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The Impacts of Plasticisers on Root Growth and Plant Systemic Signalling

By Freya Cook

This thesis is submitted for the degree of MSc by Research in Plant Sciences

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Table 1: Table of abbreviations commonly used throughout the text.

Full name	Abbreviation
Dimethyl phthalate	DMP
Acetyl tributyl citrate	ATBC
Benzyl butyl phthalate	BBP
Bis(2-ethylhexyl) phthalate	DEHP
Dibutyl sebacate	DBS
Diisononyl phthalate	DINP
Dioctyl terephthalate	DOTP/DEHTP
Tricresyl phosphate	TCP
Trioctyl trimellitate	TOTM
Abscisic acid	ABA
Cytokinin	CK
Brassinosteroid	BR
Gibberellic acid	GA
Jasmonic acid	JA
Reactive Oxygen Species	ROS

Abstract

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As plastic pollution continues and microplastics are generated from plastic breakdown in the environment, plasticiser leaching increases. This contaminates soils and aquatic systems, and bioaccumulates to amplify concentrations in organisms including crops grown for human consumption. Plasticisers, which are hydrocarbon chains added to plastics to increase flexibility, have been a subject of research for their impacts on mammalian health, as well as limited exploration into how they affect plant growth and physiology. To explore how plasticisers affect plant growth, and the mechanisms behind this, *Arabidopsis thaliana* was exposed to a selection of plasticisers with different uses and chemical structures. Seedlings were grown in agar containing a range of concentrations of plasticiser, and primary root length and secondary root number were recorded. The chemicals that impacted root architecture were used in further experiments, these were benzyl butyl phthalate (BBP), dimethyl phthalate (DMP), and dibutyl sebacate (DBS). Calcium signalling using bioluminescence, and auxin signalling using fluorescence were measured. 80 µg mL^{-1} of plasticiser had no impact on calcium signalling, but 800 µg mL^{-1} DMP induced higher calcium signalling over time and a signalling peak that was absent in other plasticiser and control experiments. In response to reactive oxygen species stress, plants grown in BBP and DMP had lower peak calcium signalling but elevated prolonged calcium signalling. Auxin signalling was elevated in the primary root of DMP-treated plants, and the secondary roots of those treated with BBP, DBS and DMP. These results implicate auxin accumulation and hydrogen peroxide stress in causing changes in root architecture observed from chronic plasticiser exposure, as well as demonstrate that DBS is harmful to plant growth, despite being previously characterised as a green alternative to plasticisers. This study provides a basis for further experiments into plasticiser interactions in soils and their effects on long-term plant development.

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Declaration

This thesis is my own work and has not been submitted in substantially the same form for the award of a higher degree elsewhere. All work other than my own is referenced fully.

Freya Cook

Lancaster, UK, December, 2023

Chapter 1: Introduction.

1.1. Microplastics in Ecology.

Microplastics, which are plastics that measure less than 5 mm in diameter, have been shown to have an impact on aquatic and terrestrial systems. The effects of microplastics on aquatic environments has been extensively researched, this stems from chemical leaching and the possibility for aquatic fauna to confuse plastic particles for food (Galloway *et al*., 2017). Both of these have the potential to alter entire aquatic ecosystems and devastate food webs, which could harm fish farming through bioaccumulation of microplastics or creating toxic farming environments (Lin *et al*., 2022). Terrestrial systems present different challenges. As the soil and particulate debris provide dilution for plastic particles, plastic is less likely to be confused for food (de Souza Machado, *et al*., 2018a). However, introducing plastic particles into a system that is composed of soil and debris can change the bulk density (the weight of a given volume of soil) of the substrate and still have leaching potential for the plastics (de Souza Machado, *et al*., 2018b). Changing the density of substrate can have impacts on plant development and biota that rely on the soil for shelter, by affecting soil porosity and microbial communities which reflect soil fertility (Wang *et al*., 2020).

The impacts of microplastics on terrestrial plant growth could be caused by a range of factors, which can be difficult to separate however the mechanisms by which microplastics can enact changes in plant growth are as follows; chemical toxicity, nutrient immobilisation, plant substrate structural changes, hydrodynamic changes, plant ecological changes and soil biota and microbiota balance (Rillig *et al*., 2019).

Plants have been shown to be impacted differentially depending on the types and sizes of the microplastics present in the soil, and the effects also vary between species. Microplastics have been shown to decrease soil bulk density and increase rhizospheric soil density (de Souza Machado *et al*., 2019). After a month and a half, plants exposed to microplastics have decreased root diameter and higher total root length compared to the control. This can be reflective of plants adapting to optimise their foraging performance in accordance with the altered substrate (Pagès, 2011), as a consequence of microplastic presence. Microplastics can also affect plant communities by decreasing biomass of dominant species with increasing microplastic diversity, increasing species evenness (He *et al*., 2023). The effects of the microplastics vary in intensity depending on the microplastic size, type and presence of plants, for example polyamide beads could be responsible for enriching soil nitrogen, resulting in a

two-fold increase in the nitrogen concentration in leaves (de Souza Machado *et al*., 2019). Microplastic effects on root symbioses, dry plant biomass and water content also vary between microplastic type.

Microplastics have been shown to have detrimental effects on plant ecosystems, and a causal model has been proposed for how impacts of plastic pollution can be pleiotropic (Figure 1). There is a cascade of events from each property that microplastics can impact, including soil structure as a primary factor from which hydrodynamics are changed, increasing the availability of water to plants which can impact their homeostasis. (de Souza Machado *et al*., 2019)

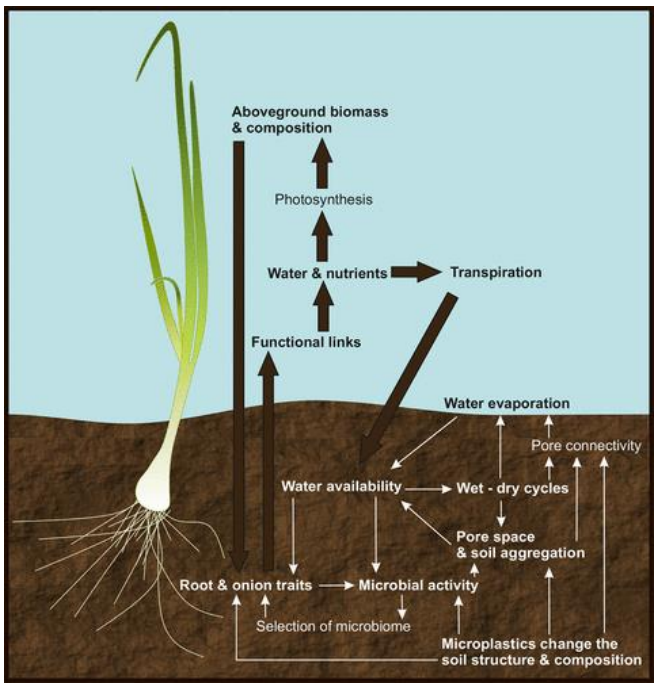


Figure 1: Schematic of the causes and effects of microplastics in soil. White arrows represent effects within the soil biophysical environment, brown arrows show plant responses and effects. Reproduced from de Souza Machado *et al*., 2019.

