Review on the adaptations of wheat and barley associated with waterlogging resistance.

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Abstract

The effect waterlogged soil can have on plant production is a serious concern for UK wheat and barley farmers. Inhibition of aerobic respiration due to the hypoxic condition of waterlogged soil causes detrimental effects to field crops leading to a loss in yields. Plants can respond to this abiotic stress through regulation of their morphological structure, energy metabolism or via hormonal and signalling adaptations. This review aims to outline the adaptations wheat and barley undertake in morphological structure, respiration, reactive oxygen species damage and plant hormone biosynthesis when subjected to waterlogging stress. Finally, we indicate the areas in which waterlogging research still needs further investigation to better understand waterlogging tolerance in these plant species.

Introduction

The development of agriculture has allowed humanity to survive throughout the ages and increase in population. The first sign of crops contributing to mankind's development can be seen in the 'Neolithic Revolution' (Hole, 1984) which involved the transition from hunter-gatherer to farmer. A key component to the progression to and from the 'Neolithic Revolution' were cereal crops which produced a reliable food source for the growing population.

The early cultivated forms of cereals were selected by farmers from wild populations based on the yields provided or any other desirable structural trait (Shewry, 2009). In the case of wheat, cultivars showing beneficial traits were repeatably selected becoming the recognisably "cultivated forms" such as the diploid einkorn or the emmer tetraploid both of which data indicates originated from south-eastern Turkey from natural populations (Shewry, 2009). However, common bread wheat was developed through cultivation via the hybridization of emmer with the grass *Triticum tauschii* (Shewry, 2009). The modern cultivated barley *Hordeum vulgare* L was bred from the *Hordeum spontaneum* C.Koch, domesticated in locations such as Tibet, Central Asia or the Near East Fertile Crescent (Wang et al., 2015). The importance of these cereals is still observed today as both species are key food staples (Food and Agriculture Organization of the United Nations, 1995). Current figures show the global production of wheat during the market year of 2020/2021 was 772.64 MMT (million metric ton) (Shahbandeh, 2021) and barley of 156.41 MMT in 2019/2020 (Shahbandeh, 2020). Within the UK the production of wheat and barley stated by the Department for Environmental Food & Rural Affairs for the year 2020 was 10.1 and 8.3 million tonnes respectively (Department for Environment Food and Rural Affairs, 2020).

However, this total production of wheat in the UK is a ~40% reduction to the previous year (Department for Environment Food and Rural Affairs, 2020). Coincidentally, the average yearly rainfall within the UK in 2020 increased 324.5 mm over what fell in

2019 based on data published on Statista (Madhumitha, 2021). Although there is no evidence this is causal, it is likely to be more than just a coincidence. Increases in rainfall or patterns of rainfall are associated with flooding of farmlands and the development of waterlogged soil, both of which can lead to dramatic decreases in crop yields. Waterlogged soil occurs when water replaces the gas spaces found around the root zone of plants (Sasidharan et al., 2017). Reduced gas spaces disrupt oxygen exchange between the plant tissues and its environment and when this occurs, plants experience a hypoxic environment (oxygen levels lower than 21% v/v in air). Under hypoxic conditions wheat and barley yields can be significantly reduced - up to 25% - and the yield reduction is correlated with the duration of waterlogged soil (Herzog et al., 2016). This reduction in yield is associated with a considerable reduction in gas diffusion rates leading to impaired root function, stomatal limitation, and loss in energy production (Herzog et al., 2016). To adapt to this hypoxic environment, plants develop various morphological responses to ameliorate the effects of the waterlogged soil. These adaptations include a change in energy production methods using fermentation in exchange of aerobic respiration, the development of root aerenchyma to increase gas exchange between plant cells and the production of adventitious roots to replace previously established root systems.

The increased rainfall observed in the UK during 2020 is not an isolated event. The occurrence of waterlogged soil has increased worldwide over the past decade due to higher intensity rainfall and unpredictable weather patterns (Hirabayashi et al., 2013). Moreover, increased waterlogging events are expected to persist within the coming years due to climate change. As explained above, waterlogging can significantly affect crops’ growth rate and production. If maintained in the upcoming years due to climate change, reduced production because of waterlogging events can pose a serious threat to human survival. The world population is currently estimated to reach ~10 billion people by 2050 (FAO, 2017). It has been estimated that in order to meet demands, global yields of cereal crops would have to increase by 1.2-1.3% per year (Fischer et al., 2014), however, once climate effects are taken into account this required increase in yield is raised to 1.7% (Reynolds et al., 2016). To meet the required food demands for the human population, we require a further understanding of how climate change will affect crop yields. Although the current focus is being placed on water scarcity (Bita and Gerats, 2013) and subsequent heat stress to be experienced by crops leading to large scale decline in yields (Fahad et al., 2017), there is one paradox that must still be considered. The paradox of global warming is that because of increased changeability and unpredictability of the weather patterns, increased occurrences of drought in one place are matched with the simultaneous increase in precipitation rates across other areas meaning we must expect a rise in occurrences of waterlogged soils and flooding as well as increased drought and water scarcity.

This review aims to review the current knowledge of how morphological and metabolic adaptations increase the survivability of wheat and barley and to provide a general overview of what traits might be changed to improve future crops, so they are better able to cope with waterlogging. Herein we will focus on the UK's two major arable crops, wheat and barley, and explore the structural, energetic, hormonal and signalling adaptations they have developed to cope with waterlogging stress.

Structural adaptations

As mentioned, the hypoxic conditions imposed on plants by waterlogged soil can lead to a variety of stresses and even cause plant death. Most plants suffer greatly because these conditions affect the diffusion rates of gasses such as oxygen ($O_{2)}$ and carbon dioxide (CO_2) through their stems and root systems. This reduction in diffusion rate leads to a significant decrease in their metabolic functions (see sections below on ethylene and ROS scavenging). However, plants can develop a series of morphological changes to assist with oxygen deficiency. The main morphological adaptation plants undertake are the development of adventitious roots (ARs), the development of aeration tissues (i.e. aerenchyma), or creating barriers to radial oxygen loss (ROL) (Gunawardena et al., 2001; Nishiuchi et al., 2012; Yamauchi et al., 2014b). Here, we will define what these structures are, where they are located, and how they develop in response to waterlogging stress.

Production of ARs can be considered a typical morphological response during extended waterlogging (Steffens and Rasmussen, 2016). They are produced from the hypocotyl or the stem nodes (Figure 1) where they can promote gaseous exchange and absorption of nutrients and water; these are new roots formed at the surface or just below the soil surface that replaces the primary roots that have died due to hypoxic stress allowing the plant to maintain metabolic functions (Rasmussen et al., 2017). In wheat, their formation is controlled by reducing the formation or elongation of other roots (such as axial or lateral roots) and altering hormone biosynthesis at the location in the stem nodes where the AR will emerge (Nguyen et al., 2018). These roots often develop aerenchyma and are connected to the shoot to help improve the diffusion of gas (Sauter, 2013; Steffens and Rasmussen, 2016).

