors have been shown to also exist in plants. Information on their inner workings are scarce but it is proposed that via temperature increases, a hairpin structure modification leads to increased translation of a thermomorphogenesis associated gene [45]. Moreover, under conditions of moderate to extreme heat stress, it is known that an unfolded protein response can be triggered. Lastly, because of the stable and temperature sensitive nature of the membrane, it is suggested that changes in temperature leading to altered membrane fluidity trigger membrane property sensors that act as thermosensors [44]. However, their existence is yet to be discovered.

5.3 pH

Much is known about the importance and effects of extracellular pH on plant physiology, but the sensing mechanisms that govern it still remain obscure. Recent research has uncovered the use of cell-surface peptide-receptor complexes as extracellular pH sensors in *arabidopsis thaliana* [46]. These complexes have been shown to function by detecting and integrating signals via use of a pattern triggered immunity (PTI) strategy. Furthermore, upon successful sensing, PTI was shown to trigger rapid extracellular pH alkalinization in cells surrounding the root apical meristem (RAM) region. Suggesting a RAM localised, growth and immunity regulating pH sensor exists. Taken together, these results describe one strategy plants can use to sense changes in extracellular pH. Further work is needed to verify its existence across plant species as well as identifying other potential strategies utilised.

6 Molecules involved and spatial distribution

6.1 Calcium

6.1.1 Salt Stress

Calcium plays an important role in the onset of salinity stress. It has been shown that upon salinity stress there is a surge in cytosolic Ca_2+. It is now known which spikes in cytosolic Ca_2+ act as a trigger for the SOS pathway, resulting in salinity tolerance [19]. Cytosolic Ca_2+ acts as a trigger for SOS3/CBL4 sensors for basal Na+ extrusion initiation. Similarly, under high salinity stress, the SOS3/CBL8 sensor is triggered to activate enhanced Na+ extrusion [19]. In addition to serving as a local signalling molecule, Ca_2+ also serves a role in signal propagation, Choi et al.(2013) observed that upon induction of salt stress at the root tip, plants elicited long distance, wave-like Ca_2+ signal transduction stemming from the root, and travelling all the way to the shoot. Additionally, gene analyses indicated an increase in transcript abundance related to stress markers in the shoot shortly after stress induction [5]. This result further confirms the use of Ca_2+ as a signalling agent.

6.1.2 Temperature

As previously mentioned, calcium serves multiple roles in stress modulation. During the onset of heat stress, root calcium levels had a direct impact on heat tolerance and growth in potato plants (*Solanum tuberosum L.*) [47]. Moreover, in heat stressed tobacco plants (*Nicotiana tabacum L.*), application of $CaCl_2$ to the leaves resulted in an improved photosynthetic rate and increased levels of ROS regulating enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) amongst others [6]. These results clearly point towards calcium's direct involvement in the regulation and modulation of molecular processes responding to heat stress. While Ca_2+ involvement in stress response is undeniable, information regarding spatiotemporal distribution is rare. Recently, information regarding calcium dynamics in response to heat stress has been obtained via development and use of radiometric calcium reporters. Weignand et al. (2021) have developed a genetically encoded calcium reporter that enables comparison of Ca_2+ dynamics in various types of plant tissue and subcellular locations. Results displayed increases in cytosolic

Ca_2+ within leaf tissue following heat stress exposure. Three different Ca_2+ influx signatures were obtained [48]. Similarly, these Ca_2+ signatures were shown to differ within pollen tubes [48]. Taken together these results highlight the nonuniform behaviour of calcium signalling and lead to speculation on the extent of use of Ca_2+ as a signalling agent.

6.1.3 pH stress

Because of its broad role in stress modulation, Ca_2+ levels respond in various forms as a result of altered environmental conditions. pH is not an exception, clear ties linking Ca_2+ and pH have been previously described in plants. For example, research investigating the effects of low pH on *arabidopsis thaliana* roots uncovered a link between low pH and Ca_2+ requirements. Seedlings exposed to a low pH solution (pH: 4.5) required greater concentrations of in solution Ca_2+ than seedlings exposed to a higher pH (pH: 5.3) in order to maintain root end viability [32]. These results suggest that pH has a direct effect on calcium stores within the roots by potentially altering Ca_2+ uptake and transportation mechanisms. Further supporting Ca_2+ protective role under low pH conditions, it was found that elevated extracellular Ca_2+ stopped cellular damage in a pH sensitive stop1 knockout mutant in *arabidopsis thaliana* primary roots [49]. Transcription factor stop1 is responsible for the regulation of multiple genes participating in signalling pathways involving aluminium and pH regulation [50]. Similarly, because of Ca's role in signalling, it is likely that levels fluctuate as a response to pH stress. This has been observed in sodium shocked seedlings, cytosolic Ca_2+ levels have been shown to increase in response to low pH conditions [51]. However, research investigating the spatial distribution of Ca_2+ following low pH exposure is forthcoming.

6.2 ROS

6.2.1 Salt Stress

In plants, ROS are commonly produced at basal levels in metabolically active organelles such as, chloroplasts, mitochondria, and peroxisomes. Similarly, ROS comes in various forms, hydrogen peroxide and superoxide anion being amongst the most prevalent. While commonly perceived as destructive, ROS serves multiple roles in organisms, some of the main ones being triggering oxidative damage, and serving as a signalling molecule in response to various biotic and abiotic stressors. Furthermore, effects of ROS on plant species are dependent on accumulation, which are further influenced by the balance of ROS production and ROS scavenging activities [52]. Moreover, ROS production has been shown to typically increase in response to stimuli strength.

In the presence of salinity and osmotic stress, ROS production increases as well as ROS accumulation associated damage [53]. Similarly, SOS2 was shown to interact with nucleoside triphosphate kinase 2 (NADPK2), a known H_2O_2 signalling protein, in the presence of salinity stress [54]. In sos2-2 ndpk2 double mutant experiments, plants displayed an increase in salinity sensitivity [54]. Curiously, the increased sensitivity did not result in the hyper-accumulation of H_2O_2, which would seem to suggest that H_2O_2 serves a role other than toxicity induction in salinity stress related responses [54]. Versules et al. (2023) also found two SOS2-interacting proteins, Catalase (CAT) CAT2 and CAT3 which further connect SOS2-ROS signalling activity.

6.2.2 Temperature

When exposed to heat stress, plants have shown increases in ROS levels, which can trigger cell death via oxidative stress if left unaddressed. In 14-day old *arabidopsis* seedlings exposed to heat stress, ROS production was observed within protoplasts, mitochondria, and chloroplasts [55]. Interestingly, in heat stressed *Nicotiana tabcum*, external addition of antioxidants such as ascorbate and superoxide dismutase, as well as inhibitors such as actinomycin D and cycloheximide decreased the extent of cell death [56]. This result is possibly linked to regulatory effects of antioxidants on ROS molecules. However, even though heat induced ROS stress often leads to cell damage and eventual death, ROS also serves roles in signalling, especially in response to abiotic factors [57].

6.2.3 pH

Just like changes in temperature or salinity, changes in pH exhibit a wide range of responses such as cellular damage and arrested growth/development in plants. One of the most common responses induced by pH stress is ROS accumulation. In rice (*Oryza sativa L.*), low pH (pH: 3.5) triggered the production and accumulation of hydrogen peroxide (H_2O_2) [58]. Similarly, at a high pH (pH: 10.87) rice seedlings exhibited root damage and growth inhibition as well as increased ROS accumulation [59]. Interestingly, ROS accumulation behaviour is not conserved across plant species. In *arabidopsis thaliana*, seedlings exposed to low pH (pH: 4.6) displayed a decrease of superoxide radicals coupled with no changes in H_2O_2 levels along the transition and elongation root zones of primary roots [60]. These results indicate that ROS accumulation varies per plant species and further research is necessary to untangle the variables responsible for accumulation.

6.3 ATP

6.3.1 Salt Stress

Adenosine triphosphate (ATP) is commonly regarded as the currency in cells, though it has been demonstrated to display varying roles in responding to external stimuli. In melatonin treated sweet potatoes (*Ipomea batatas*(L.) Lam) subjected to salinity and osmotic stress, H+- ATPase and intracellular ATP activity increased within the plasma membranes of both root and leaf tissue [61]. In oat leaves (A. sativa L.), decreased expression of ATP regulating-related proteins was observed following long-term salinity stress exposure [62]. Furthermore, there are multiple studies that tie ATP and associated molecules in roles pertaining to responding to various external stressors [63, 64, 65, 66]. Even though extensive literature exists linking ATP to stress responses, information regarding ATP localization following stress induction is scarce.

6.3.2 Temperature

Research linking the role of ATP with regard to stress induced by temperature fluctuations is sparse. However, it has been shown that increased heat sensitivity was observed in d-subunit mitochondrial ATP synthase knockout lines (ATPd), resulting in reduced growth and abnormal leaf morphology [67]. These results suggest ATPd plays a major role in normal mitochondrial ATP synthase function, and hints at a potential role in heat stress tolerance.

6.3.3 pH

Links between ATP and pH have been unravelled in plants. In Red sage (*Salvia miltiorrhiza*) root cultures, application of extracellular ATP (eATP) led to an increase in medium pH [68]. Interestingly, this increase in pH was suppressed via use of eATP inhibitor RB and a calcium chelator EGTA [68]. These results suggest eATP signal transduction pathways are dependent on plasma membrane localised Ca_2+. Similarly, another existing link between ATP and pH lies in the processes surrounding lactate and ethanol production. In pea seed (*Pisum sativum* var Alaska) and parsnip root (*Pastinaca sativa*) cell extracts, excessive lactic acid production was prevented by ATP and pH controlled lactate dehydrogenase activity [69]. These links further illustrate the extent at which ATP and pH are related.

Stressors	Effects on Plant Physiology	Molecules Involved	Localization of the stressor	Supporting literature
Temperature (Heat Shock)	• Limits growth, productivity and metabolism • Increased water loss • Seed germination inhibition	1. Ca2+ 2. Fe2+ 3. ROS	1. Cytosol 2. Cytosol 3. Multiple subcellular compartments (chloroplast, peroxisomes, apoplast and mitochondria)	1. 26,48 2. 27,8 3. 84,85,86
Salinity	• Reduced growth, biomass and fruit number and size • Limited nutrient and H2O absorption	1. Ca2+ 2. ATP 3. ROS	1. Cytosol, Cortical and Endodermal cell layers 2. Root and leaf plasma membrane	1. 5 2. 61
Osmotic	• Increased risk of infection • Decreased primary production	1. Phytohormones (ABA,SA,JA,ET)	1. Unknown	1. 87
pH	• Decreased/arrested growth • Decreased nutrient availability • Altered microbial diversity	1. Peroxidases, ROS 2. Ca2+	1. Root tip 2. Root tip	1. 60 2. 33

Figure 2: Abiotic stimuli and molecules involved.