1.2. Plasticiser Pollution.

Plasticisers are polymers that are added during plastic production to increase plastic flexibility. Plasticisers range in their chemical composition, however, can be defined into classes based on their structure. These include phosphates, citrates (ATBC), orthophthalates (BBP, DMP, DEHP, DINP), organophosphates (TCP), sebacates (DBS), terephthalates (DOTP), trimellitates (TOTM) and adipates (CEFIC, 2018). Plasticisers have common properties such that they can function the same in industrial processes, including their viscosity, water solubility, high boiling points, low vapour pressures and lipophilic structure (Net *et al*., 2015). During plastic production,

plasticisers are not chemically bonded to the plastic's polymer matrix meaning that they can leach from the plastic into the surrounding environment as they migrate within the plastic due to weathering and heat (Luo *et al*., 2022).

As plastics continue to pollute the environment through anthropogenic activities, the amount of plasticiser that leaches into terrestrial, aquatic and atmospheric systems increases. Plasticisers have been detected in up to 100% of terrestrial soil samples from China, Europe and India, in which the mean concentration of plasticisers is 63.2mg kg^{-1} (Billings *et al*., 2021). In particular, di-iso-butyl phthalate, di-n-butyl phthalate, bis (2-ethylhexyl) phthalate and di-n-heptyl phthalate have detection frequencies of 100% in soil samples (Billings *et al*., 2021). Plasticisers can have marked effects on plant and human health, although these vary due to the differences in their chemical structure. Classes such as adipates, sebacates, trimellitates, terephthalates, citrates and organophosphates are considered to be less hazardous than phthalate plasticisers (Bui *et al*., 2016) and are proposed as alternatives to plasticisers that are more commonly used and have more hazardous properties. Hazardous plasticisers commonly used in industrial processes are often orthophthalates which are phthalic acid esters (PAEs). As a result of studies into the toxicity of plasticisers, a number have been restricted to certain concentrations for commercial use. These include bis (2-ethylhexyl) phthalate, dibutyl phthalate, benzyl butyl phthalate and diisobutyl phthalate (ECHA, 2018). Despite this, due to their continuous use in the past there is still terrestrial pollution from these chemicals, and other plasticisers that have not yet been categorised as too toxic for continued use are still being manufactured and may cause unknown harm.

The chemical properties of plasticisers and low cost cause them to be in high demand for industrial processes involving plastics, as such they had an 8.4 million tonne global market estimated per year (CEFIC, 2018). 13% of this was used in consumer goods and industrial applications, and 8% was used in other applications including medical equipment (CEFIC, 2018). Plasticiser content in plastic items can be from 10 – 70% w/w (Hansen *et al*., 2013), therefore environmental pollution and leaching can lead to high concentrations of plasticisers in the environment. This means that human exposure to plasticisers can be very high depending on geographical location and plastic usage. High concentrations of bisphenol analogue plasticisers have been found in hair, urine, drinking water and air samples taken from residents in Hong Kong, with concentrations up to 36.5 ng kg^{-1} being found in urine samples (Li *et al*., 2021). The frequency of detection was 81% for phthalate metabolites in urine samples, which was lower than those found in Japan and German during previous studies that investigated other plasticisers (Li *et al*., 2021).

The13ffectts of plasticisers on human health led to the characterisation of many plasticisers as endocrine disruptors. More specifically, plasticiser exposure has been associated with human health problems including risks of cancer, obesity, thyroid disorders, diabetes, altered reproductive health, immune function and neurodevelopment (Lee *et al*., 2015; Miao *et al*., 2017; Callaghan *et al*., 2020; Burgos-Aceves *et al*., 2021; Bereketoglu and Pradhan, 2022). This was shown through multiple exposure routes including airborne, oral, and dermal routes which induced health risks from a range of plasticisers, some of which have been restricted for commercial use (Miao *et al*., 2017). Plants are also susceptible to similar exposure routes by leaching from plastic pollution (Gulizia *et al*., 2023), and the accumulation of plasticisers in plant tissues from these routes can cause bioaccumulation in humans if crop species are affected (Kumari and Kaur, 2020). Less is known about the mechanisms by which plasticisers impact plants than humans, therefore this is the subject of this thesis.

1.3. Plasticulture and Plastic Pollution.

Plasticisers are used in agriculture in plastic films that maintain humidity in greenhouses, as well as mulch film. The humidity maintained by this plastic can also insulate heat, which makes plasticisers such as PAEs used in agriplastics more easily released into soils because the strength of bonds between the PAEs and plastic are weakened (Shi *et al*., 2019). This means that crops in particular will be impacted by PAE exposure and may increase the bioaccumulation of phthalates that can impact human health, which is also shown via increased carcinogenic risk for adults consuming crops that are exposed to plasticisers (Shi *et al*., 2019). In particular, DEHP has potential carcinogenicity which is exacerbated for children and those living in the countryside compared to those in the city (Shi *et al*., 2019).

Fertilisers, including sludge organic fertilisers which can contain PAEs, have been used to ease the environmental and geopolitical pressure on global food security to maintain agricultural output to feed the growing population (Cusworth *et al*., 2022). Sludge organic fertilisers are made of sewage sludge which does not require high amounts of energy to produce, and is a form of waste utilisation (Jamil Khan *et al*., 2006). The transition to organic fertilisers, however, also presents a problem with PAEs, as sludge organic fertilisers (SOFs) are a large source of phthalates in soils (Hui *et al*., 2021). SOFs are made by composting and processing sewage sludge by-products from sewage treatment. In conditions with long-term elevated nitrogen concentration (300 kg N ha^{-1} yr^{-1}), as is often the case with crop fields to improve yield, dissolved organic matter increases in soils. SOF application causes plasticisers to have increased accumulation in soils and crops with enriched nitrogen concentrations, with the total PAE concentrations in soil elevating from 0.96 mg kg^{-1} under normal conditions to 1.86 mg kg^{-1}

under high nitrogen (Hui *et al.*, 2021). The accumulation of PAEs was 30% higher than observed in normal nitrogen conditions, exceeding the United States allowable soil concentration standard. DEHP has the highest accumulation and PAE accumulation in the roots is 4 times higher with SOF application compared to the control. 60% of the PAEs are concentrated to the top 60 cm of soil profile (Hui *et al.*, 2021). This imposes an increased human health risk and carcinogenic risk when SOFs are applied to soil, especially in soils with a high nitrogen concentration which can be caused by SOF application itself. Characterising the effects of PAEs from various sources is therefore vital to determine whether alternatives must be investigated in combination with other ecological properties.