A significant factor that allows adventitious roots to maintain gaseous exchange rates within plants under waterlogged soil, is their aerenchyma content which augments the uptake and diffusion rate of O_2 (Visser and Voesenek, 2005). Aerenchyma are tissue cavities produced by programmed cell death in cortical cells of plant roots and shoots. They allow for O_2 transport across waterlogged and non-waterlogged tissue while also being able to discharge CO_2, playing a vital role in maintaining physiological metabolism in plant cells (Drew et al., 2000; Yamauchi et al., 2013). There are two main types of aerenchyma: schizogenous or lysigenous aerenchyma. Schizogenous aerenchyma develops spaces for gas diffusion via cell separation and differential expansion without causing cell death (Evans, 2004). Lysigenous aerenchyma, however, forms space for gas diffusion via programmed cell death and is commonly seen in cereal crops (Arikado and H., 1955; Trought and Drew, 1980). Lysigenous aerenchyma (Figure 2) in the root cortex forms when the contents of the cell that form the aerenchyma are digested leaving behind only the cell wall (Evans, 2004). However, some lysigenous aerenchyma cells may remain intact in order to function as radial bridges, therefore, retaining root structural rigidity. The development of lysigenous aerenchyma is only triggered in dryland cereal crops such as wheat and barley when roots are faced with poorly aerated soils e.g., waterlogged soils, and do not develop in well-drained soils (Trought and Drew, 1980).

Aerenchyma provides a robust way to allow gaseous exchange throughout the plant cells under waterlogged conditions, however, they can lead to a loss of O_2 via both consumption for respiration and through lateral leakage into intercellular spaces of the rhizosphere (Yamauchi et al., 2018). This loss of oxygen through aerenchyma is termed radial oxygen loss (ROL). To counteract this oxygen loss and allow further adventitious root growth, plants can form a ROL barrier on the root exterior that reduces ROL (Pedersen et al., 2021). This barrier is formed by the addition of suberin within the walls of the hypodermis and exodermis cells (Kotula et al., 2009). This barrier development is observed in rice as the accumulation of suberin in the outer layers of cell walls allow it to withstand submerged conditions (Nishiuchi et al., 2012). In regards to this review's focus i.e., wheat and barley, the formation of the ROL barrier with suberin is not possible, potentially due to specific genetic variations between rice and these cereals (Ouyang et al., 2020), although this is not yet fully understood but can also be attributed to the differences in root physiology between these plants (Kreszies et al., 2018). However, it is interesting to note that wheat has the ability to develop the ROL barrier when it is crossed to *Hordeum marinum* (Malik et al., 2011; Watanabe et al., 2013). Although ROL barrier development is not normally possible, wheat and barley plants still change their morphology in order to decrease ROL by regulating the surface area and volume (SA: V) of their adventitious roots. Thin roots are found to have high SA : V causing higher rates of oxygen diffusion to hypoxic/anoxic soils therefore by increasing the thickness of roots reduces the SA : V values leading to a reduction in ROL within wheat and barley (Pedersen et al., 2021).

Energy production adaptations

Energy production the most crucial factor for plant development and growth, however under hypoxic conditions the energy metabolism of plants are stunted leading to a reduction in the plant's capacity to develop and survive (Loreti et al., 2016). Plant energy metabolism is primarily based on the mitochondrial electron transport chain, where oxygen is used as an electron acceptor to produce adenosine triphosphate (ATP). Under normal soil conditions dissolved oxygen (in water) is found at approximately 0.23 mol/ m^3, however, under waterlogged conditions, this oxygen concentration is reduced to 0.05 mmol/m^3 (Armstrong, 1980; Bailey-Serres and Voesenek, 2008). Depletion of oxygen availability, therefore, affects the amount of ATP a plant can produce via mitochondrial respiration. To maintain energy levels a plant would rely on the ATP production of glycolysis and ethanol fermentation, however, such modes of metabolism are not as efficient as mitochondrial respiration. Using 1 mol of glucose in mitochondrial respiration can produce 36 to 38 mol of ATP, in contrast glycolysis and fermentation (Figure 3) can only produce 2 mol ATP (Melkonian and Schury, 2019). To supplement this glycolysis and ethanol fermentation must be accelerated to provide the necessary ATP. Production of ATP via anaerobic fermentation can follow two different paths, through lactate dehydrogenase (LDH) producing lactic acid or via pyruvate decarboxylase (PDC) resulting in acetaldehyde that is reduced to ethanol by alcohol dehydrogenase (ADH) (Zabalza et al., 2009). Both ADC and PDC are key to ethanol fermentation and their activity is used as indexes reflecting a plants tolerance to waterlogging. To sustain the plants under hypoxic conditions the expression levels of both ADH and PDC along with related enzymes must be regulated accordingly. Through a genome-wide analysis of waterlogged barley, it was shown that the expression levels of both *ADH* and *LDH* was induced and expression levels for them were higher in the waterlogging-susceptible Yerong species in comparison to a waterlogging-resistant variety Deder2 (Borrego-Benjumea et al., 2020). However, the study by Borrego-Benjumea et al., (2020) surprisingly showed that in barley varieties *PDC* expression, which is involved with the first steps of fermentation, was mostly downregulated. These alternative methods of energy production do assist the plant to survive longer periods under waterlogged conditions (Zabalza et al., 2009). Yet if the hypoxic environment persists for extended periods of time the formation of toxic substances such as lactic acid, alcohols and aldehydes produced by these modes of energy production can lead to a reduction in plant health and eventually cause death (Pan et al., 2021).

Impact of ethylene

The phytohormone ethylene (ET) has been associated with the regulation of plant growth and senescence relative to its concentration and timing within plant species (Iqbal et al., 2017). Within plants, ET is synthesized from 1-aminocyclopropane-1-carboxylic acid (ACC) (Houben and Van de Poel, 2019). The accumulation of ET is an important mechanism in waterlogging resistance in plants (Hartman et al., 2021), however, its production is an oxygen dependant reaction, where ACC is converted to ET by ACC oxidase (ACO) (Houben and Van de Poel, 2019). This requirement for oxygen is overcome by the continuous transport of ACC from the hypoxic root system to the aerobic region of the plant where the oxidation reaction can be achieved (Pan et al., 2021). It has been previously shown that ET induces the formation of aerenchyma (Gunawardena et al., 2001) and that its inhibition can block the aerenchyma development under hypoxic conditions within various plant species (Jackson et al., 1985; Konings, 1982). To understand if this connection existed within wheat, Yamauchi et al., (2014b) researched the effects of wheat seedling pre-treated with an ethylene precursor (ACC) under aerated soil conditions or stagnant (to simulate waterlogged soil) conditions. Results from pre-treatment of seedlings with ACC under stagnant conditions showed greater positive results in comparison to the non-pre-treated seedlings (Yamauchi et al., 2014b). These experiments also showed that the percentage of aerenchyma development in first seminal roots was also higher in pre-treated samples grown in stagnant conditions (Yamauchi et al., 2014b). ET aids in the development of the lysogenic aerenchyma by stimulating programmed cell death (Sasidharan and Voesenek, 2015).

Simultaneously the accumulation of ethylene also contributes to the development of adventitious roots within cereals. Research investigating the effects of inhibition on ET biosynthesis showed that this can impede the development of adventitious roots (Vidoz et al., 2010). The role of ethylene has also been connected with the development of adventitious roots in rice and their ability to withstand submerged conditions (Hattori et al., 2009; Van Der Straeten et al., 2001). Although the connection between ET and adventitious root development has been deeply researched in connection to surviving submergence, and connections with specific transcription factors and related genes have been demonstrated, most of this work has been done in other species such as rice (Fukao et al., 2019)rather than in wheat or barley. However, Yamauchi et al., (2014a) analysed the effects of pre-treatment to ACC could have on wheat seedling grown initially in aerated soil, then transferred to stagnant i.e. hypoxic soil conditions. Their work showed that wheat seedlings when pre-treated with ACC would develop adventitious roots sooner in comparison to controls, these pre-treated seedling adventitious roots would be larger in diameter in comparison to non-ACC pre-treated seedlings.