7 Missing links

While there is no doubt significant progress has been made regarding the unveiling of the inner mechanisms governing stress responses, there are still plenty of mysteries yet to unveil. Many of the current studies target specific molecules and pathways involved in stress responses. This research strategy often overlooks the mixed effects of other present molecules. While detailed, it fails to provide the bigger picture. This limitation stems from the lack of technology capable of providing information on that level. However, recent technological advances have greatly increased experimental throughput, making it possible to use and adapt to adjacent fields of study. A great example of this is mass spectrometry (MS).

7.1 MALDI-MSI

According to Merriam-Webster, mass spectrometry (MS) is defined as an "instrumental method for identifying the chemical constitution of a substance by means of the separation of gaseous ions according to their differing mass and charge". Due to its ease of use and availability, MS has become a common technique employed by many research groups. It yields information on sample composition by vaporising and ionising a sample via laser or heat. Which then travelling through a vacuum chamber where the vaporised sample is ionised. Then passes through a mass analyzer that determines molecular weight in the form of mass to charge m/z and approximate quantity. Even though all mass spectrometers serve the same purpose, they do not function the same way, they employ different ionisation techniques and utilise various types and quantities of detectors that allow for the identification of a wide array of molecules. Moreover, even though MS methods yield detailed information on sample composition, there are two key pieces of information it fails to provide 1) Location of molecules with respect to the sample 2) Time molecules become visible. These shortcomings led to the development of a new field in MS, Mass Spectrometry Imaging (MSI).

MSI is a relatively new branch of mass spectrometry, developed in 1997 by Richard Caprioli [70]. In time-course experiments, MSI provides spatiotemporal distribution information of various molecular species within a sample with single/couple cell resolution in the form of an image. This is accomplished by using one of two techniques, Microprobe or Microscope [12]. In Microprobe, a small section of the sample is initially ionised, afterwards, a mass spectrum is generated for that specific section, spatial coordinates are saved in conjunction with the spectra. After the whole sample has been analysed, an image depicting locations of desired masses with respect to the sample can be generated [12]. The second technique, Microscope, takes a different approach by utilising ion-optical microscope elements to transfer ions onto a position-sensitive detector and thus creating an image [12].

In plant research, MALDI-MSI has seen limited use in comparison to other eukaryotic organisms, mainly due to the lack of protocols and insufficient spatial resolution. More recently, many advances have been made in the field addressing some of those concerns, however research is still forthcoming. Currently, MALDI-MSI has been used to map metabolites, lipids, ginsenosides, oligosaccharides as well as some peptides and proteins in various regions of plants [11]. Most of the studies conducted thus far are based on creating metabolomic and proteomic profiles. Fewer studies focus on molecular responses elicited in response to an external stressor. Gaining such information is key to understanding signalling processes surrounding cell death and stress perception

and modulation.

7.2 MALDI-MSI Work flow

MALDI-MSI was developed in the late 90s, and overtime protocols have been created to streamline the process. The workflow described below is a general description and various modifications can be implemented to tailor its use to a particular project.

7.2.1 Flash Freezing and Cryosectioning

Just as important as conducting MALDI-MSI analyses is taking the proper steps when conducting tissue sample preparation. An important step in this process is flash freezing, in which the tissue sample is frozen. It usually involves taking the sample shortly after it has been obtained and using a method to rapidly freeze the sample, usually submersion in liquid nitrogen. By freezing the sample, the degradation of molecular species is prevented, this is especially important when analysing molecules that are volatile and rapidly degraded [71].

The next step in sample preparation is cryosectioning. Cryosectioning is conducted as it is impossible to analyse whole tissue samples and an area of focus must be chosen. Parameters influencing cryosectioning include temperature inside the cryomicrotome, tissue thickness and sectioning width. All the aforementioned parameters will greatly influence the detected results in MSI experiments [71].

7.2.2 Matrix Selection and Application

After cryosectioning, matrix sublimation follows, this step is crucial as it is the last step before laser ionisation and has great influence over results obtained from the MSI instrument. An ideal matrix application step will provide both, good ionisation, and reproducible results. To obtain good ionisation, adequate amount and homogeneous matrix deposition is required, these parameters along with matrix chemistry affect matrix crystallisation, which in turn affects ionisation efficiency and greatly impacts visibility of molecular species. Various matrix deposition mechanisms exist such as direct spray, dry coating, and a two-step method [71]. Because of the complex nature of matrix selection and deposition, result replication is often difficult and established protocols are scarce. More research investigating matrix selection and deposition is required to fully reap the benefits of MALDI-MSI.

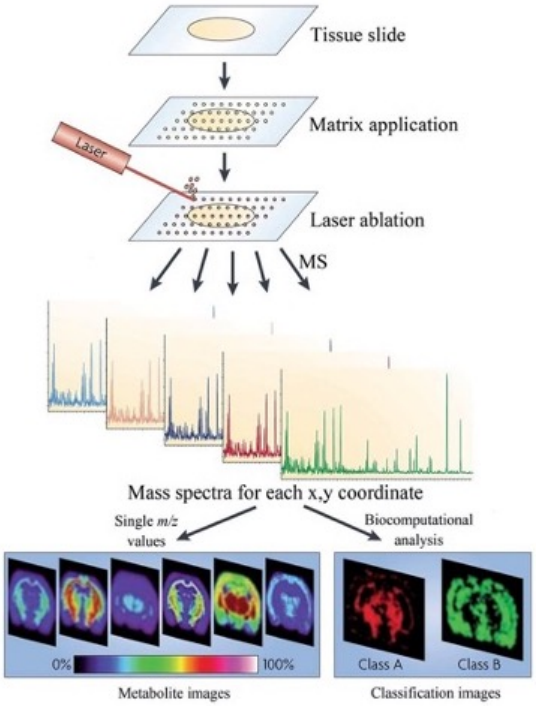

Figure 3: MALDI-MSI workflow. Reprinted from Advances in mass spectrometry-based metabolomics for investigation of metabolites, by Ren, J., 2018, RSC Adv., 2018,8, 22335-22350.

8 Project Description

The goal of this project is to further adapt MALDI-MSI for plant based studies investigating spatiotemporal changes in response to RCD in *arabidopsis* roots. This project constitutes a novel strategy to address fundamental issues related to RCD in plants and will pave the way to future interdisciplinary research. The output of the MSc thesis will not be limited to a better understanding of the spatiotemporal changes in the plant tissue upon RCD. It will also result in the establishment of a novel pipeline that combines localised RCD induction and in situ mass spectrometry.

To achieve this, it is necessary to first develop a system that accurately and reproducibly induces localised cell death using pH as a stressor. Gracas et al. (2021) developed a method that induces semi-localised cell death within the primary root, along the transition and elongation zones following exposure to low pH (Figure 4). Part of this thesis work will be to reproduce these results and set up a streamlined method to trigger localised cell death in a reproducible manner using *arabidopsis thaliana*. Simultaneously, establishment of an efficient method assessing cell death is also required. The second part of this thesis work will focus on developing methods to further cement the use of MALDI-MSI for plant based studies, specifically studies investigating RCD.

8.1 Objectives

The specific objectives of this MSc are:

1. Assess the effect of low pH on growth and development of *arabidopsis* roots.
2. Establish protocols for assessing cell death in *arabidopsis* roots upon low pH and heat stress.
3. Develop a methodology to apply MALDI-MSI technology to study cell death in *arabidopsis* roots.
4. Recommend alternate methods or modifications to further the adoption of MALDI-MSI for plant based studies.

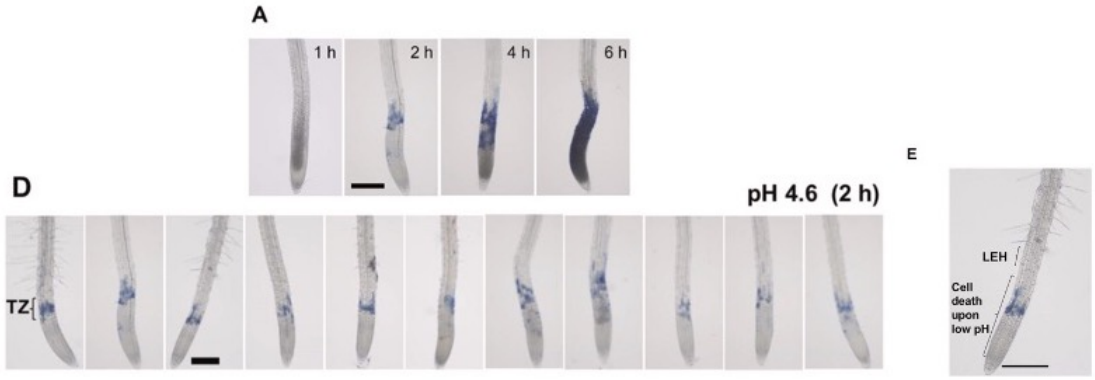

Figure 4: Low pH induces cell death in a specific subset of cells in *arabidopsis* roots. *arabidopsis* seedlings were exposed to a 0.5 mM $CaCl_2$ and 0.6 mM Homopipes buffer (homopiperazine-1,4-bis(2-ethanesulfonic acid)). Roots were stained using Evans Blue. The blue staining is indicative of cell death. Figure modified from Gracas et al.(2020).

9 Materials and Methods

9.1 Half Strength Murashinge and Skoog Medium

Arabidopsis thaliana seedlings were grown in half strength Murashinge and Skoog (MS) medium. The recipe consists of 2.15g MS salts, 5g of sucrose 1%, and 5g of phytagel (only needed if making solid media), for 1 L of media. After weighing, ingredients were mixed and placed in an autoclave for sterilisation.

9.2 Seed Sterilization

Arabidopsis thaliana seeds were sterilised using 70% ethanol, 50/50% mix of bleach and deionised (DI) water, sterile DI water. Seeds were transferred from the stock into a separate eppendorf tube. Afterwards, 1mL of 70% ethanol was pipetted into the eppendorf tube and mixed for one minute after which it was removed. Next, 1mL of a solution containing 50/50 mix of bleach and water was pipetted into the eppendorf tube and mixed for five minutes and then removed.