1.4. Hormone Roles and Crosstalk in Root Growth.

Plant root growth is a coordinated process that involves the growth of plant organs below the ground. Roots have many roles in plant homeostasis, including but not limited to nutrient, mineral and water acquisition, plant stability and communication with other plants. The root system of a plant is generally composed of a primary root and secondary roots which grow and branch from the primary root. Root development follows an apical-basal gradient (Figure 2) as cells proliferate at the apex of the root in the root apical meristem, and then elongate in the elongation zone just behind the apex (Hodge *et al.*, 2009). Root growth is maintained by a root stem cell niche that is composed of quiescent centre organiser cells and surrounding initial cells (Cruz-Ramírez *et al.*, 2013).

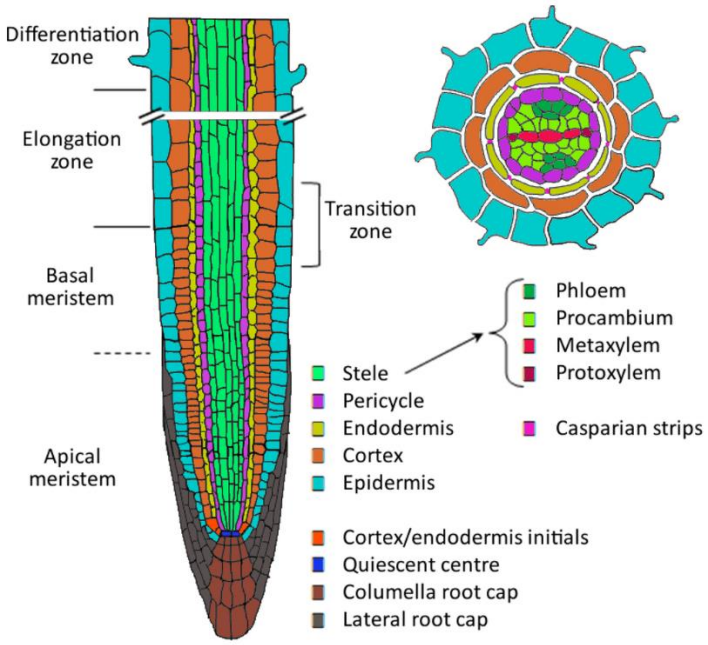


Figure 2: *Arabidopsis thaliana* root tissue organisation along longitudinal (left) and latitudinal (right) planes. Cell types are illustrated by colour. The apical meristem is where cell division is

focussed, then it slows down the basal meristem, and the transition zone is where cells begin to elongate instead of divide, moving into the elongation zone. Cells differentiate into different tissue types in the differentiation zone, as shown in the latitudinal cross section. Reproduced from De Smet *et al*., 2015.

Root system development is mediated by phytohormones, which are chemical messengers. Phytohormones involved in root growth (Figure 3) are auxin, gibberellic acid (GA_3), abscisic acid (ABA), cytokinin (CK), ethylene, jasmonic acid (JA), brassinosteroids (BR), and strigolactone (Ubeda-Tomás *et al*., 2012; Qin *et al*., 2019). Of these hormones, GA_3, auxin, CK and BR are stimulatory, whilst ABA, JA and ethylene downregulate root growth (Sharma *et al*., 2021). The stimulatory hormones can also have inhibitory effects at certain concentrations and under certain plant stresses.

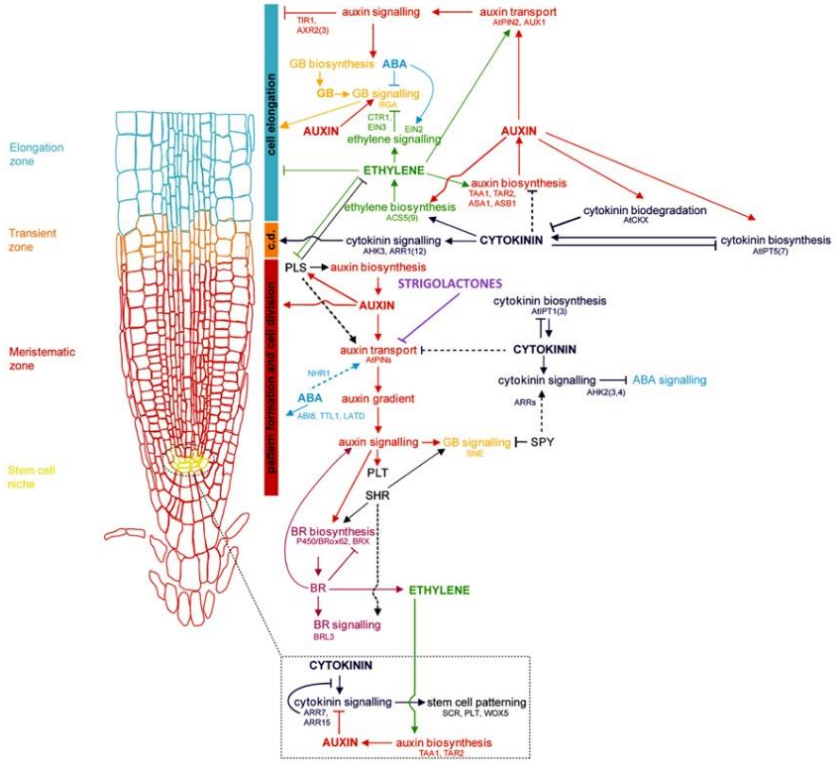


Figure 3: Schematic of the crosstalk network in hormone signalling that regulates root growth and development. Different colours show activity by each hormone, dashed lines represent relationships that have not been fully investigated or are indirect. Lines ending in bars show antagonistic relationships. Reproduced from Benková and Hejátko, 2009.