I would like to also briefly mention a potential connection between ethylene and the amelioration of damage caused by reactive oxygen species (ROS). The control of ROS via ethylene is associated with increased NADPH oxidase levels and activity(Desikan et al., 2006). The connection between ethylene-regulated oxidative stress tolerance associated with abiotic stresses such as drought, heat or salt stress has been mentioned in the literature (Peng et al., 2014; Wu et al., 2008). However, no functional link has yet been established between reduction of ROS damage and ethylene during waterlogged or flooding stress, or specifically within cereal crops. It is possible to assume that ethylene could also contribute to ROS damage amelioration under hypoxic conditions, but further research is necessary.

ROS signalling and scavenging

Production of reactive oxygen species (ROS) is a normal occurrence within plant metabolism, however, reduction in O_2 availability leads to increased intracellular ROS levels (Bailey-Serres and Chang, 2005; Pucciariello et al., 2012). The production of superoxide radicals ($^{\cdot}O_2$), hydroxyl radicals ($^{\cdot}OH$), and hydrogen peroxide (H_2O_2) leads to lipid peroxidation and delipidation of leaf membranes and oxidative damage to proteins, DNA, cell membranes and organelles due to their oxidizing activity (Sharma et al., 2012). Although the presence of ROS can lead to severe damage to plant cells, they are still considered important signalling molecules for plants under stress. For example, plant NADPH oxidase is a significant enzyme that is associated with the production of ROS and plays a role in ROS-mediated signal transduction. When looking at the expression of *ALCOHOL DEHYDROGENASE 1* (ADH1), a gene associated with ethanol fermentation in *Arabidopsis* under waterlogging stress, it has been observed that the increase in ADH1 expression is caused by the expression of the NADPH oxidase related gene AtRbohD, one of the respiratory burst oxidase homologues (Rbohs), via H_2O_2

signalling (Sun et al., 2018). Similarly, the production of this signalling molecule is connected to ET induced aerenchyma formation by modulating the transcription of hypoxia-induced genes such as *ETHYLENE-RESPONSIVE FACTORS73* (ERF73/HRE1) in *Arabidopsis* (Yang, 2014). Unfortunately, these connections within wheat or barley have not fully been annotated or understood, however, through phylogeny analysis, it has been shown that the *TaNOX2* in wheat or the *HvFRO1* in barley are similar to *AtRbohD* (Hu et al., 2018) and therefore could be the key to these responses in these cereals.

To counteract the increased ROS production due to waterlogging stress plants rely on antioxidant enzymes to maintain ROS balance, reducing oxidative damage (Hasanuzzaman et al., 2020). Genome analysis of barley under waterlogged stress has revealed the activity of numerous enzymes, such as CATALASE (CAT), ASCORBATE PEROXIDASE (APX) and SUPEROXIDE DISMUTASE (SOD) to mitigate damage (Borrego-Benjumea et al., 2020). However, based on various insights on the activity of antioxidants under waterlogging stress, it is difficult to identify a general correlation between stress tolerance and antioxidant activity, but instead, there may be a more genotype-dependent correlation. For example, Yordanova et al., (2004) reported that soil flooding led to varying levels of activity of antioxidant enzymes in barley leaves, showing that 72 – 120 h after waterlogging led to the significant decrease in the activity of SOD, meanwhile the activity of POD, CAT and APX significantly increased. The changes in POD activity over time was also observed by Borrego-Benjumea et al., (2020), with the Deder2 barley, where three *POD* up-regulated genes identified in their study showed increased expression at 72 h (2.56, 2.00 and 2.21 logFC) but expression decreased at 120 h (2.15, 1.48 and 1.47 logFC). It has also been shown by Luan et al., (2018) that this antioxidant response is not limited to waterlogging tolerant crops, by inducing both susceptible and tolerant genotypes of barley experiments showed that SOD, POD and Cat activities were increased in both genotypes.

Another enzyme that is believed to aid with reducing oxidative damage is GLUTATHIONE-S-TRANSFERASE (GST). GSTs are a group of multi-gene isoenzymes involved with cellular detoxification from both xenobiotic and endobiotic compounds. They catalyse the conjugation of glutathione (GSH) to the xenobiotic and/or endobiotic compound creating less toxic hydrophilic products that can be metabolised, stored within vacuoles, and/or removed from the organism. Genomic analysis has reported that several *GST*-encoding genes were significantly upregulated by waterlogging stress (Borrego-Benjumea et al., 2020). With regards to barley, research shows that *GST* expression was increased in multiple genotypes, but greater changes were observed within the more waterlogging tolerant genotypes, suggesting that a more efficient ROS detoxification occurs within them, however further research is required to understand the difference in function (Luan et al., 2018).

Conclusion

As we can see the maintenance of plant growth and development under waterlogging stress involves a combination of different factors, such as changes to metabolic, hormonal, and structural traits to improve survivability. These methods of adaptation vary greatly amongst different crops and even different varieties of the same crop. So far research on waterlogging tolerance has prioritised focusing on the morphological, physiological, biochemical, and metabolic gene signalling of plants, as outlined in this review. Based on current understanding, the most effective methods to enhance plant waterlogging tolerance would be to prevent waterlogging from occurring using improved cultivation management to reduce the level of damage crops experience under waterlogged soils, but there are also opportunities to use molecular biology, genetics, and genomics to identify key genes within plant species that regulate tolerance to this stress and once their functions have been verified, generate crops that carry the improved traits.

Based on the research results for this review, there are various identified gaps within our understanding of wheat and barley waterlogging tolerance. Firstly, research so far has focused on the vegetative growth stages of plants in waterlogged soils (Pan et al., 2021), however, very little is available in regards to the molecular responses during seed germination or the late reproductive growth stages under waterlogging stress. Furthermore, although genes have been identified that regulate waterlogging and submergence tolerance via transcriptomics, proteomics and other methods, these have focused primarily on model plants such as *Arabidopsis* (Lee et al., 2011)or other major crops such as soybean (Lin et al., 2019) or maize (Yu et al., 2015); the majority of these still require functional characterization. The same cannot be said regarding wheat or barley as only recently have researchers carried out proteomic studies of these plants under waterlogged soils. Therefore, further exploitation of genetic resources using isolated populations and natural populations should be utilized to further identify waterlogging tolerant genes. Due to the current predictions on how global warming will affect our ecosystem, major focus over the past few years has been on drought tolerance of crops and understanding the morphological and genetic adaptations contributed to drought tolerance. To illustrate this, at the time of writing performing a search on the NCBI PMC database with the terms “Drought tolerance in wheat and barley” reveal a total of 8405 hits while a search on “Waterlogging tolerance in wheat and barley” reveal 680 hits. Therefore, we can observe that there is still much left to learn about the effects this abiotic stress has on plant survival and the development of tolerance to it. However, this topic is slowly gaining more interest amongst the scientific community so we can hope that with time, we will grasp a better level of knowledge on this area in order to guarantee a stronger sense of food security, as our environment continues to change around us.