Lastly, seeds were rinsed three times with 1mL of DI water for one minute. Sterilised seeds were then either transferred into a plate containing solid or liquid MS media. Plates were then sealed using micropore tape, wrapped in aluminium foil and placed inside a fridge for two days before placing them inside the growth chamber for cultivation (16h Day program: 27% light 7.00-23.00, Temperature: 21 C°by day, 19 C°by night, Humidity: 40% by day and 60% by night).

9.3 Staining Techniques

9.3.1 Evans Blue

The Evans Blue staining protocol described pertains to use on *arabidopsis thaliana* root tissue from 5-8 day old seedlings. Evans Blue staining was created by mixing 0.25g Evans Blue stain and 100mL 0.1M $CaCl_2$ solution. Note, it is important to properly mix the solutions mentioned above as to minimise the amount of precipitate formed by the mixture. An orbit shaker set to 100 revolutions/min was used to mix the solution but other stirring methods can be used. After treatments, any leftover solution from an experiment was discarded and plant material was rinsed with DI water. Following rinsing, 4-5mL of Evans Blue solution was added to wells containing plant material for 10 minutes. The plant material-containing wells were washed three times with DI water respectively for 10-15 minutes. Seedlings were imaged with a Zeiss AXIO Zoom.v16 stereoscope 1 day after staining.

9.3.2 SYTOX Green

Protocols vary within the literature, the protocol used in this thesis was used to assess cell death in roots of 5-8 day old *arabidopsis thaliana* seedlings exposed to pH, salinity stress, and osmotic stressors. Moreover, the protocol utilised is heavily based on a protocol developed by Distéfano et al. (2022) with minor modifications [79]. To prepare the SYTOX Green Nucleic Acid Stain, a stock solution containing 5uL of SYTOX green stain and 45uL of DI water was made. Afterwards, 4uL of the 50uL solution was pipetted into each well containing plant material. It is important to note that 50uL of solution is enough for only 12 wells containing one seedling each. After pipetting 4uL of solution into the wells containing plant material, the 12-well plates were incubated in a dark environment for nine minutes. After nine minutes, each plant sample was transferred into a new well containing DI water for rinsing, this step was repeated twice, with each wash lasting a total of three minutes each. Images were obtained minutes after washing using an Invitrogen EVOS M5000 microscope. More details and information about this protocol can be found in the appendix section.

9.4 Treatments

Various pH varying buffers were used in this thesis work with the purpose of inducing cell death. Below are the protocols used for the production of the stock buffer solutions.

9.4.1 Sodium Acetate (NaAc)

To prepare 500mL of 0.1M NaAc buffer, 400mL DI water, 2.89g Sodium Acetate and 0.89g Acetic acid were used. Acetic acid and NaOH were used to balance pH to desired values.

9.4.2 Pipes/Calcium Chloride (Pipes/$CaCl_2$)

Two 100mL solutions of 0.5mM $CaCl_2$ and 0.6mM Pipes (Piperazine-N,N'-bis(2-ethanesulfonic acid)) were made using a mixture containing 0.5mL $CaCl_2$ and 60mL Pipes and 39.5mL DI water. Solutions were pH adjusted using 0.1M NaOH and 0.1M HCL.

9.4.3 2-(N-morpholino)ethanesulfonic acid (MES)

To prepare 500mL of a 0.5M MES buffer solution, 400mL DI water, 48.81g MES free acid, 0.1M NaOH and 0.1 HCL is needed. DI water was mixed with the MES free acid and pH was adjusted using NaOH and HCL, remaining volume of DI water was poured after solution was pH balanced.

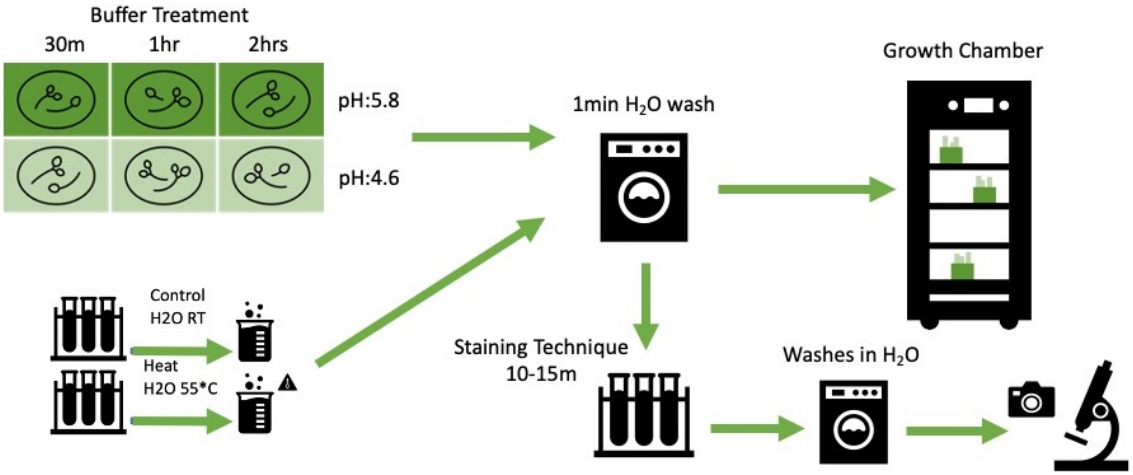

Figure 5: : Graphical illustration of experimental design used to induce and assess stress-induced cell death in *arabidopsis thaliana* seedlings.

10 Results

10.1 Primary Root Cell Death patterns in Wild-Type *arabidopsis thaliana* seedlings upon different pH buffer exposure

In an effort to trigger a pH-induced, localised cell death response as described in Gracas et al. (2021), *arabidopsis* seedlings were exposed to a variety of buffers at two pH conditions: Normal (pH:5.8) and Low (pH:4.6). This section will discuss some of the staining patterns obtained from Evans Blue and/or SYTOX Green cell death staining assays. Evans Blue staining is a technique commonly used to assess cell death in eukaryotes, it stains cells by penetrating through destabilised or ruptured membranes [88]. Stressors are known to induce membrane destabilisation in plants, resulting in an increased uptake of Evans Blue in comparison to non-treated controls.

Stained cells can be both qualitatively and quantitatively assessed using bright field microscopy and spectrometry methods respectively [88]. In this work, Evans Blue was used to observe localization of cell death following various buffer treatments. In addition to Evans Blue, SYTOX Green nucleic acid stain was also used to further confirm location and extent of cell death displayed by Evans Blue staining. SYTOX dyes are commonly used as a quick and easy method for assessing cell viability, SYTOX Green nucleic acid stains are impermeant to live cells and stain only DNA of cells with compromised plasma membranes. This type of staining technique is often paired with confocal laser-scanning microscopy technology to qualitatively and quantitatively assess extent of cell death [89].

10.1.1 NaAc

Normal and Low pH Two pH values (5.8 and 4.6) were selected to observe pH induced effects on cell death induction in *arabidopsis* roots. A "normal" pH value of 5.8 was used as a control as it is widely considered a pH value plants are commonly exposed to. Additionally, various studies investigating pH induced effects on plant physiology have utilised pH: 5.8 as a control with great success [29, 32, 33]. Furthermore, the MS growth media used for cultivation has a pH value of 5.8. As one of the objectives of this master thesis was to induce localised cell death via use of low pH, a pH value of 4.6 was selected. This decision was in part made based on the results obtained by Gracas et al. (2021). Gracas demonstrated that upon low pH exposure (pH: 4.6), *arabidopsis* seedlings displayed a unique, semi localised cell death response in cells located along transition and elongation zone (Figure 4)[33].

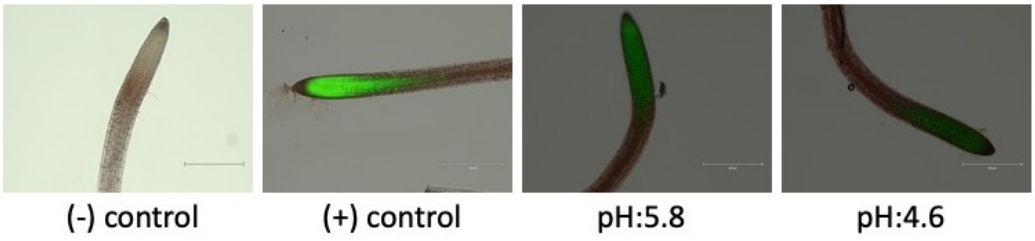

Figure 6: Cell death in *arabidopsis* primary roots following exposure to 50mM NaAc buffer under control (pH: 5.8) and Low (pH: 4.6) conditions for 30m. Controls were immersed in deionized water, (-) control was heated at 55C°for 15m. Stained using SYTOX Green nucleic acid stain. Green fluorescence indicates dead tissue.

Time dependent exposure This work mainly explores the time effects of several buffers at three different time points: 30 minutes, 1 hour, and 2 hours, though separate experiments using other time points were used.

Normal pH Upon exposure to 50mM NaAc buffer at pH:5.8, seedlings exhibited a distinct staining pattern at all three time points (Figure 7A Top) using Evans Blue stain. The pattern exhibited can be characterised by the "whitening" of the primary root cap followed by a dark blue stain indicative of cell death, located along the meristematic and elongation zones. Moreover, as exposure time increases, so does the extent of Evans Blue penetration into the primary root end (Figure 7A Top). Taken together, these results suggest a relationship exists between exposure time

and extent of cell death using NaAc buffer at pH 5.8. This is surprising since this pH value is commonly used as a control value in various studies investigating pH related responses. Similar results were obtained when primary roots were stained with SYTOX Green nucleic acid stain. Two timepoints were used testing 50mM NaAc pH:5.8, 15m (Data not shown) and 30m (Figure 6). Results obtained from 30m exposure time, indicate cell death is spread throughout the root cap, meristematic and transition zones of the primary root.

Low pH As aforementioned, three main time points (30m, 1hr and 2hrs) were used in the majority of experiments exploring pH-induced cell death. To induce a low pH response, a 50mM NaAc buffer with a pH of 4.6 was used. Following exposure to low pH conditions, seedlings exhibited a staining pattern different from the one triggered at the "normal" pH. The primary roots displayed a unique "deep blue" staining pattern when stained with Evans Blue stain. Similarly, staining was localised along the root cap, meristematic and elongation zones, and was visible throughout all time points (Figure 7A Bottom). This result suggests complete cell death of the primary root upon pH 4.6, in contrast with pH 5.8 where cell death is limited to zones above the root cap.

Bleaching In addition to displaying a different staining profile, *arabidopsis* seedlings exposed to 50mM NaAc buffer at pH:4.6 bleached post experiment (Figure 7B Bottom). A bleaching response occurred 1-2 days post experiment, and was only evident in NaAc buffers at low pH and heat treated controls.