Auxins encompass multiple compounds including indole-3-acetic acid (IAA), which is the most broadly studied auxin (Epstein and Ludwig-Müller, 1993). IAA can accelerate stem elongation but suppress root elongation at concentrations above 1 nM (Scott, 1972; Walter *et al*., 2020;

Edelmann, 2022), which is exemplified when roots grow gravitropically by decelerating growth of lower root cells via an increased IAA concentration, enabling the roots to bend downwards (Marshall Porterfield, 2002; Levernier *et al*., 2021). Endogenous IAA is regulated by cell influx, cell efflux, biosynthesis from tryptophan, and oxidative IAA breakdown (Tanimoto, 2007). IAA is mainly synthesised in aerial shoots and root tips, therefore its concentration is largely regulated via influx, efflux or oxidation in other parts of the roots (Ljung *et al*., 2005; Pěnčík *et al*., 2013). Membrane-located carrier proteins, PIN proteins, allow this transport, which follows an apical to basal polarity to facilitate cell elongation in the elongation zone (Petrášek and Zažímalová, 2006). PIN proteins are asymmetrically localised to the plasma membrane, enabling polar transport (Langowski *et al*., 2016). Calcium signalling is required to establish this polarity, as microtubule responses to mechanical perturbation are transduced by calcium and result in changes of PIN protein distributions (Zhang *et al*., 2011; Li *et al*., 2019). Auxins are heavily linked with root growth by regulating gene expression via RNA synthesis (Key and Shannon, 1964) and cell cycle transcription factors (Himanen *et al*., 2002). Elongation is regulated by auxins by initiating "acid growth", which is when cell walls become more flexible under acidic conditions caused by increasing proton pump activity (Rayle and Cleland, 1992; Lin *et al*., 2021).

Auxin is at the centre of many crosstalk signalling networks, this is because auxin promotes root growth which may need to slow in response to other hormones signalling which produces antagonistic relationships. GA_3 is an example of this, because it promotes DELLA protein degradation and selectively downregulates cytokinin-responsive transcription factors which activate auxin regulator genes (Moubayidin *et al*., 2010). BR has also been implicated in this process, as BR has the effect of reducing differentiation by downregulating CK activity and reducing proliferation by downregulating auxin activity (Moubayidin *et al*., 2010). As a result of crosstalk, mutant plants that have a reduced sensitivity to one hormone may also have reduced sensitivity to others. This is exemplified as auxin resistance that produces a dwarfed phenotype in *Arabidopsis thaliana* mutants, as well as reduced gravitropism, also show resistance to ABA and ethylene signalling (Wilson *et al*., 1990).

GA_3 stimulates root elongation in a saturation dose-response, meaning that GA_3 does not inhibit root growth at certain concentrations and instead has a limit of saturation beyond which increasing the concentration of GA_3 has no effect on root elongation (Chandler and Robertson, 1999). GA_3 biosynthesis is located in many plant tissues but is predominantly produced in the endodermis (Barker *et al*., 2021). GA_3 is able to diffuse across the plasma membrane and is transported into cells with influx transporters (Rizza and Jones, 2019).

Concentrations of GA_3 are regulated by temporal and spatial expression of biosynthetic and catabolic enzymes that control GA_3 levels in different tissues and stages of development (Yamaguchi, 2008). GA_3 acts at the meristem elongation zone and facilitates elongation by initiating the breakdown of DELLA proteins, which repress cell growth (Silverstone *et al*., 2001). DELLA proteins also upregulate cell cycle inhibitors which reduce cellular expansion and proliferation. Therefore, GA_3 activity promoting DELLA degradation directly increases root growth (Achard *et al*., 2009). GA_3 also changes the structure of the cell wall in expanding cells to enable water influx, which increases the cell volume and elongates the cells (Jones and Kaufman, 2008).

JA is heavily associated with pathogenic defence and stress responses, however has roles in root growth that involve crosstalk with other hormones such as ethylene and auxin. Amongst these, JA has a role in lateral root development through crosstalk with auxin (Jiaqiang *et al*., 2009). This occurs via jasmonate-induced auxin biosynthesis, which acts in the basal meristem to increase local auxin concentrations and influence auxin transport. JA and auxin increase mitotic activity of quiescent centre organisers, which promotes regeneration of root tissues in response to herbivory (Zhou *et al*., 2019).

Ethylene has been shown to influence root growth by increasing root width, initiating formation of ectopic root hairs, and decreasing cell elongation (Swarup *et al*., 2007). This is achieved by crosstalk with auxin by upregulating auxin biosynthesis and transport in the apex of the root. Ethylene response factors are maintained by auxin to regulate cell division patterns in early lateral root primordia (Hirota *et al*., 2007). Auxin biosynthesis is also upregulated by ethylene to initiate development of the adventitious root, this theory is supported by concentrations of both auxin and ethylene increasing when the basal root is cut and begins regrowth (Guan *et al*., 2019).

CK is synthesised in the root tip and active in the transition zone of the roots (Aloni *et al*., 2005). CK promotes vascular differentiation and root elongation, which is achieved by antagonising auxin (dello Ioio *et al*., 2007). This antagonism controls the size of the root meristem by redistributing auxin along polar transport mechanisms which promotes cell differentiation (dello Ioio *et al*., 2008; Růzĭčka *et al*., 2009). CK has also been observed restricting adventitious and lateral root growth (Bollmark and Eliasson, 1986; Debi, Taketa and Ichii, 2005). CK has been suggested as a continuous product of root statocytes, which are gravity-sensing cells that allow for immediate gravitropism due to their constant production of CK that enables directional growth (Aloni *et al*., 2004).

BR activity is restricted to the epidermis of the meristem in roots and determines the meristem size (Hacham *et al*., 2011). This occurs via controlling cell cycle progression and cell expansion before mitosis. Cell expansion by BR is achieved by cell-wall alterations, this takes place in the root elongation zone and involves inhibiting root lignification up to the mature root zone (Li *et al*., 2022). Upon entering the mature root zone, BR signalling decreases and root lignification occurs which strengthens cell walls. BR, like auxin, can have an inhibitory effect on root growth at certain concentrations and can act antagonistically towards auxin which maintains optimal root growth (Chaiwanon and Wang, 2015). BR also plays a role in gravitropic responses through an agonistic relationship with IAA, increasing polar expression of auxin and inducing formation of the actin cytoskeleton (Li *et al*., 2005; Lanza *et al*., 2012). The gravitropism and polar expression occur via BR promoting accumulation of the protein PIN2 in the tip of the root, as *PIN* genes facilitate auxin transport (Lanza *et al*., 2012).

ABA has a biphasic root growth pattern as a result of crosstalk with ethylene and auxin signalling pathways (Li *et al*., 2017). This is due to high concentrations of exogeneous ABA causing inhibition of root growth, when low concentrations promote elongation (Miao *et al*., 2021). ABA is a stress hormone and has been closely linked with mediating stomatal aperture in response to stresses like light or drought (Leckie *et al*., 1998). ABA has been shown maintain root growth under water stress (Saab *et al*., 1990). Expression of the auxin transporter *PIN2* is suppressed by ABA, therefore preventing root growth (Xie *et al*., 2021). This occurs in tandem with agravitropic root growth patterns and reduced auxin accumulation. ABA derived from foliage increases root growth, but inhibits lateral root development which is thought to be through inhibition of IAA (McAdam, Brodribb and Ross, 2016). ABA alters root growth by inducing ABA INSENSITIVE4 (ABI4) gene transcription, which in turn inhibits expression of positive cell cycle regulatory genes and prevents cell cycle progression that would elongate the root (Luo *et al*., 2023). Other research has suggested additional involvement of Ca^{2+} and reactive oxygen species in ABA mediating root growth (Sun *et al*., 2018).