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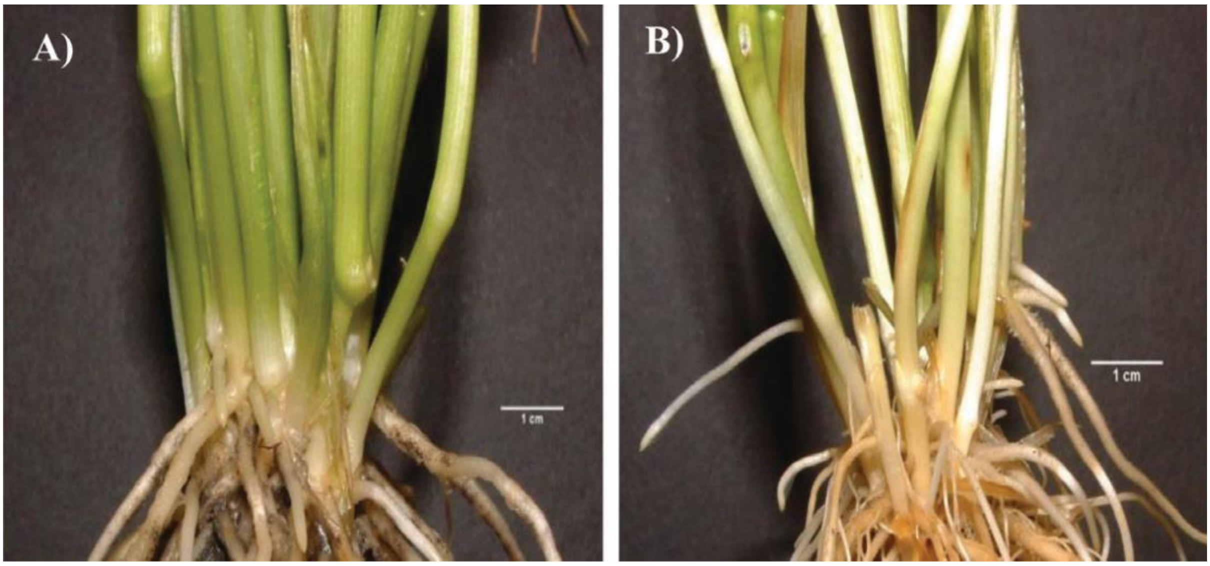

Figure 1. Comparison of adventitious root formation of wheat in control (A) and 28 d waterlogged (B) plants. Image taken from Nguyen et al., (2018)

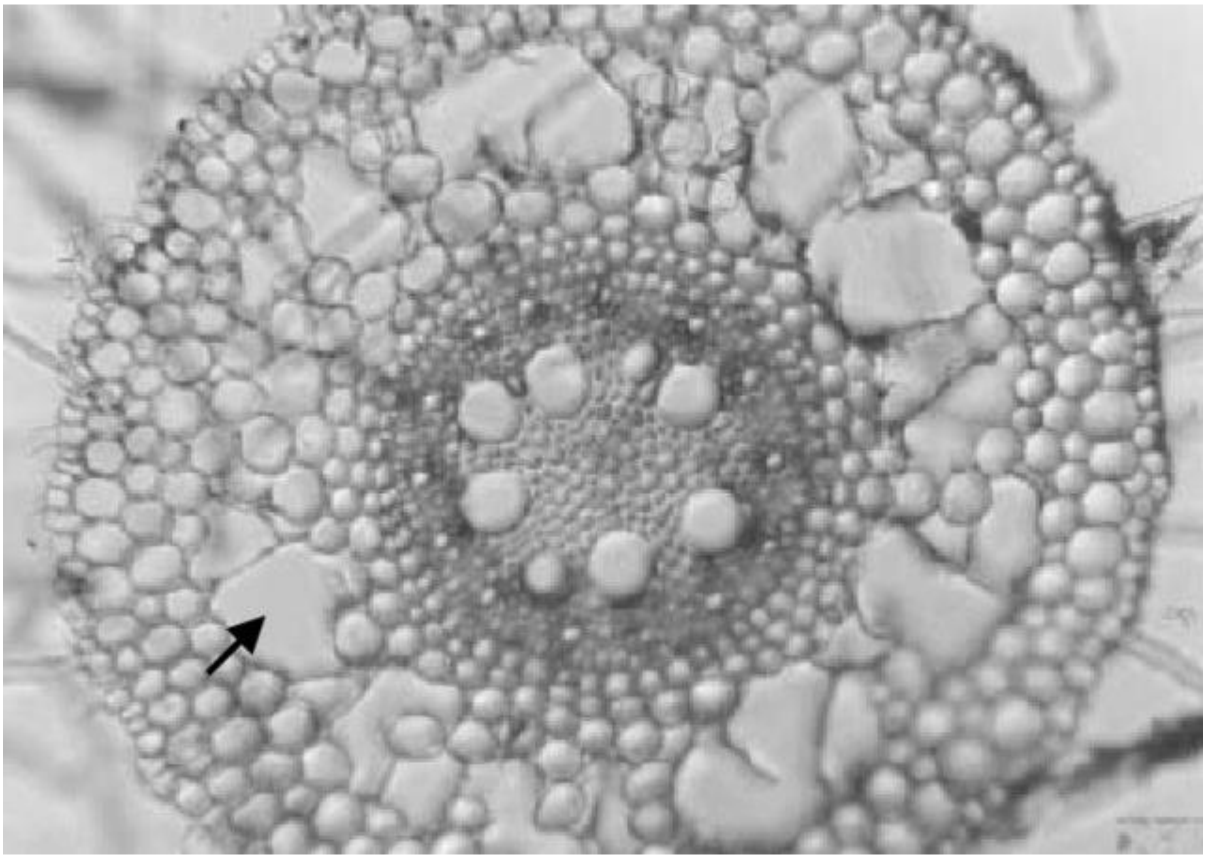

Figure 2. Waterlogged wheat lysigenous aerenchyma (indicated by arrow) formed within cortex of adventitious roots. Image taken after 14 days of waterlogging treatment. Figure taken from Malik et al., (2001)

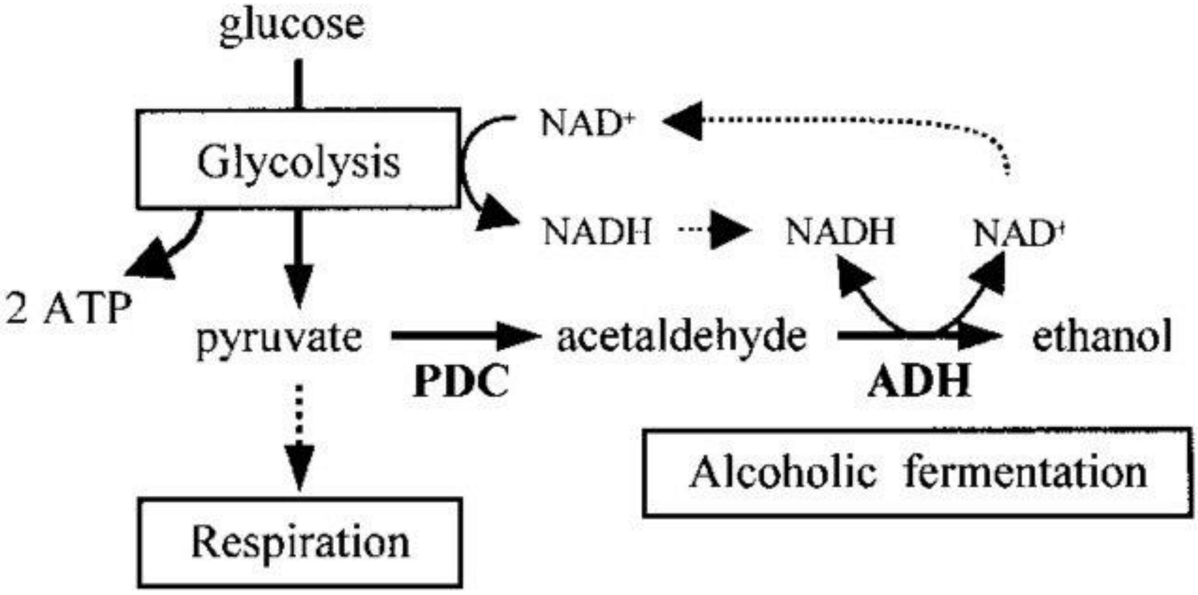

Figure 3. Simplified diagram demonstrating production of 2 ATP molecules from glycolysis combined with ethanol fermentation pathway utilising pyruvate from glycolysis. Image taken from Saika et al., (2006)

Investigating the connection between ethylene and adventitious root growth under waterlogged condition, induced by dissolved starch solution.