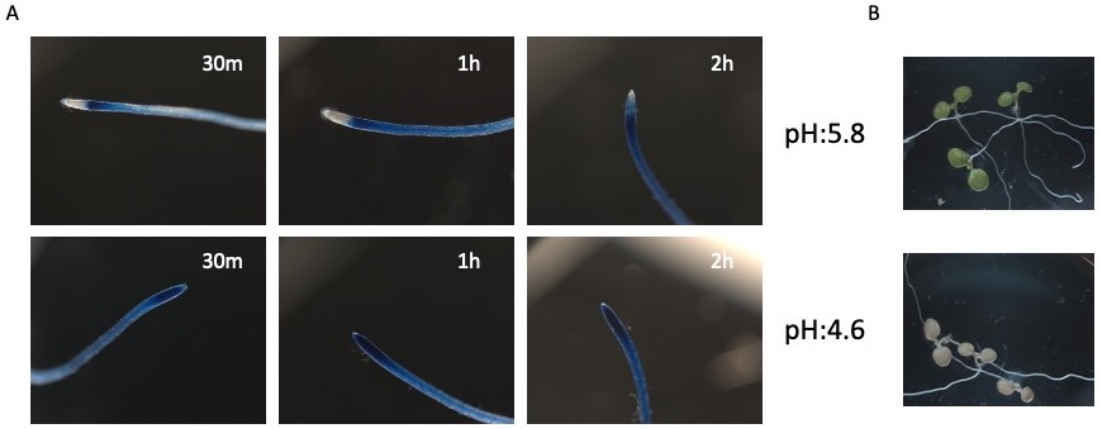

Figure 7: Cell death in *arabidopsis* primary roots following exposure to a 50mM NaAc buffer under control (Top, 5.8) and low (Bottom, 4.6) pH conditions. A: *Arabidopsis* seedlings exposed to 50mM NaAc buffer at 30m, 1h and 2hrs. Seedlings were stained using Evans Blue stain to evaluate cell death. Blue indicates dead tissue. B: Picture showing entire seedlings after exposure to different pH treatments.

Concentration dependent exposure

Normal pH In addition to time, the effects of varying concentrations of NaAc buffer were also investigated to assess whether the observed phenotype could be related to osmotic stress. NaAc buffer pH:5.8 was tested at concentrations of 10 and 50mM in *arabidopsis thaliana* seedlings for one hour (Figure 8 Top). Various experiments displayed a relationship between buffer concentration and Evans Blue uptake. As the concentration of NaAc buffer increased, so did absorption of Evans

Blue dye. This result indicates that as buffer concentration increases, it leads to increases in cell membrane disruption and consequently cell death. This can be in part due to osmotic and/or sodium toxicity effects.

Low pH Experiments testing several buffer concentrations at low pH conditions were also tested. Evans Blue staining patterns remained the same throughout different concentrations (Figure 8A Bottom). Furthermore, the staining pattern obtained at a low pH can be described as a deep blue colour located along the primary root end, and is suggestive of complete primary root death.

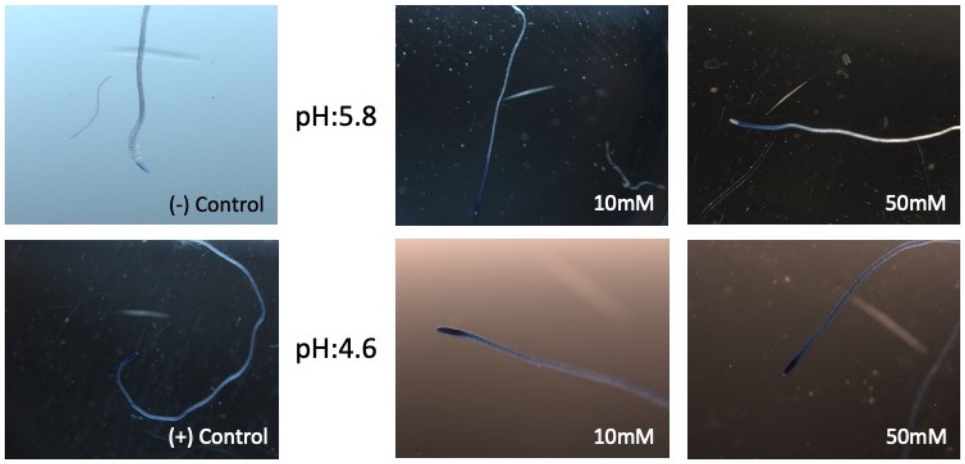

Figure 8: Cell death of *arabidopsis* primary roots after 1h of exposure to NaAc buffer under control (pH:5.8) and low (pH 4.6) conditions at varying concentrations. *Arabidopsis* seedlings were exposed to NaAc buffer at control (pH: 5.8, Top) or low (pH: 4.6, Bottom) at different concentrations for 1 hour. Control seedlings were submerged in deionized water, (-) control was heated at 55C°for 15m. Seedlings were then stained using Evans Blue to evaluate cell death. Blue indicates dead tissue.

10.1.2 Pipes/$CaCl_2$

Multiple buffers were tested to assess cell death extent in an attempt to replicate results obtained by Gracas et al. (2021). A buffer closely resembling the one utilised by Gracas et al. (2021) consisting of 0.6mM Pipes and 0.5mM $CaCl_2$ solution was tested at two pH conditions (Normal: 5.8 and Low: 4.6). Tested pH values were chosen based on observed results from Gracas et al. (2021).

Time dependent exposure

Normal pH At a pH of 5.8 *arabidopsis* seedlings exposed to a Pipes/$CaCl_2$ buffer showed inconclusive Evans Blue staining patterns (Figure 9A Top). Seedlings displayed minimal staining and staining intensity was lower compared to seedlings treated with NaAc buffers. Furthermore, no evident staining patterns were observed at any time point. Moreover, after buffer treatment, seedlings were transferred back into a growth enhancing environment to assess post exposure growth behaviour. Results indicated seedlings resumed normal growth via mainly primary root (Figure 12).

Low pH At low pH conditions, seedlings exposed to Pipes/$CaCl_2$ buffer displayed similar results as the Pipes/$CaCl_2$ pH: 5.8 treated seedlings (Figure 9B). Additionally, no clear Evans Blue staining patterns were observed at the tested time points. Moreover, when transferred back into growth enhancing conditions, seedlings displayed similar growth responses as seedlings exposed to pH: 5.8, and no primary root death was evident (Figure 12). In conclusion, no concrete results linking pH-induced cell death using Pipes/$CaCl_2$ buffer were observed.

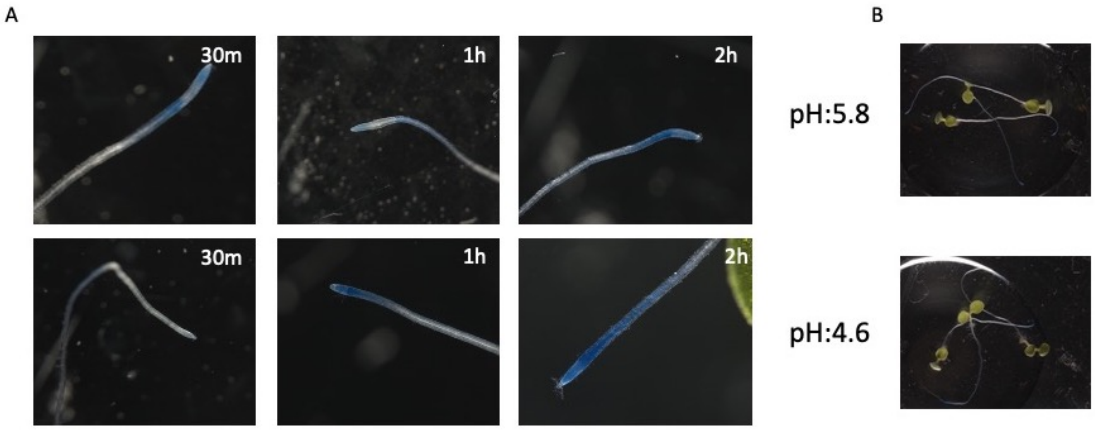

Figure 9: Cell death in *arabidopsis* primary roots following exposure to a 0.6mM Pipes/0.5mM $CaCl_2$ buffer under control (Top, 5.8) and low (Bottom, 4.6) pH conditions. A: *Arabidopsis* seedlings exposed to Pipes/$CaCl_2$ buffer at 30m, 1h and 2hrs. Seedlings were stained using Evans Blue stain to evaluate cell death. Blue indicates dead tissue. B: Picture showing entire seedlings after exposure to different pH treatments.

10.1.3 MES

The effects of MES buffer on pH-induced cell death were also investigated in this work. MES buffer is a common buffer extensively used in the plant research community. Therefore, it was selected to address its effect on *arabidopsis* seedling primary roots. Extensive research suggests a pH value of 5.7 is suitable for use in *arabidopsis thaliana*. In this work, a 10mM and 50mM MES buffer with two pH values (Normal: 5.7 and low: 4.6) were used.

Time dependent exposure Similar to previous experiments, *arabidopsis* seedlings were exposed to a 10mM and 50mM MES buffer at two pH values for 30 minute, 1 hour and 2 hour intervals. Similarly, extent of cell death was evaluated using Evans Blue staining and a post-exposure growth assay.

Normal pH At pH: 5.7, MES treated seedlings displayed similar staining patterns as ones treated with NaAc buffer pH: 4.6. Seedlings displayed a deep blue staining pattern indicative of primary root death at all time points (Figure 10A Top). Moreover, the extent of cell death was similar throughout all time points, suggesting deleterious effects of MES buffer at 50mM concentration occur at pH: 5.7. Further confirming these results, a post buffer exposure assay revealed a stress response was triggered at all tested time points (Figure 13).

Low pH When exposed to pH: 4.6, *arabidopsis* seedlings displayed an indistinguishable Evans Blue staining pattern compared to MES buffer treated seedlings at pH: 5.7 (Figure 10A Bottom). Similarly, seedlings also responded by exhibiting stress responses in the form of adventitious rooting (Figure 14).