Strigolactones, derived from carotenoids, are predominantly synthesised in the roots following phosphate deficiency then transported (Kohlen *et al*., 2011). Strigolactones control adventitious root formation (Rasmussen *et al*., 2012; Fan *et al*., 2023), secondary stem growth (Gomez-Roldan *et al*., 2008; Agusti *et al*., 2011), auxin regulation (Hayward *et al*., 2009; Zhang *et al*., 2020a), vertical plant growth (de Saint Germain *et al*., 2013), and vascular tissue composition (Zhao *et al*., 2023). Adventitious root formation is inhibited by reduced expression of *CYCLIN B1* induced by strigolactone, restricting initial cell divisions that lead to adventitious roots (Rasmussen *et al*., 2012; Sun *et al*., 2019). This is in direct antagonism with auxin which

promotes adventitious root initiation. Strigolactone has been shown to promote primary root growth and suppress lateral root formation (Ruyter-Spira *et al*., 2011; Zhang *et al*., 2020a). This occurs from downregulation of PIN proteins, impeding auxin transport. Conditions with limited phosphate caused more rapid lateral root growth in wild-type *Arabidopsis thaliana* compared to mutants with strigolactone deficiency (Ruyter-Spira *et al*., 2011). The relationship between phosphate levels and strigolactone activity may be linked with mycorrhizal symbiosis, as arbuscular mycorrhizal symbiosis leads to increased inorganic phosphate sequestration from soils (Ruyter-Spira *et al*., 2011). Strigolactones have been shown to stimulate *Gigaspora* cellular proliferation (Besserer *et al*., 2006) and induce hyphal branching (Akiyama, Matsuzaki and Hayashi, 2005), therefore under phosphate deficient conditions plants with elevated strigolactone activity can upregulate mycorrhizal symbiosis and provide additional phosphate to supplement the environmental phosphate deficiency (Yoneyama *et al*., 2007). The response of upregulated SL from phosphate deficiency is also conserved in plants that do not host arbuscular mycorrhiza (Kohlen *et al*., 2011).

Though not a plant hormone, calcium is involved in root growth by modulating hormone accumulation and transducing hormone signals, including auxin, through distinct calcium signatures that produce specific cellular responses (X. P. Zhang *et al*., 2020). The release of calcium from nuclear membrane-localised ion channels in root cells has been correlated with auxin homeostasis and meristem development (Leitão *et al*., 2019). Calcium chelating agents have also been shown to prevent gravitropic root growth, suggesting that calcium is required for gravitropic root growth (Evans, 1986). Calcium application to one side of the root cap at the tip of the root caused growth towards the calcium source, and polar transport of both calcium and auxin along the elongation zone in response to gravistimulation. This suggests that calcium has a role in auxin signalling since auxin transport inhibitors also prevented polar calcium transport (Evans, 1986).

Hydrogen peroxide (H_2O_2) and calcium crosstalk can occur on a cellular level in plants during root growth and development. H_2O_2 has been implicated in mediating calcium influx into root cells which causes root elongation in a mechanism that mimics the action of ABA (Han *et al*., 2015). Conversely, H_2O_2 has been shown to counteract ABA effects on seeds by inducing germination via triggering calcium influx which creates a positive feedback loop, further elevating H_2O_2 and sustaining the calcium signature which modulate GA_3 and ABA levels (Cheng *et al*., 2022). Calcium and Calmodulin may be downstream signalling molecules of H_2O_2 and nitrous oxide, and are implicated in adventitious root stimulation by auxin (Liao *et al*., 2012).

1.5. Plant Stress Responses.

Plants use hormones to mediate their stress responses, with hormones often responding to different stresses. Osmotic stress has been illustrated as a factor that activates crosstalk between hormones, this is mediated by many hormones including ABA, CK, ethylene and auxin. In response to osmotic stress, ABA upregulates auxin transport by *AUX1* and *PIN2*, which causes root elongation in immature rice and *Arabidopsis thaliana* (Xu *et al*., 2013), CK and ethylene are also downregulated because these inhibit ABA signalling (Sharp, 2002; Nishiyama *et al*., 2011).

JA and ethylene signalling are integrated by auxin in response to environmental changes via transcription factors. One example of this is in response to necrotrophic fungi, which cause plant disease, EIN3 and EIL1 transcription factors are upregulated which are also crucial in ethylene activation (Zhu *et al*., 2011; Binder and Jez, 2020). These transcription factors upregulate *TAA1* transcription which increases auxin biosynthesis, this process involves AAR1 which is a cytokinin transcriptional regulator (Yan *et al*., 2017; Vaseva *et al*., 2018).

As a secondary messenger, calcium is involved in transducing signals from stimuli which includes stress perception (Figure 4). Abiotic stresses can be transduced by mechanosensitive channels due to touch sensitivity or membrane fluidity being affected which trigger these channels (Tsugama *et al*., 2018). Cold stress perception is mediated by COLD1 (chilling-tolerance-divergence 1), a transmembrane protein, and receptor-like kinases which also sense changes in membrane fluidity (Ma *et al*., 2015). Reactive oxygen species (ROS), in particular H_2O_2 are detected by a leucine-rich repeat receptor kinase (Fichman *et al*., 2022). Once the cell membrane perceives a stress factor, second messengers are upregulated within the cell which can include Ca^{2+}, lipids and cyclic nucleotides (Yip Delormel and Boudsocq, 2019). In the case of Ca^{2+}, distinct signatures are produced which causes a cascade of phosphorylation or dephosphorylation of proteins that influence gene expression (Das and Pandey, 2010). This produces stress-tolerant phenotypes as gene expression can result in cellular responses ranging from upregulation of growth hormones for root architecture changes, to producing ROS scavenging enzymes that prevent cellular damage (Reddy *et al*., 2011; Savchenko and Tikhonov, 2021).