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Key words: Adventitious roots, waterlogging, ethylene, abiotic stress, Arabidopsis thaliana

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Abstract

Plants display a variety of morphological and structural adaptation to stress survival. These adaptations are varied and differ between species, however, structural adaptations are often seen to be similar across various species. Waterlogged soils cause plants to face hypoxic stress due to the reduction in gas exchange surrounding the root system. This abiotic stress is of growing concern around temperate regions due to the increase in rainfall associated with climate change. A method in which plants adapt to waterlogged soil is via the development of adventitious roots. Adventitious roots are those that develop after prolonged waterlogging and are located around the hypocotyl or stem nodes of plants typically on the surface or directly below the soil surface and help to promote gaseous exchange and nutrient absorption. Under waterlogged stress plants begin to produce large amounts of the plant hormone ethylene, this hormone is associated with regulation of plant growth and senescence, simultaneously it is known that the hormone is connected to environmental stress adaptations. In this case the hormone is connected to adventitious root development. For this project we aim to test the development of adventitious roots on an ethylene insensitive Arabidopsis mutant against (ein2) against the wild type and a mutant with impaired ethylene biosynthesis. Simultaneously we aim to test an innovative protocol using dissolved starch to simulate the hypoxic conditions of waterlogged soils. We take into consideration any and all shortcomings such a protocol may have and aim to assess its viability and suggest further improvements for future research.

Intro

The human population is expected to grow to roughly 10 billion citizens by the year 2050 (FAO, 2017), this high increase in population leads to growing concerns regarding food security and availability. In order to match food demands, global yields of cereal crops alone would be required to increase by 1.2-1.3% per annum(Fischer et al., 2014). However, these estimates do not take into account the impact of climate change, which when considered would raise the minimum increase per annum to 1.7%(Reynolds et al., 2016). Focus is currently being given to the effects water scarcity will have on food production and how heat stress leads to large scale decline in yields(Bita and Gerats, 2013; Fahad et al., 2017), however, the opposite of this biotic stress should still be considered. Global warming poses one significant paradox in that because of its high variability and the overall unpredictability of weather patterns, drought occurrences in one location are usually matched with a simultaneous increase in precipitation rates across other areas. Therefore, it is reasonable to expect increased occurrences of waterlogged soils and flooding as well as drought and water scarcity. Such an increase in waterlogged soils has already been observed globally over the past decade due to the increased intensity of rainfall and unpredictable weather patterns (Hirabayashi et al., 2013). Waterlogged soil affects crop yield due to the replacement of gas spaces surrounding plant roots with water (Sasidharan et al., 2017), which leads to disruption of oxygen exchange between plant tissues and its environment; generating a hypoxic environment (oxygen levels lower than 21% v/v in air). Hypoxic environments have been shown to significantly reduce yields of cereal crops – up to 25% - and yield reduction shows correlations with the duration of waterlogging (Herzog et al., 2016).

To survive under these conditions plants employ various mechanisms to ameliorate their circumstances, these can be either structural or metabolic adaptations. For this paper, we will be focusing on structural adaptations, specifically adventitious root development. The hypoxic conditions imposed on plants in waterlogged soils affect the diffusion rates of gasses e.g. oxygen (O_2) and carbon dioxide (CO_2) through their stems and root systems. A reduction in gaseous exchange leads to negative implications to plant metabolic function. Adventitious root (AR) development is considered a typical morphological response to prolonged waterlogging (Nishiuchi et al., 2012; Steffens and Rasmussen, 2016). These types of roots are produced from the hypocotyl or stem nodes of plants typically on the surface or directly below the soil surface and help to promote gaseous exchange and nutrient absorption (Rasmussen et al., 2017; Steffens and Rasmussen, 2016). The development of these roots is controlled by both the reduction in axial and lateral root development along with alterations in hormone biosynthesis located near the nodes where AR develop (Nguyen et al., 2018).

Plant hormones (phytohormones) regulate a variety of plant metabolic functions, such as shoot and root growth, fruit development, senescence as well as playing role in the symbiosis between plants and microbes (Foo et al., 2019). As mentioned plant hormone biosynthesis plays a role in the development of adventitious roots, for this morphological adaptation auxin and ethylene play key roles. Ethylene is also associated with the regulation of plant growth and even plant senescence, however, the hormone is strongly associated with adapting the plant to environmental stress(Pan et al., 2021). The synthesis of ethylene is achieved by the oxygen dependant reaction where 1-aminocyclopropane-1-carboxylic acid (ACC) is converted to ethylene by ACC oxidase (ACO) (Houben and Van de Poel, 2019). The accumulation of ethylene is highly associated with plants response to waterlogging and plays a significant role in the development of adventitious roots in order to increase a plants survivability under a hypoxic environment (Hartman et al., 2021). The way in which ethylene helps the development of adventitious roots under waterlogged soils is associated with how it interacts with the phytohormone auxin (Rasmussen et al., 2017). Auxin is a well know plant hormone involved with the promotion of cell division leading to elongation in plant cells i.e. plant growth, and it is often produced within the tips of growing stems and roots. Auxin and ethylene form reciprocal interactions within root systems, whereby ethylene accumulation leads to an increase in auxin transport from plant shoots and/or enhances its biosynthesis in root caps through increasing the expression of tryptophan aminotransferase genes (involved in auxin biosynthesis); in retrospect, auxin is able to up-regulate ACC synthase gene expression forming an auto-enhancing cycle (He et al., 2011; Stepanova et al., 2007, 2005). However, this cycle can cause alteration in the basipetal auxin movement due to disturbances in the overall auxin gradient within the plant (Růžička et al., 2007; Vandenbussche et al., 2012). Under hypoxic conditions of waterlogged soils, the accumulation of ethylene inhibits the basipetal auxin movement (potentially associated with alterations to internal auxin gradient due to the increase in auxin production) causing the development of adventitious roots higher up in the plant hypocotyl (Rasmussen et al., 2017)

The role of ethylene goes further than the stimulation of adventitious roots. Within a hypoxic environment, the accumulation of ethylene leads to a range of rapid ethylene-dependent signalling, for example within 1 hour of submergence a large accumulation of Ethylene Insensitive 3 (EIN3), a principal transcriptional regulator of ethylene signalling was noticed in Arabidopsis root tips (Hartman et al., 2021). This signalling pathway enables the activation of hypoxia tolerance genes believed to regulate prolonged hypoxia survival. These core genes regulate waterlogging survival processes of fermentation, oxidative damage repair, aerenchyma and adventitious root development (Pan et al., 2021). For example, in the development of aerenchyma in waterlogged wheat, there is evidence indicating that when seedlings were pre-treated with an ethylene precursor i.e. ACC, and grown in stagnant soil conditions (simulating hypoxic conditions) led to increased development of aerenchyma in roots in comparison to those that didn't receive the pre-treatment (Yamauchi et al., 2014). The majority of research into hypoxic tolerance of plants has been targeted at submergence tolerance in *Oryza sativa* and *Zea mays* (Morrell and Greenway, 1989)and some work done on *Arabidopsis thaliana* and *Rumex palustris* (van Veen et al., 2013).