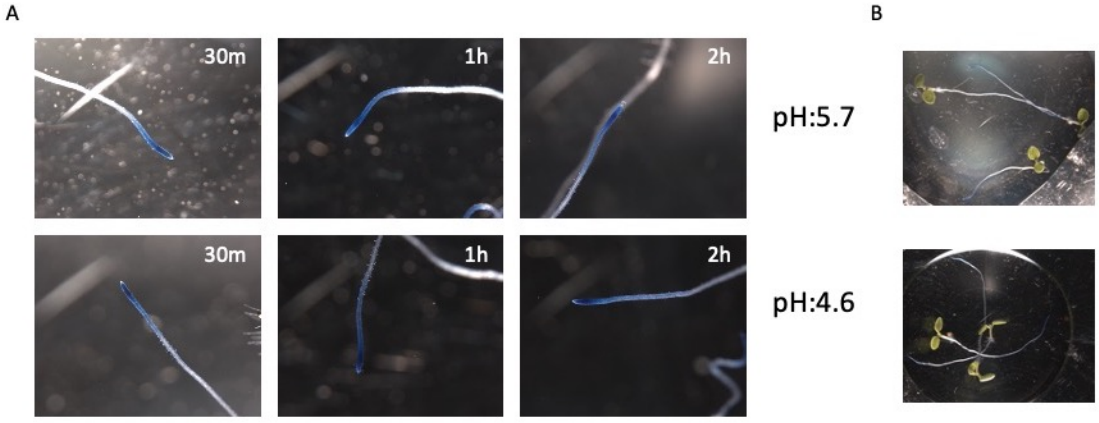

Figure 10: Cell death in *arabidopsis* primary roots following exposure to a 50mM MES buffer under control (Top, 5.7) and low (Bottom, 4.6) pH conditions. A: *Arabidopsis* seedlings exposed to 50mM MES buffer at 30m, 1h and 2hrs. Seedlings were stained using Evans Blue stain to evaluate cell death. Blue indicates dead tissue. B: Picture showing entire seedlings after exposure to different pH treatments.

Concentration dependent exposure An experiment was conducted assessing concentration effects on cell death induction using a 10mM and 50mM MES buffer at three time points (15, 30 and 60 minutes). Experiment only utilised MES buffers at pH: 5.7

Normal pH At pH: 5.7, *arabidopsis* seedlings all displayed Evans Blue staining patterns indicative of primary root cell death (Figure 8A Top). Moreover, supporting this observation, post buffer exposure growth assays revealed arrested primary root growth and triggering of a stress response in the form of increased adventitious rooting (Figure 13). Moreover, the stress response was visible in both 10mM and 50mM concentrations and at all tested time points.

10.2 Changes in Root Morphology

As mentioned above and evidenced by various experiments, several staining patterns were obtained from exposing *arabidopsis* seedlings to various buffers and pH conditions. Most notably, a deep blue colour was observed in meristematic and elongation zones in NaAc (Figure 7) and MES (Figure 10) treated seedlings. Leading to speculation as to the actual meaning of the cell death displayed at the primary root end.

To test the relationship between the staining patterns observed and post exposure growth effects, an assay testing post experiment growth behaviour was developed. After any given buffer treatment, half of the treated seedlings were stained using a cell death assay and the other unstained half were transferred back into a growth media containing plate and placed back inside a growth chamber to observe post-experiment growth behaviour.

Results obtained showed a clear relationship between the deep blue Evans Blue staining pattern and primary root death (Figure 17). Primary roots displaying a deep blue colour, namely NaAc and MES treated seedlings failed to continue growing post buffer exposure when placed back in a growth enhancing environment. Moreover, morphology changes were also evident in stained areas compared to untreated primary roots (Figure 17). Primary roots displaying a deep blue staining pattern displayed altered primary root end morphology and arrested growth compared to their untreated counterparts. These results led to the conclusion that seedlings displaying a deep blue Evans Blue staining pattern also suffered from primary root death.

A common response to stressors is the propagation of adventitious roots [90]. In plants, this response is commonly exhibited as an attempt to increase chances of survival. In this work, adventitious rooting was triggered via use of various buffers under varying pH conditions and exposure times. This section will discuss observed adventitious rooting responses in the buffers utilised.

10.2.1 NaAc

Adventitious rooting was observed in NaAc buffer treated *arabidopsis* seedlings under several circumstances. Stress responses associated with adventitious rooting were observed in all seedlings exposed to pH: 5.8 at all tested time points, and buffer concentrations (Figure 11). Moreover, seedlings treated with 50mM NaAc buffer exhibited an adventitious rooting stress response two days post experiment (Figure 19). Under low pH conditions, *arabidopsis* seedlings only displayed adventitious rooting at concentrations of 10mM (Figure 11). Upon exposure to buffer concentrations of 50mM at pH: 4.6, seedling leaves bleached and no further growth was observed in the leading days (Figure 20).

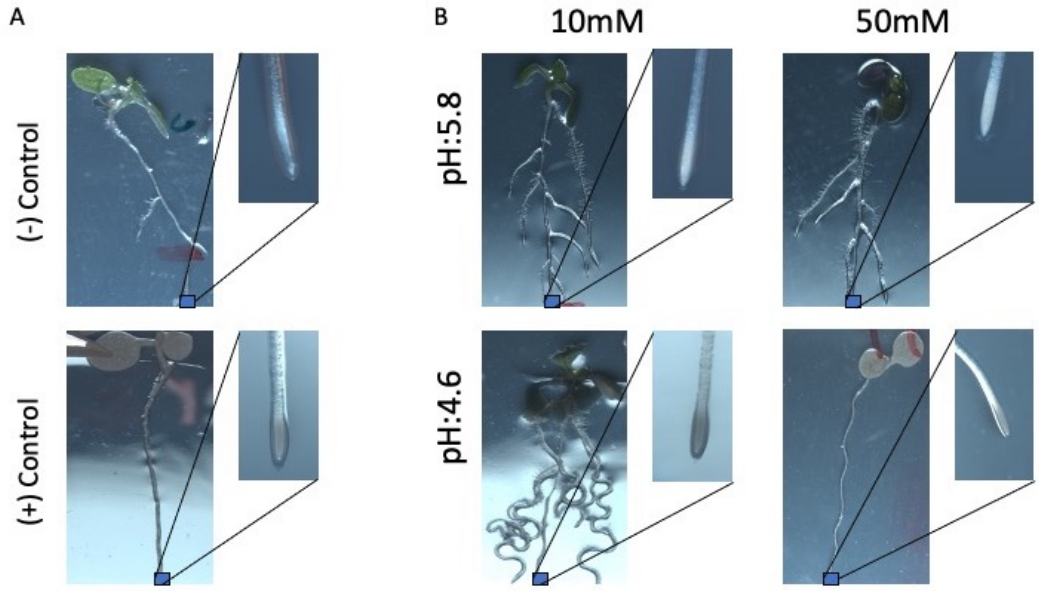

Figure 11: Growth of *arabidopsis* primary roots after of exposure to NaAc buffer pH 5.8 and pH:4.6 at varying concentrations. *Arabidopsis* seedlings were exposed to NaAc pH 5.8 (Top) or 4.6 (Bottom) at different concentrations for 1h. (-) Control was treated with deionized water, (+) control was heated at 55C°for 15m in deionized water. Seedlings were then transferred to an $\frac{1}{2}$ MS plate and kept in the growth chamber for 10 days. Pictures were taken 7 days after transfer.

10.2.2 Pipes/$CaCl_2$

In treatments using Pipes/$CaCl_2$ buffer, no changes in root morphology was observed at any pH or tested time point (Figure 12). Additionally, as previously mentioned, no distinguishable cell death staining patterns were observed (Figure 9). Moreover, primary root growth continued post exposure in unstained seedlings (Figure 12). Thereby, no conclusion can be made on the effects of Pipes/$CaCl_2$ buffer on adventitious rooting responses.

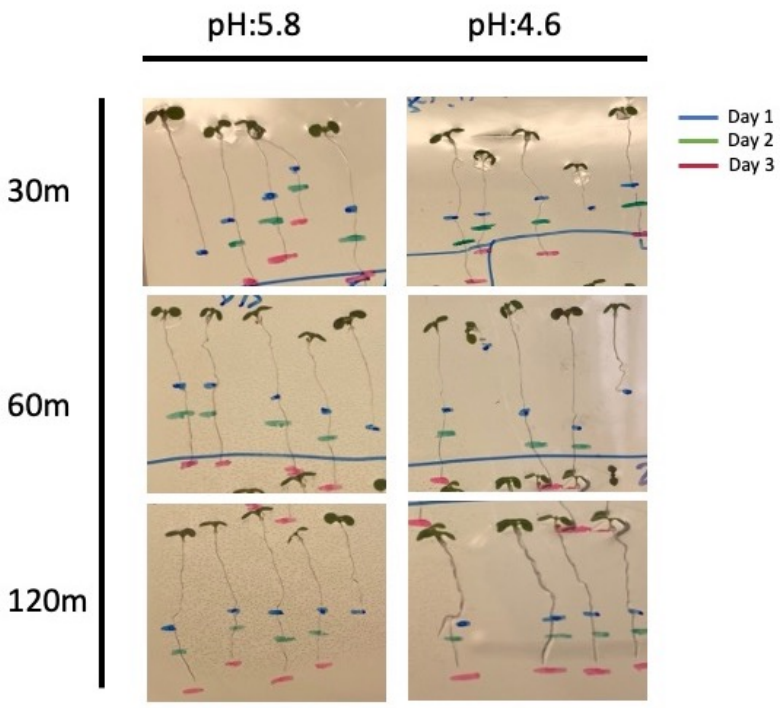

Figure 12: Post Pipes/$CaCl_2$ buffer exposure growth behaviors in wild-type *arabidopsis*. Pipes/$CaCl_2$ treated seedlings were exposed to normal (pH:5.8) and low (pH:4.6) pH conditions at varying time points. After buffer exposure, seedlings were transferred into $\frac{1}{2}$ MS media containing plates. Images were taken 3 days after buffer exposure; colored lines indicate daily primary root growth.

10.2.3 MES

In MES treated *arabidopsis* seedlings, stress induced adventitious rooting was observed at all tested timepoints (15, 30, 60, 120 minutes) and pH values (5.7 and 4.6) (Figure 14). Coupled with Evans Blue data (Figure 10), these results suggest MES buffer triggers stress-related responses, and exposure results in primary root death in *arabidopsis* seedlings.