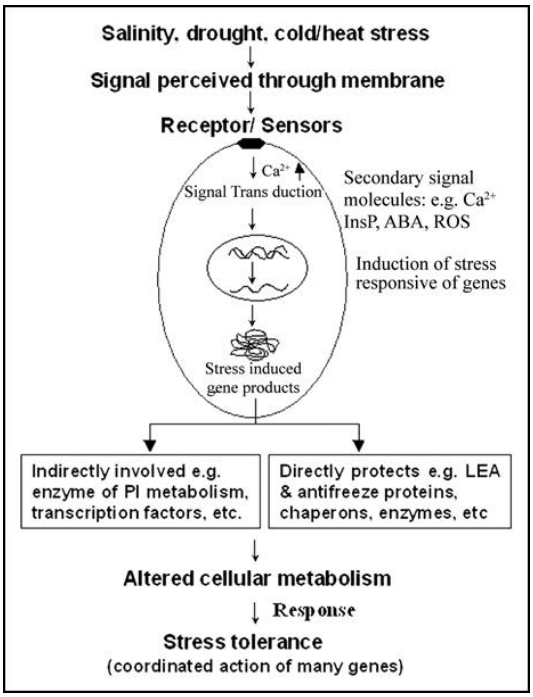


Figure 4: Calcium signalling pathway from extracellular stress detection to stress response and tolerance. Transmembrane proteins detect exogenous stress, often by transmembrane proteins, which causes second messenger generation to transduce the stress signal. This causes altered gene expression to upregulate stress response pathways, producing a stress-tolerant phenotype. LEA, late embryogenesis abundant; InsP, inositol phosphate. Reproduced from Tuteja and Mahajan, 2007.

Salt stress in particular has been shown to arrest primary and lateral root growth in which the lateral roots enter a prolonged quiescent phase. This quiescent phase is induced by increased ABA concentrations (Duan *et al*., 2013) in which the quiescent centre is shielded from damage by programmed cell death induced by ROS accumulation in the root tip (West *et al*., 2004; Hasanuzzaman *et al*., 2021). This occurs until the root meristem decreases in size, and the high ABA concentration also disrupts auxin distribution which inhibits root growth (Bloch *et al*., 2019). Partial growth recovery occurs in the recovery phase when crosstalk occurs and JA, GA_3 and BR concentrations increase whilst ABA decreases (Duan *et al*., 2013). Salinity influences root architecture by producing fewer secondary roots and reducing primary root growth in *Arabidopsis thaliana* (Iglesias *et al*., 2010). This process is mediated by calcium transients which transduce stress signals and cause hormone signalling pathways to activate (Dinneny, 2015).

Whilst ROS are present in plant cells at low concentrations and are often used as signalling molecules, high levels of ROS induce oxidative stress (Mittler *et al*., 2004). This can cause

programmed cell death, decreased growth rate and mitochondrial protein degradation (Sweetlove *et al*., 2002; Biswas, Terada and Mano, 2020) Plants respond to oxidative stress by using ROS scavenging mechanisms. This involves enzymatic and non-enzymatic pathways that reduce ROS levels in cells and mitigate cellular damage (Savchenko and Tikhonov, 2021). ABA is involved in the enzymatic pathway, as ABA induces gene transcription mediating ascorbate-glutathione activity (Ghassemian *et al*., 2008). Oxidative stress can be induced by other stresses, including severe salt and osmotic stresses as plants activate the same gene expression pathways for oxidative stress when other stresses become chronic (Claeys *et al*., 2014). These examples illustrate the interplay between hormones to produce stress responses, which could be active when plants are exposed to plasticisers to produce changes in phenotype due to plasticiser stress.

1.6. Plasticiser Influence on Plant Physiology.

The impact of plasticisers on plant physiology has been investigated in many studies, revealing that many different plasticisers impede plant growth and development. However, there is a lack of knowledge in how plasticisers influence hormones and subsequent developmental pathways that arise from altered hormone activity, which are at the centre of plant growth.

Dimethyl phthalate (DMP) has an impact on vegetable crop germination and plant growth. Physiological indexes in leaves are altered following 7 days of DMP exposure in excess of 30 mg L^{-1} (Zhang *et al*., 2015). These indexes are increased proline, peroxidase, catalase and hydrogen peroxide concentrations, and decreased chlorophyll content. Seed germination is also delayed with DMP exposure (Zhang *et al*., 2015). Root growth is inhibited above 30 mg L^{-1}, which is speculated to be caused by the initial growth stages of seedlings being inhibited by DMP treatments. The ultrastructure of the chloroplasts is altered, including disorganised lamellae, damaged thylakoid membrane structures, and increased starch grain size and volume (Zhang *et al*., 2015). This is thought to be caused by reactive oxygen species accumulating.

Plasticisers di-n-butyl phthalate (DBP), and Bis (2-ethylhexyl) phthalate (DEHP), are frequently used in polyvinyl chloride (PVC) production. Increasing DEHP and DBP concentrations leads to decreases in a range of physiological parameters, including net photosynthetic rate, stomatal conductance, maximal photochemical efficiency, transpiration rate, photochemical quenching, non-photochemical quenching, photosynthetic electron transport rate, fresh biomass, dry biomass and effective quantum yield of photosystem II in wheat seedlings (Gao *et al*., 2016). There are increases in intracellular concentration of CO_2, and initial chlorophyll fluorescence. These changes together indicate that damage to the plant is caused by DEHP and DBP. These

impacts span photosynthetic activity and root development which reflect holistic effects of plasticisers on plant growth (Gao *et al*., 2016).

BBP, which has been restricted for commercial use, was studied for its effects on *Hordeum vulgare* seedlings and caused significant increases in stress indexes (Kumari and Kaur, 2019). This includes superoxidase dismutase, catalase, guaiacol peroxidase, glutathione reductase and ascorbate peroxidase, which are indicators of oxidative stress as a response to the presence of reactive oxygen species in plant tissues. Cytotoxicity was also observed showing deformed stomata and low cell viability, which was assessed via plasma membrane integrity (Kumari and Kaur, 2019). There was also an increase in soluble sugars and protein content in response to BBP exposure. These increased measures of plant stress and abnormal physiology of the barley seedlings highlight that BBP is harmful to plants and induces stress response pathways.

PAEs have a high toxicity to lettuce, as characterised by the effects of DnBP and DEHP on biomass, soil microbial communities, photosynthetic parameters and physiological measures of *Lactuca sativa* (Ma *et al*., 2018). The results showed that different soil pH conditions yielded different toxicity of DnBP and DEHP, and under neutral soils water content influenced the phytotoxicity of PAEs (Ma *et al*., 2018). Moreover, increasing concentrations of PAEs were negatively correlated with leaf area, biomass, vitamin C, soil microbial parameters and superoxide ion levels. DnBP was shown to have a more inhibitory effect on soil microbial flora, carotenoid and Chlorophyll a concentrations as well as an overall higher toxicity at different soil pH conditions (Ma *et al*., 2018). This illustrates the pleiotropic effects of plasticiser toxicity, as the ecosystem as a whole can be altered by only a few factors.