Based on the knowledge we have on how ethylene contributes to waterlogged soil survivability; it would be safe to assume that the removal of this plant hormone would reduce the rate at which they develop structural adaptations and reduce their survival rate. The development of adventitious roots is a simple to quantify adaptation and can be done within a short period of time using Arabidopsis. We aim to compare the development of such roots between ethylene insensitive (ein2) Arabidopsis mutants versus wild type Arabidopsis growing in either normoxic or hypoxic conditions. To simulate the hypoxia-induced by waterlogged soils, two main techniques can be utilized, the use of a hypoxic chamber (He et al., 2007)or more commonly the use of specialised growth media which is then flushed with nitrogen gas to deoxygenate the media (Yamauchi et al., 2014). Simultaneously for this project, we aim to test the efficacy of an alternative, low cost and easily accessible method in inducing hypoxia in plants through the use of the dissolved starch solution. Previous work on flooding tolerance in maize was done using this method of inducing hypoxia within potted plants (Mano et al., 2006), we are now aiming to see if such a method could show significant results within a plant cell culture. If this altered protocol proves successful, we assume that adventitious root development will be reduced within the ethylene insensitive plants in comparison to their wild type controls.

Methods

Seeds source and growth conditions

For this experiment, Arabidopsis (*Arabidopsis thaliana*) wild type (Col-0) seeds, along with ein2 (CS66117) and R002 (CS8102) mutant seeds, were obtained from NASC (Nottingham Arabidopsis Stock Center). Unless otherwise stated, all plants were grown under 16-h/8-h day/night conditions at 22°C.

Seeds to be used for plant cell culture were the first surface sterilized in 5% bleach for 5 min and then rinsed with distilled water 4 times. For the root growth assessment, between 5 and 8 seeds were sown on circular Petri dishes containing a 0.7% agar with 4.4g/L Murashige and Skoog basal medium powder (Sigma-Aldritch) then left to vernalize for 3 d at 4°C under. Afterwards, plates were left to grow for 4 d in dark conditions to promote etiolation. Plates were then returned to 16-h daylight conditions at 22°C for another 12 days. During this 12 day growth period, plants were separated between normal watering conditions and waterlogged conditions. Waterlogged conditions were induced by watering petri dishes with a 0.2% dissolved starch solution to a point where a small film of water could be seen above agar. The number of adventitious roots was then counted and measured.

Counting of adventitious roots was only done for roots grown above the root-shoot junction and was achieved by the use of a stereoscope and all images were taken using an Honor Pro 20 phone camera.

Results

Adventitious root development

As stated in the methods roughly 5 to 10 seeds were sown in each petri dish with a total of three petri dishes used for each mutant and treatment type. Seeds were sown and watered under a flow hood to prevent contamination, however, once treatments began with 0.2% dissolved starch solution a large majority of petri dishes became contaminated. Due to the potential effects, this could cause on root growth, dishes with severe contamination were discarded, and unfortunately, due to time constraints, the second batch of samples was not possible to produce. Therefore, for the analysis of adventitious root growth and development, three Arabidopsis seedlings from each variant and treatment type were selected. Each plant was then viewed under a stereoscope and all adventitious roots grown along the hypocotyl and above the shoot/root junction were counted regardless of length, and images were taken using a mobile phone camera. The main assumption made for these experiments was that plants with hindered ethylene metabolism or detection within their signalling mechanisms would suffer some level of impairment in the development of adventitious roots under waterlogging stress. Here we aimed to test this hypothesis through an unorthodox method in instigating a hypoxic environment within a plant cell culture. After the specific growth period for the experiments, it was observed that both waterlogged and non-waterlogged plants did develop adventitious roots to some degree based on our selection criteria (Figure 1 & 2).

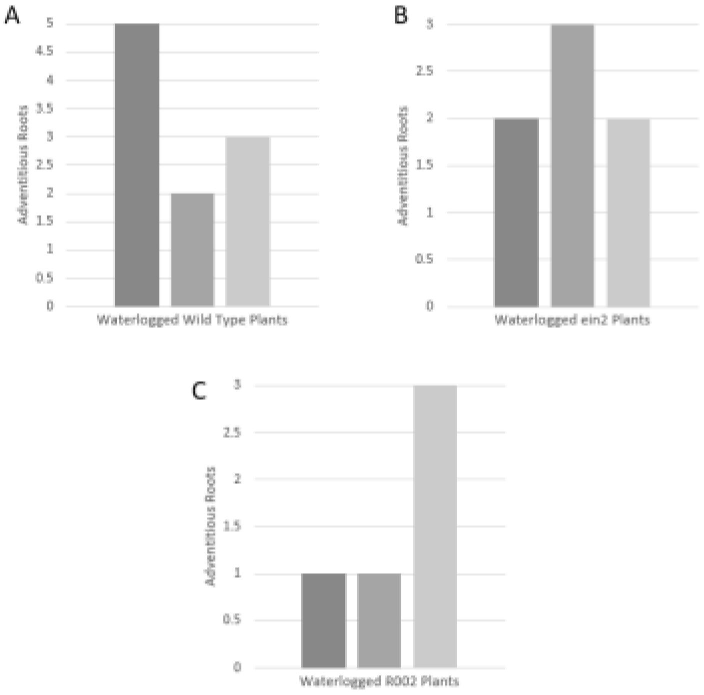


Figure 1: ***Adventitious root count of waterlogged Arabidopsis plants.*** *Collected data showed that Wild Type (A) Arabidopsis produced the highest amount of adventitious roots after waterlogging treatment (average of 3.3 roots). As expected,*

the R002 Arabidopsis mutant developed the least number of adventitious roots (average of 1.7 roots). The ethylene insensitive mutant ein2 fell directly between both Wild Type and R002 mutant in the total number of developed roots (average of 2.3 roots).

The ein2 mutant Arabidopsis plants (CS66117) are considered to be ethylene insensitive and were therefore chosen as the main mutant to test our hypothesis. However, based on the observed data (Figure 1 & 2) the mutant demonstrated a small difference in adventitious root growth between the waterlogged and non-waterlogged plants (Table 1). Waterlogged treatment of this mutant did however show other signs of growth impairment. For example, during growth stage 1.04 identified by the 4 rosetta leaves, a slight yellow colouration could be observed from the first two leaves which were not observed in the non-waterlogged plant. This colouration difference was considered as an indicator of internal cell damage from the hypoxic environment; possible oxidative damage (Figure 3).

The wild type Arabidopsis plants in contrast to the mutants, showed an increase in adventitious root development when grown under waterlogged conditions based on the data observed (Figure 1). However, similar to the mutant Arabidopsis plants the difference in the amount of adventitious root development between treated and untreated plants were low (Table 1). Yellow colouration of leaves was also observed for the waterlogged wild type Arabidopsis plants. Although not a focused factor for this experiment, it is interesting to note that the seminal and lateral root development below the shoot/root junction was of higher quantity to the mutant plants.