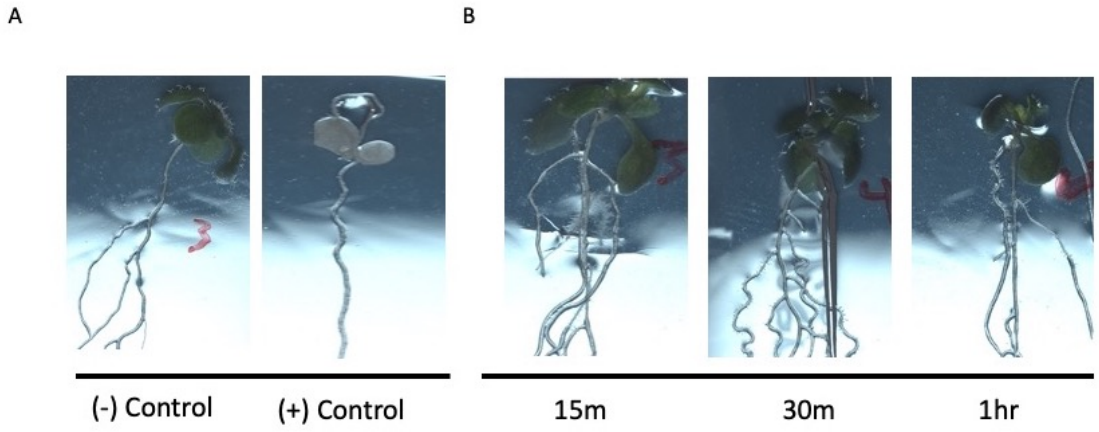

Figure 13: (A) Control and (B) post 50mM MES buffer (pH:5.7) exposure growth behaviors in wild-type *arabidopsis* roots. Images were taken 7 days after transfer to $\frac{1}{2}$ MS media plate. A: Negative (Left) and positive (Right) controls. (-) Control was heated at 55C°for 15m in deionized water prior to imaging. B: Post MES buffer exposure stress response at 15, 30m and 1hr.

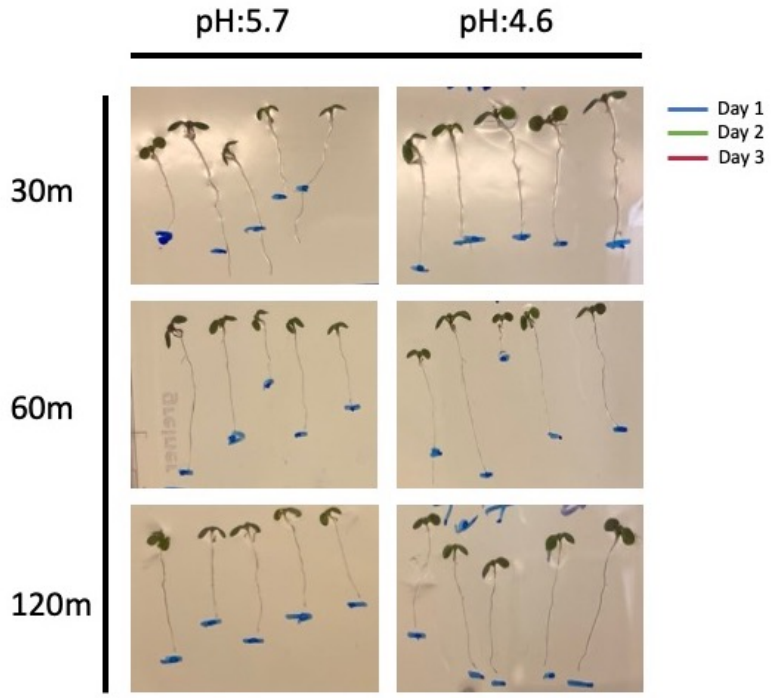

Figure 14: Post 50mM MES buffer exposure growth behaviors in wild-type *arabidopsis*. MES treated seedlings were exposed to normal (pH:5.7) and low (pH:4.6) pH conditions at varying time points. After buffer exposure, seedlings were transferred into $\frac{1}{2}$ MS media containing plates. Images were taken 3 days after buffer exposure; colored lines indicate daily primary root growth.

10.3 Sample Preparation: Cryosectioning and Staining

Prior to conducting MALDI-MSI studies, sample cryosectioning and morphology stainings must be conducted. Cryosectioning and staining steps were conducted at the Cellular and Molecular Imaging Core lab (CMIC) in St. Olav's Hospital, Trondheim.

10.3.1 Sample Preparation

Sample preparation began with the flash freezing of the tissue sample. Tissue samples were first mounted on a glass slide and then covered in a proprietary embedding media. After embedding, tissue samples and embedding media were flash frozen using liquid nitrogen. After which the sample was removed from the slide and mounted on a cork square (Figure 16A), and dipped in liquid nitrogen. After a couple minutes samples were removed and stored for sectioning.

10.3.2 Cryosectioning and Morphology Staining

Cryosectioning was made possible via use of a Fisher Scientific Epredia™ CryoStar™ NX70 Cryostat. *Arabidopsis* samples were sliced in 10-15uM sections and mounted on glass slides for morphology studies. Hematoxylin and eosin (H&E) staining is a staining technique commonly used in histology studies, H&E stain binds to nuclear DNA and ribosomal RNA and turns purple. In this thesis it was used to assess morphology of sectioned plant material by providing contrast (Figure 16B). Upon H&E staining, sectioned plant material became visible. Moreover, due to complications in the sectioning step, no complete sections of plant material were obtained. Instead, fragments of plant material were deposited onto glass slides (Figure 16B).

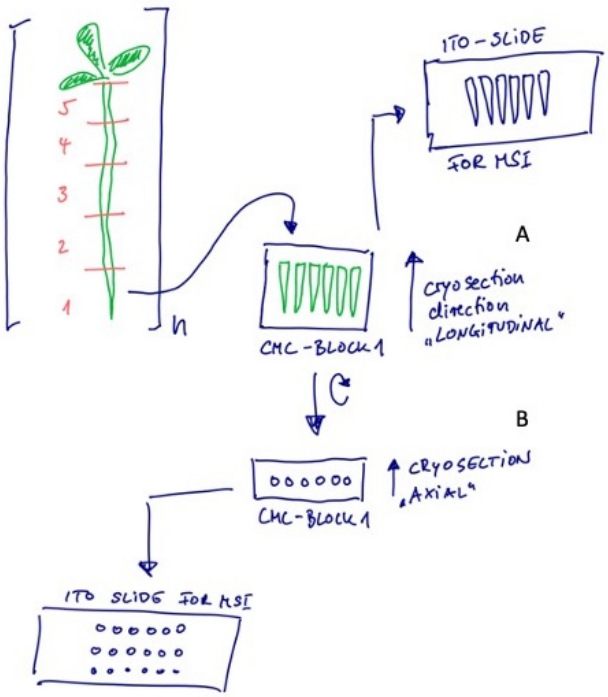

Figure 15: Scheme illustrating cryosectioning directions for plant material. A: Longitudinal, B: Axial. Illustrated by Sebastial Krossa.

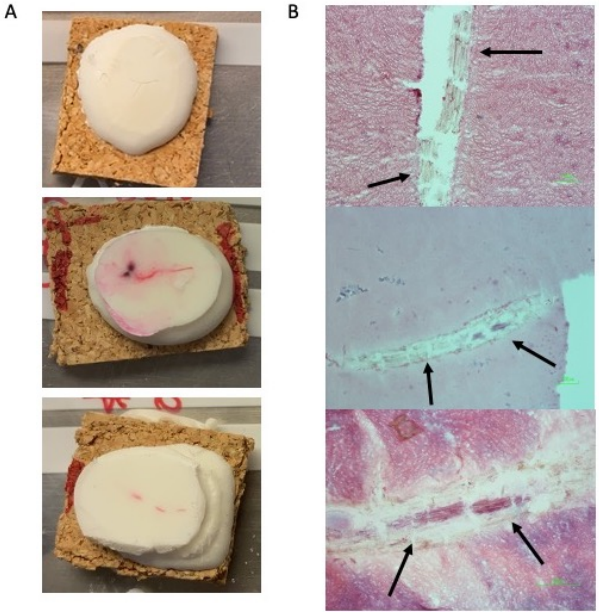

Figure 16: Sample preparation and H&E morphology stain post cryosectioning. A: Plant tissue embedding pre (Top and Middle) and post (Bottom) cryosectioning. B: H&E staining of sectioned plant material. Arrows point towards sectioned plant material.

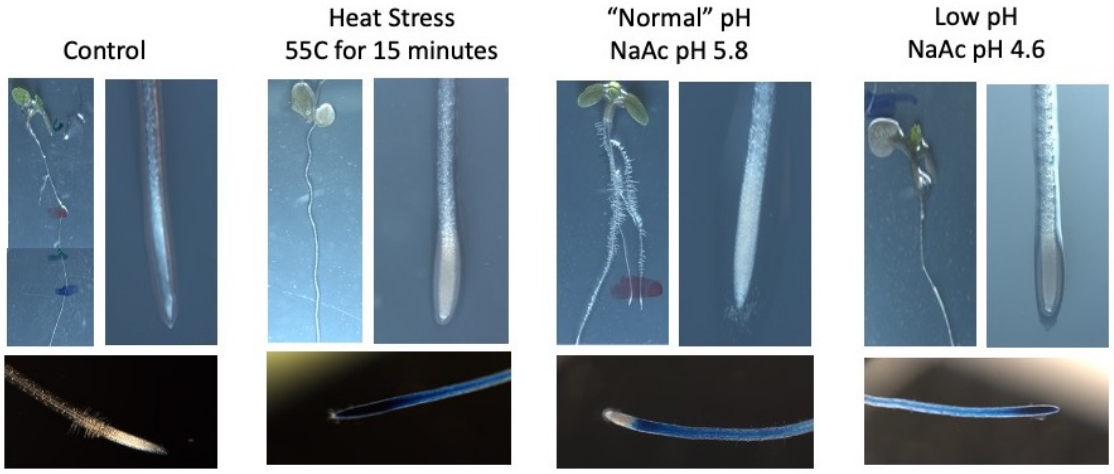

Figure 17: Summary of growth behavior (Top Left) and primary root morphology (Top Right) responses to abiotic stimuli in *arabidopsis*. Primary root cell death staining patterns (Bottom) in response to various abiotic stimuli. Stained using Evan's blue stain. Blue is indicative of tissue death.

11 Discussion

11.1 Buffer Selection

Buffer selection is often one of the most overlooked aspects of experimental design. It has become increasingly easy to utilise buffers that have been commonly used throughout the literature to test variables such as: concentration, pH, and temperature induced effects, and lose sight of the potential buffer chemistry induced effects. Moreover, literature reviewing the effects of various buffers on plant physiology is limited. In this work various buffers, concentrations, pH values, and exposure times were tested in an attempt to induce a localised pattern of cell death.

One of the main observations was the lack of consistent responses throughout the various experiments. As evidenced by the various staining patterns observed (Figure 7,10,9) following buffer exposure it became increasingly clear that buffer chemistry plays an important part in cell death induction, and they are not all the same in function. For example, even though the staining response was similar in both low pH MES and NaAc-treated seedlings at a concentration of 50mM, they exhibited different post-exposure growth patterns (Figure 10,7). Given these results, careful consideration should be given to buffer selection, and interpretation of results obtained.