1.7. Plasticiser Alternatives.

The evidence for toxicity of plasticisers to humans, plants and wider ecosystems has led to the exploration of alternatives to plasticisers in plastic production. This includes plasticisers that are still phthalic acid esters but have been characterised as less harmful to endocrine signalling (Qadeer *et al*., 2022) However, many of these compounds have been shown to still have profound effects. For example, bisphenol-A (BPA) was replaced with other bisphenol analogues that had effects on endocrine activity similar to those that made BPA a restricted plasticiser (Rochester and Bolden, 2015; le Fol *et al*., 2017). In contrast, in plants, adipates which are alternatives to PAEs have been shown to have either low or insignificant toxicity to watercress and radish, in terms of plant root length, seed germination and above-ground height (Vikhareva *et al*., 2021). Similarly, a range of 12 organophosphate esters (OPEs) applied

to zucchini, soybean, tomato, lettuce and carrot have been shown to translocate into plants from the soil with negligible effects on plant growth (Hu *et al*., 2021).

Plant-based plastic alternatives have been developed using arrowroot starch biofilms and glycerol acting as a plasticiser. The resultant films had increased flexibility and improved environmental shielding capacity compared to other starch-based biofilms and underwent rapid degradation following burial in soil, with over 70% of the biofilm degraded in as little as 22 days for biofilm with 15% glycerol content (Tariqueet *et al*., 2021). Moreover, processes that mimic the effect of plasticisers have been explored such as copper-free azide-alkyne click chemistry (Earla and Braslau, 2014). The compounds used to achieve this have been shown to have the same effect as the most commonly used plasticisers in plastic production, however they do not have the same chemical leaching potential which causes the endocrine disruption observed from traditional plasticisers. Alkyne click chemistry can be completed at room temperature and covalently bonds the plasticiser mimics to the plastic, eliminating leaching potential and lowering production costs (Earla and Braslau, 2014).

1.8. Thesis Aims.

Several classes of plasticiser have been shown to effect different facets of plant physiology, including photosynthesis, and root growth. However, a holistic study spanning different plasticiser groups and their impacts on plants is essential to assess the effects of increased societal and agricultural use of plastics on health and global food security. The objective of this study is to identify the molecular mechanisms by which plasticisers effect root development and growth. This study aims to:

1. Test effects of plasticisers on root growth.
2. Investigate plasticiser effects on calcium signalling.
3. Assess impacts on hormone signalling.

The hypothesis is that plasticiser treatments will have a negative impact on root growth, in particular the orthophthalates. These have been characterised as having a prominent effect on root growth in existing literature. Orthophthalates are also predicted to have the highest impact on hormone and messenger signalling, which will be shown by altered fluorescence in genetically modified *Arabidopsis thaliana* plants. The signalling molecules selected that will show variation from plasticiser exposure will be auxin and calcium, because auxins are heavily involved in root growth, and calcium is a heterogeneous messenger for transducing a range of signals from different stimuli therefore many stresses caused by plasticiser exposure can be transduced by calcium and cause changes in its distribution.

The impacts of plasticiser on calcium signalling may increase or decrease sensitivity to stimuli depending on the mechanism of how plasticisers affect plant physiology. Plasticiser uptake could damage response pathways and limit the plants' signalling capacity when stressed, alternatively it could dampen responses by giving plants chronic stress which may make them less responsive to acute treatments because there is a higher threshold for plants to be considered as stressed. It could also increase sensitivity by constitutively activating pathways for stress responses so the plants are primed to respond to stress and can rapidly upregulate physiological responses.

Auxin signalling may be upregulated because plants are responding to plasticiser stress by altering root growth, this is illustrated when plants detect salinity stress in which the roots exhibit tropism and grow away from salt sources to maintain homeostasis and mitigate extended exposure to high salt conditions (Galvan-Ampudia *et al*., 2013). Auxin may also be elevated due to damaged polar transport mechanisms that cause auxin accumulation. Increased auxin production or accumulation would be illustrated as increased fluorescence the roots of auxin reporter lines. Alternatively, downregulation of auxin would occur as similarly during salt stress plants can respond by downregulating lateral root growth which is mediated by ABA (Bloch *et al*., 2019), an antagonist of auxin which would cause decreased auxin signalling.

Chapter 2: Methods.

2.1. Chemicals.

All chemicals used were from Sigma Aldrich unless otherwise stated. Plasticisers were selected for a range of molecular weights, chemical structures and groups (Table 2).

Table 2: Chemical structures of plasticisers used, including number of benzene rings, chemical and linear formulae, chemical group, and molecular weights.

	Benzene ring	Chemical formula	Linear formula	Group	Molecular mass (Da)
ATBC	0	$C_{20}H_{34}O_8$	$CH_3CO_2C[CO_2(CH2)_3CH_3][CH_2CO_2(CH_2)_3CH_3]_2$	Citrate	402.48
BBP	1	$C_{19}H_{20}O_4$	$2\text{-}[CH_3(CH_2)_3O_2C]C_6H_4CO_2CH_2C_6H_5$	Orthophthalate	312.36
DBS	0	$C_{18}H_{34}O_4$	$[\text{-}(CH_2)_4CO_2(CH_2)_3CH_3]_2$	Sebacate	314.46
DEHP	1	$C_{24}H_{38}O_4$	$C_6H_4(COOC_8H_{17})_2$	Orthophthalate	390.56
DINP	1	$C_{26}H_42O_4$	$C_6H_4(CO_2C_9H_{19})_2$	Orthophthalate	418.61
DMP	1	$C_{10}H_{10}O_4$	$C_6H_4\text{-}1,2\text{-}(COOCH_3)_2$	Orthophthalate	194.18
DOTP	1	$C_{24}H_{38}O_4$	$C_6H_4\text{-}1,4\text{-}[CO_2CH_2CH(C_2H_5)(CH_2)_3CH_3]_2$	Terephthalate	390.56
TCP	3	$C_{21}H_{21}O_4P$	$(CH_3C_6H_4O)_3PO$	Organophosphate	368.36
TOTM	1	$C_{33}H_{54}O_6$	$C_6H_3\text{-}1,2,4\text{-}[CO_2CH_2CH(C_2H_5)(CH_2)_3CH_3]_3$	Trimellitate	546.78

2.2. Root growth assay.

Arabidopsis thaliana ecotype Colombia-0 (Col-0) seeds were surface sterilised in an Eppendorf tube suspended in 1 mL 70%/0.1% (v/v) ethanol/Triton X for 5 minutes, rinsed with 1 mL 95% (v/v) ethanol, and expelled onto Whatman filter paper with 0.5 mL 95% (v/v) ethanol and allowed to dry. Once dry, 100 seeds were sown onto Petri dishes with control medium (CM) containing 0.8% (w/v) agar, 0.6% (w/v) sucrose and ½ Murashige and Skoog with vitamins to pre-germinate seeds. This media provides a consistent, nutrient rich, moist environment that allows for non-invasive analysis of root architecture. All seeds and seedlings were grown at 22 ± 2°C day/20 ± 2°C night, and a 16 hour photoperiod with 33 ± 2 w m^{-2} irradiance in Controlled Environment (CE) Room 9 at Lancaster Environment Centre, Lancaster University.