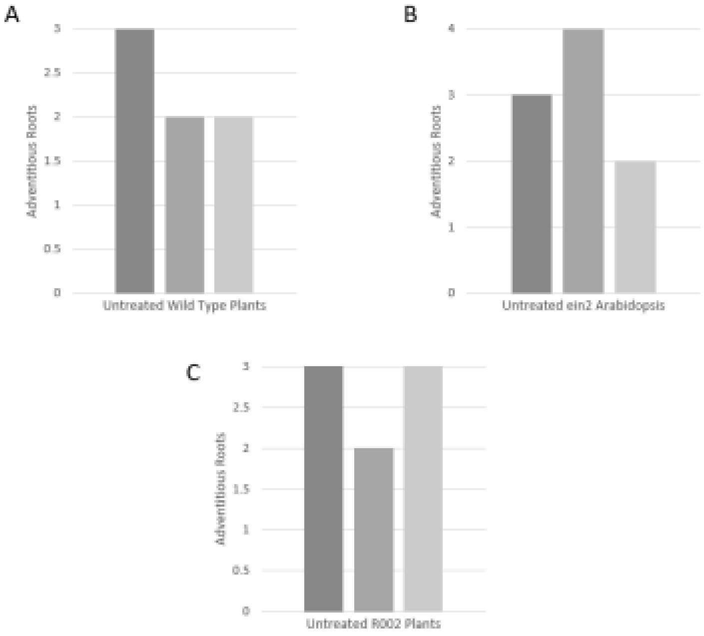
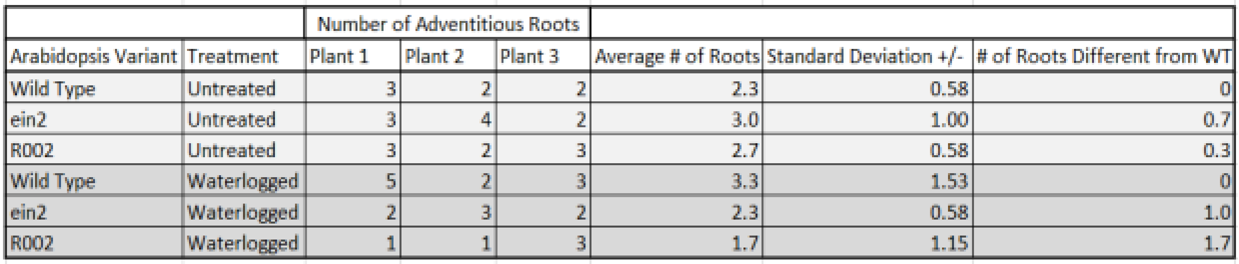


Figure 2: ***Adventitious root count of untreated Arabidopsis plants****. Collected data showed that the ein2 Arabidopsis mutant developed the highest amount of adventitious roots (average of 3 roots), however the difference between ein2 and the Wild Type (average of 2.3 roots) or R002 (average of 2.7 roots) mutant was low.*

The R002 Arabidopsis mutant was selected as a positive control for this experiment due to it having no detectable alcohol dehydrogenase; a major enzyme involved in ethylene biosynthesis (Zabalza et al., 2009). It was assumed that this mutant would serve as a suitable positive control to compare with the ein2 mutant. It was assumed that this mutant would have the lowest amount of adventitious root development under waterlogged treatment, which our results confirmed (Table 1). Similar to the ein2 mutant, these plants also demonstrated yellow colouration in the leaves under waterlogged treatment (Figure 3)

Table 1: Table showing number of adventitious roots developed for each Arabidopsis variant along with the difference of each sample from Wild Type.



		Number of Adventitious Roots					
Arabidopsis Variant	Treatment	Plant 1	Plant 2	Plant 3	Average # of Roots	Standard Deviation +/-	# of Roots Different from WT
Wild Type	Untreated	3	2	2	2.3	0.58	0
ein2	Untreated	3	4	2	3.0	1.00	0.7
R002	Untreated	3	2	3	2.7	0.58	0.3
Wild Type	Waterlogged	5	2	3	3.3	1.53	0
ein2	Waterlogged	2	3	2	2.3	0.58	1.0
R002	Waterlogged	1	1	3	1.7	1.15	1.7

Overview of starch protocol

Based on these results, we can confirm that there was a difference in the development of adventitious roots from the Arabidopsis plants when watered with a 0.2% starch solution. However, due to the small differences between the number of roots formed between treated and untreated plants, several questions are generated on whether this method of inducing hypoxia was the true reason for the difference or if the difference is significant at all (see Discussion). As mentioned above this protocol did lead to numerous amounts of sample loss due to bacterial contamination. Further testing and improvement of this protocol would be necessary to correctly determine its utility with plant cell cultures.

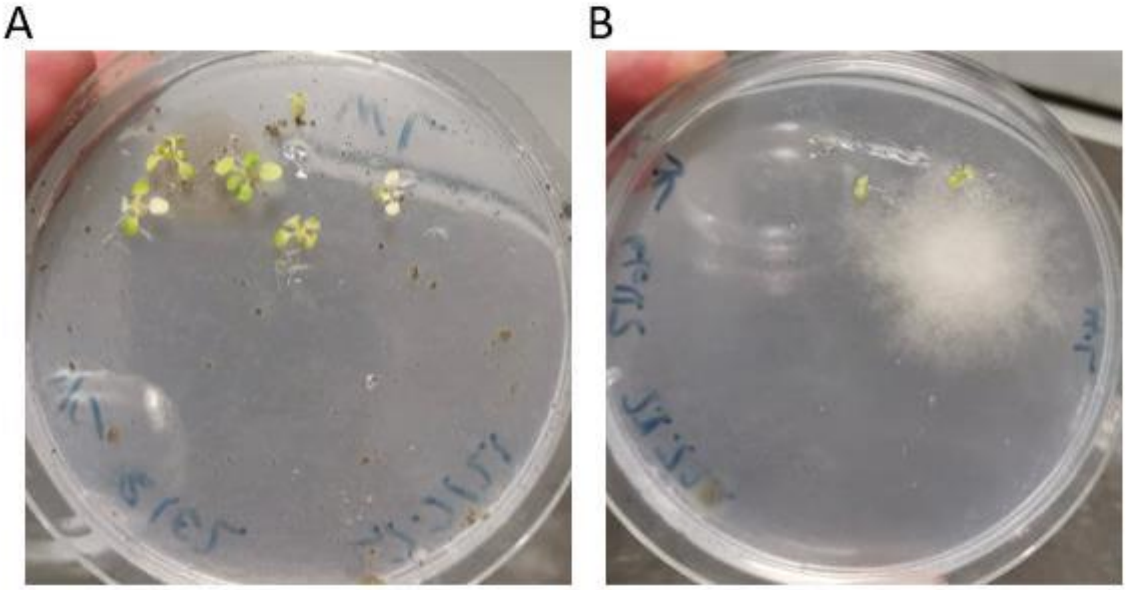


Figure 3: Image demonstrating yellow colouration of Arabidopsis leaves due to waterlogged treatment (A) and an example of bacterial contamination (B).

Discussion

The objective of this project was to both identify if ethylene insensitive mutants of Arabidopsis would show inferior adventitious root development under waterlogged conditions in comparison to its wild type counterpart, and to see if such a result could be observed using a simple low-cost protocol. In retrospect, we have observed that under waterlogged conditions ethylene insensitive mutants do on average produce less adventitious roots (Table 1). Both mutants demonstrated lower counts of adventitious roots in comparison to WT. But the ein2 mutant still produced more adventitious roots than the R002 mutants, this is attributed to the fact that ein2 mutants are ethylene insensitive, meaning that the signalling pathways regarding ethylene are in a sense disrupted, yet they are still able to have some level of ethylene within their cells, unlike the R002 mutants which possess no alcohol dehydrogenase and therefore are unable to produce ethylene. However, due to various implications, time constraints and the aim to keep costs low, it is hard to define if these results can be considered significantly valuable. The reason for this assumption can be attributed to a variety of factors. The most obvious one would be regarding how a large amount, of experimental samples, was lost due to the bacteria contamination issue (Figure 3). Although work was done in sterile conditions it was unfortunate to lose a large majority of our samples, and regrettably, the time constraints for this project did not allow for a second run in order to collect further experimental data.