11.2 Buffer induced Cell Death

11.2.1 NaAc

Unexpected results obtained by exposing *arabidopsis* seedlings to a seemingly "normal" pH value of 5.8 in NaAc buffer led to questioning concerning the type of stress seedlings were actually subjected to. Extensive cell death mainly located at the primary root end was observed in both Evans Blue and SYTOX green cell death assays (Figure 7,6). Moreover, in post-exposure assays conducted, the primary root end in NaAc treated seedlings changed both in morphology and colour when compared to the untreated control (Figure 11). These results along with the development of an adventitious rooting response indicated NaAc buffer at pH: 5.8 was not a stable pH condition for *arabidopsis* seedlings. One of the hypothesised culprits for the unexpected cell death patterns was osmotic stress. To test the effect of osmotic stress on seedlings, multiple concentrations of NaAc buffer were tested at different time points (Figure 8). Results displayed similar staining and post-exposure growth phenotypes at all tested concentrations (Figure 8,11). Interestingly, when concentration of NaAc buffer pH: 4.6 was dropped to 10mM, seedlings exhibited the same stress response as pH: 5.8 treated seedlings at all concentrations. This result suggests concentration of NaAc buffer and pH act in concert to induce stress responses and cell death in seedlings. In a similar study surveying the effects of MES buffer on root growth, it was found that a 1% concentration MES buffer was sufficient to significantly inhibit root growth [91]. Taken together, these results demonstrate that root cells are capable of sensing and responding to the slightest change in osmotic differences.

Another plausible and likely culprit for the cell death shown at the normal pH is sodium toxicity. Sodium stress has been long known to cause arrested growth as well as cell death in primary roots in *arabidopsis* [21, 92]. Moreover, a major characteristic sodium stress is the development of adventitious roots. Even though experiments using NaCl at similar concentrations were not

conducted in this work, research indicates NaCl exposure can lead to adventitious rooting responses in *arabidopsis* [21, 92]. Furthermore, *arabidopsis thaliana* seedlings have been shown to undergo sodium stress associated responses at concentrations as low as 20mM using NaAc [93]. Similarly, In this work, extensive cell death was observed at the primary root end when utilising a NaAc buffer at concentrations as low as 10mM. These results 1) confirm sodium is a potent stressor at low concentrations and 2) suggest the paired ion has a minimal effect on inducing stress.

Another potential explanation for the cell death exhibited is pH stress. A clear relationship exists between seedlings treated using pH: 5.8 and 4.6. Seedlings treated at low pH exhibited complete, non-reversible cell death compared to their pH: 5.8 counterparts (Figure 11). Furthermore, Gracas et al. (2021) observed cell wall disturbances along with arrested elongation in *arabidopsis* seedlings exposed to a low pH solution [33]. However, the lack of literature on effects of NaAc buffer on plant cell death and physiology make it difficult to draw conclusions.

In conclusion, unravelling the exact mechanisms by which cell death occurred in NaAc treated seedlings is not a straightforward process. It is likely that a combination of the factors listed above led to the distinct cell death patterns and post-exposure phenotypes observed in the NaAc treated seedlings. Further research investigating the mechanisms by which NaAc buffers induce cell death and associated stress responses is required to fully unravel the results obtained in this work.

11.2.2 MES

Because of its effective buffering capacities at pH ranges 5.5-7.0, MES buffer has been extensively used in plant media cultures at concentrations of 2.5-10mM [91]. However, studies assessing its impact on root growth are concerningly limited. In this thesis work, the impact of MES treated *arabidopsis thaliana* seedlings on root growth and adventitious rooting was investigated. *Arabidopsis* seedlings were exposed to two concentrations (10mM and 50mM) at various time points (15, 30, 60 and 120 minutes) (Figure 14). The most notable result was the arrested primary root growth and development of adventitious roots (Figure 13). The aforementioned results were observed at all concentrations, pH, and time points tested. Similarly, a separate study investigating the effects of MES buffer at concentrations of 0-1% found root growth suppression, decreased meristem length, and root hair number at 1% MES concentrations [91].

pH induced MES buffer effects pH induced buffer effects were investigated by subjecting *arabidopsis* seedlings to MES buffer at pH: 5.7 and 4.6, as seedlings in both treatments stained deep blue. No significant staining pattern differences were detected using Evans Blue cell death staining assay (Figure 10). Furthermore, seedlings transferred back into a growth enhancing environment post buffer exposure, displayed similar responses, namely arrested primary root growth and adventitious root development in both tested pH values (Figure 14). This data suggest pH is an unlikely cause for the arrested growth and adventitious root development observed. However, further research observing the relationship between MES buffer and pH has to be conducted. This can be accomplished by quantifying responses such as primary root growth, number and length of adventitious roots to name a few.

Concentration induced effects In addition to the two pH values tested, two concentrations (10mM and 50mM) were used to test concentration induced effects in MES buffer treated seed-

lings. No major differences were observed in cell death staining assays and post-exposure growth behaviour at both concentrations. These results suggest that after exposure to a certain concentration, responses to MES buffers become uniform in nature. According to Kagenishi et al. (2022), deleterious effects of MES buffer in *arabidopsis* begin at concentrations of 1% MES [91]. These results suggest a 10mM MES buffer was sufficient to induce non-reversible cell death. Further experiments testing lower concentrations are required in order to uncover the relationship between MES concentration and exposure-related effects. In conclusion, based on the results obtained in this work, caution should be exercised when interpreting results utilising MES buffer as either a control or treatment.

11.2.3 Pipes/$CaCl_2$

In an effort to replicate results obtained by Gracas et al. (2021), *arabidopsis thaliana* seedlings were treated with a 0.6mM Pipes 0.5mM $CaCl_2$ buffer to induce a localised cell death response. Gracas et al. (2021) reported that exposure to low pH (4.6) Homopipes/$CaCl_2$ buffer for 2 hours resulted in a localised cell death response along the transition and elongation zone (Figure 4). Since no Homopipes reagent was readily available, it was substituted by Pipes in the experiments. Furthermore, results were inconsistent amongst each treatment group (Figure 9). Lacking any localised and specific staining patterns, no relationship could be observed from the use of Pipes/$CaCl_2$ buffer for cell death induction. The difference in results could be attributed to the use of Pipes instead of Homopipes reagent. However, further testing and method adjustment is necessary to potentially replicate the results described.

11.3 Incorporation of MALDI-MSI for Plant Based Research

Part of the aim of this master thesis was to explore the potential use of MALDI-MSI technology for use in plant cell death studies. Unfortunately, due to time constraints and tissue sectioning issues, it was not possible to conduct MALDI-MSI experiments. However, this section aims to provide the reader with information concerning steps prior to conducting analyses. More specifically, sample preparation and sectioning.

One of the first steps when conducting MALDI-MSI experiments is sample preparation. During sample preparation, the sample is flash frozen and embedded in a media for cryosectioning. The first issue encountered was the extensive handling of plant tissue. Because of its uneven nature, mounting root tissue on a flat glass slide is a major challenge that involves extensive handling of the sample. In turn, this extensive handling may potentially affect the obtained results. One potential solution is changing the direction root tissue was sectioned. In this work, root material was sectioned in parallel to the direction of root growth into longitudinal sections of 10-15uM. Moreover, because the root tissue was unevenly seated on the glass slide, it led to uneven sectioning (Figure 16). By changing the direction of sectioning to horizontal the problem of uneven sectioning is minimised (Figure 15).

Furthermore, another major challenge presented during the cryosectioning stage was the actual sectioning of the plant tissue. Because seedlings are small in size and diameter, it is incredibly difficult to effectively section off plant material. Plant tissue used in this work ranged from 5-7 days old, prior work successfully using MALDI-MSI for root analysis utilised 42 day old plants

[94]. In addition to using mature plant material, other plant research groups have had success using MALDI-MSI by implementing strategies such as: use of specialised matrices and electronically controlled deposition methods, laser beam size reduction or a different ionisation method LDI [95, 96, 94]. Similarly, given the manual operation of the cryosectioning equipment, further developments streamlining the cryosectioning process via use of AI or cameras are necessary for effective sectioning at smaller scales.

In conclusion, even though several challenges remain in the road to making MALDI-MSI a standard technology for plant based studies, significant progress has been made thus far. By streamlining sample preparation steps and meticulously testing variables involved in tissue cryosectioning it is possible to effectively utilise MSI technology and harness it to its full potential. This will in turn amplify the generation of data and aid in increasing our current knowledge of plant biology

12 Supplementary Information

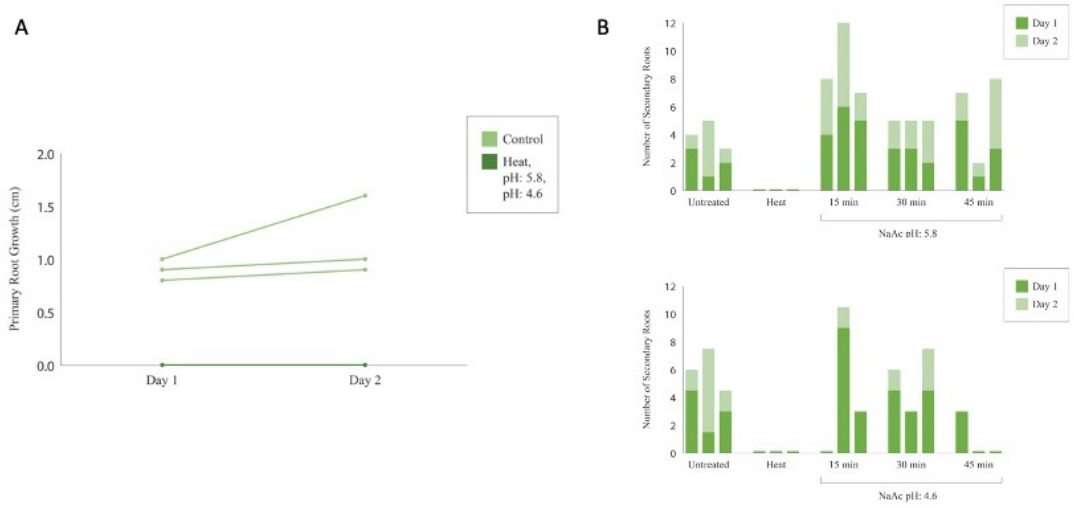

Figure 18: Primary (A) and secondary root growth behavior (B) 2 days post 10mM NaAc buffer exposure. A: Post experimental primary root growth under control (Light green) and abiotic factors (Dark green). B: Post experimental secondary root growth under untreated and abiotic factors (Top: pH:5.8) (Bottom pH:4.6).