Due to the innovative nature of this experiment, it lacked a key factor normally attributed to more conventional methods in testing the effect of hypoxia in plants, the main factor in question is the confirmation that the treatment truly induced a hypoxic environment. Normally this would be confirmed by the results. However, due to the small differences observed between treated and untreated plants (Table 1), we cannot guarantee if the protocol was successful in inducing hypoxia. By conventional methods, we refer to the precise control of the stagnant deoxygenated environment which is normally achieved either via mechanical regulation of gaseous exchange rates (He et al., 2007; Kölling et al., 2015) within the growth environment, through the regulation of chemicals in a hydroponic system (Carbonare et al., 2019), or finally through the removal of oxygen from the growth media entirely via the use of nitrogen gas (Yamauchi et al., 2014) to induce hypoxia.

As mentioned in the introduction, to test the effect of hypoxia on plant development, researchers either utilize complex hypoxic chambers which can precisely regulate and maintain the hypoxic condition throughout the experimental process or utilize a unique media recipe to enhance growth and then flush this set media with a chemical to reduce the oxygen exchange rate. An example of the hypoxic chamber can be seen with the Low Pressure Plant Growth System (LPPG) (He et al., 2007), a fully automated system capable of regulating the gas concentrations, moisture content, and pressure in growth chambers. In this method, every detail is precisely recorded and maintained so that the room for experimental error significantly decreases and you are able to accurately determine the plant response to specific environmental factors. Such high levels of precision were not possible with our protocol as maintaining such a system would be of too high a cost for this project.

Improvements

As with any innovative work, once it is complete regardless of the result considerations regarding improvements are made so that if a repeat was to occur the many shortcomings could be avoided and the quality of the experiment can be improved. For this experimental protocol, a series of considerations have been taken. For instance, after the collection of data, it was considered whether the results would have been of better value if the plants had a longer growth period before being subjected to the dissolved starch solution. During the waterlogging treatment process of the seedlings, constant care had to be taken so that the leaf tissue was not stuck to the agar media in order to prevent any interference with shoot development as it would be in this surrounding area where we would identify the development of adventitious roots. Assuming our method of inducing hypoxia truly functions, having older plants would have provided greater ease in both the identification and counting of adventitious roots, therefore, reducing further the risk of human error in counting the roots as there would have been a greater distinction between the root types. It is suggested that a possible increase of a minimum of 20 days growth, therefore reaching growth stage 1.08 (Boyes et al., 2001), before initiating treatment would have allowed significant time for the plant to both adjust well enough to the agar media, as well as allowed for better selection of seedlings to use for treatment and finally provided significant time for normal root development to occur.

Following on from this, we came to understand that there was little information on how these Arabidopsis mutants would develop within the agar media. Perhaps it would have been beneficial to grow each seed variant previously simply under normal conditions over the desired period within the agar media and take note of the precise time taken for the plants to reach specific growth stages(Boyes et al., 2001). With this background information, we could then grow a second batch and induce that to the waterlogging treatment with a better understanding of the ideal time and growth stage to induce the treatments and collect better results. Although initially considered, for this experiment it was decided against using any additives to the agar media which would have assisted in root development i.e. sucrose addition (MacGregor et al., 2008). Perhaps including root development enhancers in the media would facilitate the development of the root system during the early growth stages of the plant, then when it has reached the desired growth stage, plants can be transplanted into neutral media without any sucrose and experience the waterlogging treatment. Doing this would provide us with better-developed plants, a more defines root/shoot distinction and potentially the opportunity to test another consideration that being using a range of dissolved starch solutions to see the differences in effect on Arabidopsis. As mentioned the inspiration for using dissolved starch originated from a different work using such a method on maize to identify QTL controlling flood tolerance (Mano et al., 2006), in which they tested a range of different dissolved starch concentrations. For our work, we decided to use the 0.2% concentration as it caused a hypoxic effect on the maize experiment but was not too severe to cause large damage to plants and since our work was to test this method on seedlings we did not wish to overstress them during early development. As stated an occurring issue faced was that during the watering process of the plates, it was common for the plant leaves to fall and stick to the agar after being sprayed. The necessity to move the plant with sterile tweezers to return them upright involved quite delicate handling to not disturb the placement of the plant or roots themselves since the agar was very delicate. Perhaps utilizing parafilm to prevent contact between media and leaf tissue would be an ideal solution (MacGregor et al., 2008)

Outside of these practical adjustments to improve result quality, we can also consider the inclusion of other factors to try and further validate the results give by this method and further pursue the understanding of how ethylene contributes to the adventitious root response. A major way in doing so would be to also include samples with some form of auxin inhibition or even use mutants with genes removed associated with both auxin and ethylene biosynthesis or signalling. As mentioned before both auxin and ethylene form a feedback loop during hypoxic stress that triggers the development of adventitious roots (He et al., 2011; Stepanova et al., 2007, 2005). By testing samples with inhibited auxin signalling or biosynthesis we can gain further knowledge on the connections between these hormones and stress responses. Simultaneously in order to get further validation of this method, it would be advised to also run a few sample plates flushed with nitrogen gas beforehand to create a stagnant condition. By running these samples alongside the dissolved starch waterlogged plates, we can compare the development of plants under this innovative method against a more established method. We may also consider combining both methods and see what results we can obtain from the combination.

Conclusion

Overall, our primary goal was to gain further insight into the importance of ethylene to waterlogging tolerance. Although our data does indicate a reduction in the amount of developed adventitious roots and therefore would suggest the importance of ethylene in this phenomenon, we believe further testing is necessary to further validate our results. The importance of ethylene in the survival of waterlogged soils and the genetic connections to these hormones biosynthesis has been of great focus in terms of understanding submerged tolerance in rice with the remaining focus being on maize, Arabidopsis or soybean. However, in regards to the genetic data available, functional characterization and clear identification of signalling pathways still require further study. Our global environment is forever becoming harder to predict, and this poses a great risk to future food security. Our goal was to simply identify if ethylene plays a role or not in waterlogging tolerance if further data confirms its importance more than the next steps into understanding how to improve our food sources must be taken. Further steps can be to identify levels of gene expression relating to ethylene biosynthesis in plants when grown under waterlogged conditions. Normally this would be done using model plants such as Arabidopsis, but perhaps this work should be pursued using plant species of more significance in the human diet, e.g. wheat, maize and soy. If key genes can be identified and characterized perhaps progressing into developing new mutants with improved genetic tolerance to waterlogging would provide a new gateway into food security. Interestingly, this field of research i.e. waterlogging tolerance has prioritised the vegetative growth stages of plants in waterlogged soils, yet, low amount of research has been done regarding the effects waterlogging has on germination, seedling stages or late reproductive stages. Therefore, there is much left to comprehend in terms of tolerance to this abiotic stress, especially when focusing on crops that do not grow naturally in submerged fields as is the case for rice.

Regrettably, the work done for this project did not yield results that we believe provides significant advances in this field, however, it has provided a good opportunity to explore this area of science associated with waterlogged tolerance and provided many ideas into ways of further researching the importance of plant hormones in plant survival.

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