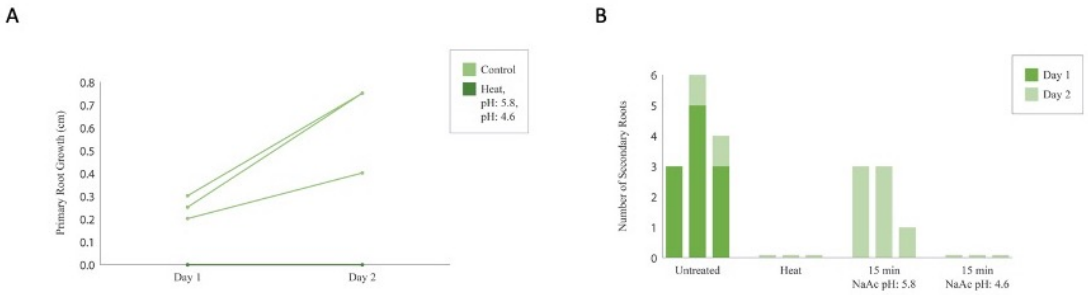

Figure 19: Primary (A) and secondary root growth behavior (B) 2 days post 50mM NaAc buffer exposure. A: Post experimental primary root growth under control (Light green) and abiotic factors (Dark green) at control (Light green). B: Post experimental secondary root growth under untreated and abiotic factors at pH: 5.8 and 4.6.

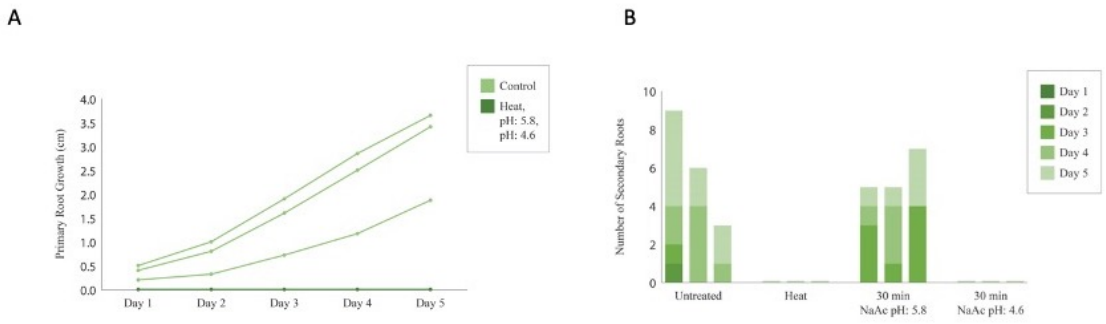

Figure 20: Primary (A) and secondary root growth behavior (B) 5 days post 50mM NaAc buffer exposure. A: Post experimental primary root growth under control (Light green) and abiotic factors (Dark green) at control (Light green). B: Post experimental secondary root growth under untreated and abiotic factors at pH: 5.8 and 4.6.

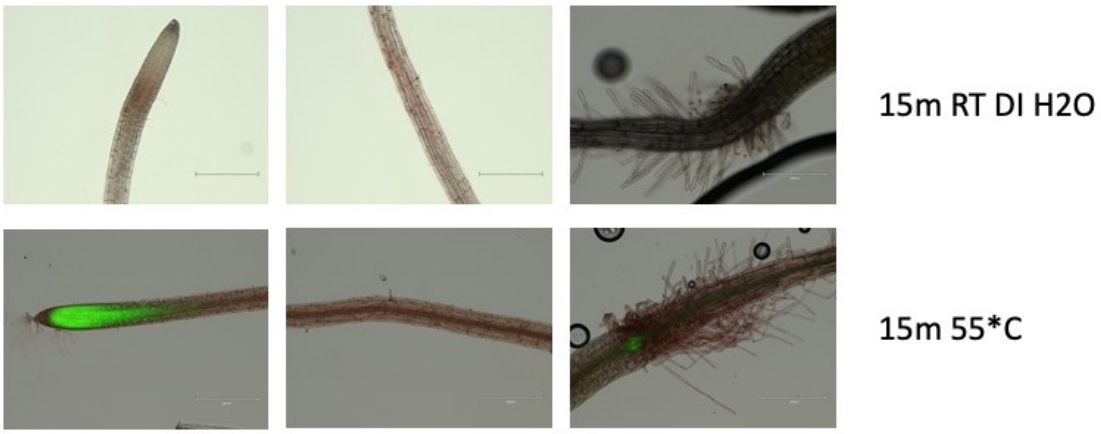

Figure 21: (A) Spatiotemporal analyses of cell death in *Arabidopsis* roots under control (Top, room temperature) and heated (Bottom, 55C°DI H_2O). Stained using SYTOX Green nucleic acid stain. Figures are from same experiment displayed in [Figure 6].

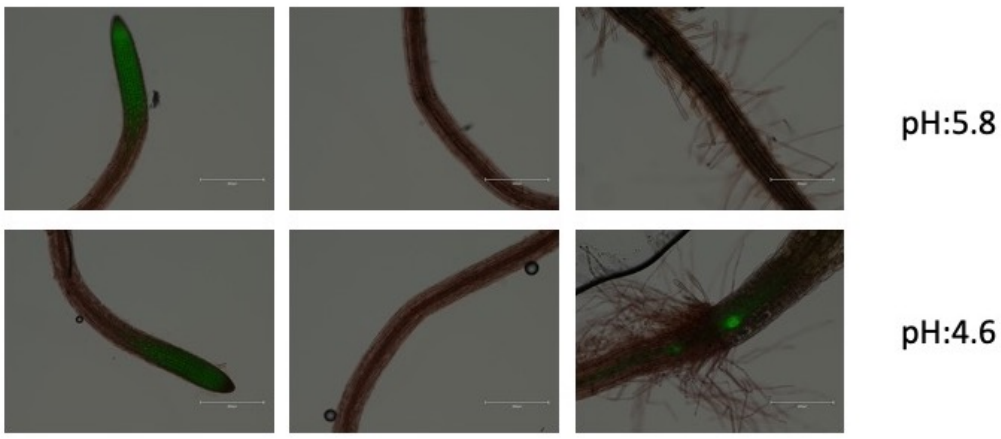

Figure 22: Spatiotemporal analyses of cell death in *Arabidopsis* roots following exposure to 50mM NaAc buffer for 30m normal (Top, 5.8) and low (Bottom, 4.6) pH. Stained using SYTOX Green nucleic acid stain. Figures are from same experiment displayed in [Figure 6].

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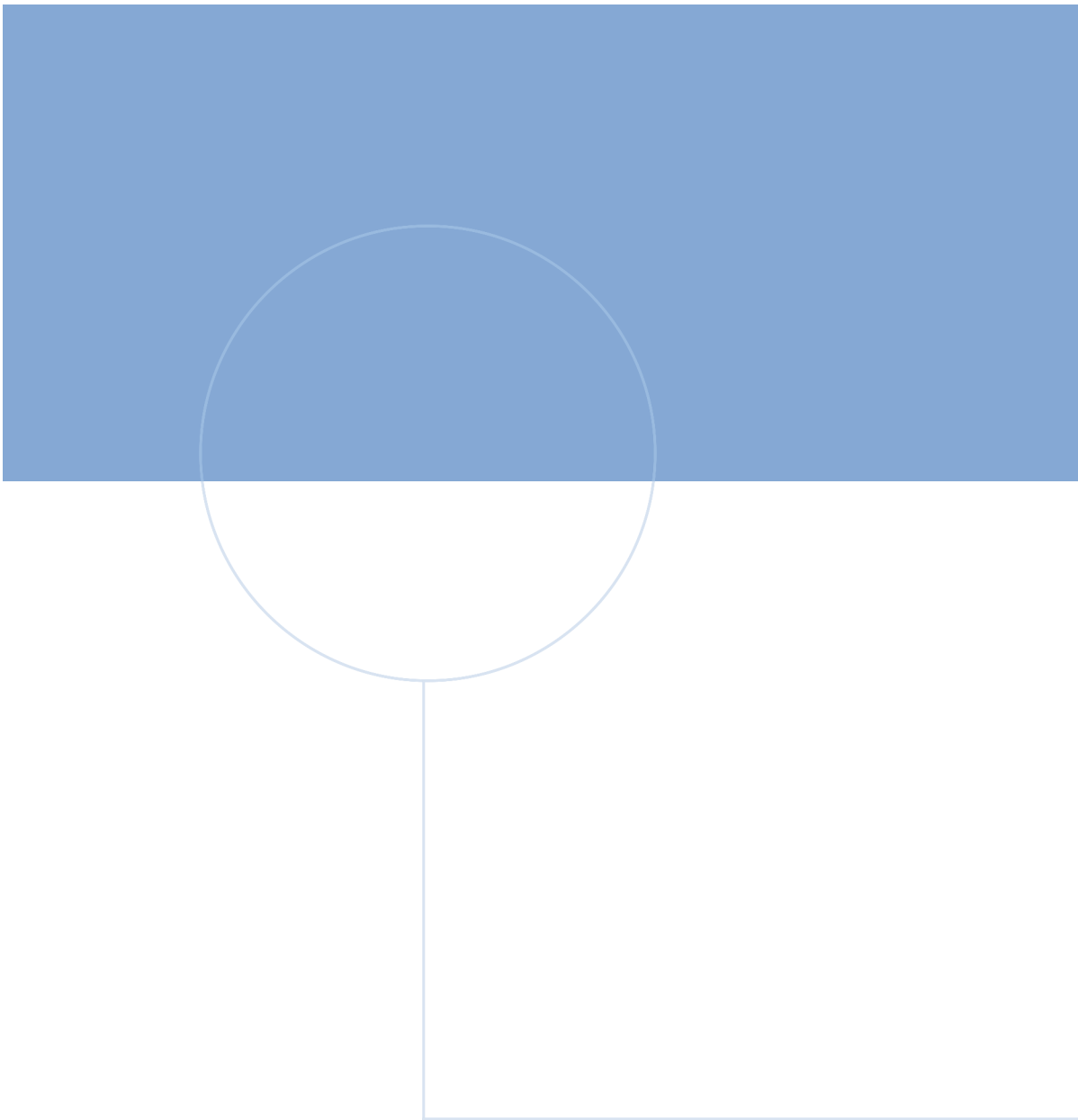

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