Seeds were maintained in the CE room for 3 days, then 8 germinated seeds were transferred to each fresh Petri dish containing CM with or without plasticiser. Plasticiser concentrations were selected based on Billings *et al*. (2021) using recorded plasticiser presence and

concentrations in soil samples. The concentrations used were 5 µg mL^{-1}, 10 µg mL^{-1}, 20 µg mL^{-1}, 40 µg mL^{-1} and 80 µg mL^{-1} of plasticiser dissolved in ethanol by serial dilution to give a maximum concentration of ethanol of 1% (v/v). Seedlings were also grown on CM containing 1% (v/v) ethanol to control for the impacts of the highest concentration of plasticiser solvent on root growth, as well as 50 mM NaCl, which inhibits root growth (Zhu *et al*., 1997; Liu *et al*., 2015), as a negative control. NaCl was selected because of its ability to dissolve in media and impact root architecture in similar ways to those predicted in this study (Liu *et al*., 2015). In all cases, one plate of seedlings grown on CM in the absence of 50 mM NaCl, ethanol or plasticiser was included alongside each treatment as a control to ensure that different batches of agar did not have an impact on root growth. As the seedlings grew, root length was marked on the petri dish every 2 days until seedling were 10 days old. The seedling roots were then pictured using a Samsung S20 FE mobile phone and measured using ImageJ to ascertain root length and number of secondary roots. The threshold for a secondary root instead of a secondary root bud was defined as secondary branching above 0.5 mm. Root architecture ratio was calculated as the number of secondary roots per cm of primary root within each treatment and concentration. Root length inhibition was measured as the percentage difference in primary root length between the control treatment and 80 µg mL^{-1} of the chosen plasticiser.

2.3. Measurement of cytosolic Ca^{2+} using the recombinant aequorin system.

Whole seedling cytosolic Ca^{2+} was measured by luminometry (Short *et al*., 2012). Seeds of *Arabidopsis thaliana* Col-O constitutively expressing the apoaequorin gene (Col-Aeq) (Knight *et al*., 1991) were surface sterilised and grown on CM, as in the root assay methodology (see Section 2.2). 10-day-old seedlings were carefully removed from the agar and incubated in 4 µM coelenterazine, 1.5% (v/v) methanol at 20 ± 2°C for a minimum 16 h in the dark to reconstitute the aequorin. Individual seedlings were transferred to 14 mm luminometer cuvettes containing 0.5 mL RO water, the cuvette was inserted into the luminometer in the dark and the aequorin bioluminescence recorded. The baseline bioluminescence was measured for 5 minutes, and then 0.5 mL of the test chemical was injected into the cuvette and the bioluminescence recorded for a further 50 minutes. Ice-cold 0.8M $CaCl_2$ / 20% (v/v) ethanol was then injected to discharge the remaining aequorin. The Ca^{2+}-dependent aequorin bioluminescence was subsequently calibrated using an empirically derived formula (Knight *et al*., 1991; Knight*et al*., 1996) to allow the magnitude of the Ca^{2+} increase in response to test chemicals to be calculated.

Plasticisers that had a significant impact on root growth were selected for studies of seedling cytosolic Ca^{2+}. To assess the immediate, acute effects of plasticiser exposure, seedlings were treated with either 80 µg mL^{-1} or 800 µg mL^{-1} of plasticiser. This was compared to the effects of RO water as a negative control, H_2O_2 (1 mM, 10 mM and 100 mM) which induces an increase in cytosolic Ca^{2+} (Short *et al*., 2012) as a positive control, and 1% (v/v) ethanol to control for the maximum concentration of solvent used in the plasticiser treatment. To assess the effects of long-term, chronic exposure to plasticisers, seedlings were grown on CM containing 80 µg mL^{-1} of plasticiser and the impact of ROS on Ca^{2+} signalling was recorded. Seedlings were exposed to 1 mM, 10 mM or 100 mM H_2O_2 and the H_2O_2-induced increase in cytosolic Ca^{2+} compared to that in control seedlings that had no chronic plasticiser treatment.

2.4. Measurement of cytosolic auxin using the DR5::GFP reporter system.

Auxin levels were visualised in the roots of seedings of an *Arabidopsis thaliana* DR5::GFP reporter line (Benková *et al*., 2003). Seeds were surface sterilised and grown as in the root assay methodology (see Section 2.2) in CM, in the presence or absence of individual plasticisers shown to have a significant effect on root growth to assess the plasticiser's long-term, chronic effects. 10-day-old seedlings were carefully removed from the agar and mounted on 75 x 26 x 1 mm microscope slides with 0.5 mL of tap water, and covered using number 0 thickness coverslips. The DR5::GFP fluorescence (395 nm excitation, 510 nm long pass emission) was observed using an Nikon epifluorescence microscope using a 40x lens. Images were captured with GX Capture software (Version 7.3.1.7) at 1 S, 220 mS and 260 µS exposure, maximum gain, low frame speed, and 2584 x 1936 resolution. Images were analysed using Fiji to quantify the relative fluorescence of root tissue using the integrated density and mean grey value, comparing areas that had auxin fluorescence to surrounding tissue with autofluorescence.

2.5. Statistical Analysis.

The impact of plasticisers compared to control treatments was assessed using SPSS 29.0 software with an alpha of 0.05 for all analyses. A repeated measures ANOVA was used to assess the impacts of concentration on incremental root growth. Secondary root length and primary root length were evaluated with univariate ANOVAs. Univariate ANOVAs were also used for the luminometry data and fluorescence data. Regression analyses were used to compare root architecture ratio to plasticiser concentration. Paired T-test was used to compare primary to secondary roots within each test treatment for relative fluorescence. Tukey's post hoc test was used, with homogeneity tests to group data by statistical similarity.

Chapter 3: Results.

3.1. Plasticisers Inhibit *Arabidopsis thaliana* Root Growth.

In order to determine whether plasticisers alter root growth, *Arabidopsis thaliana* seedlings were grown on agar containing 5 – 80 µg mL^{-1} of plasticiser. This concentration range and plasticiser selection were chosen based on Billings *et al*. (2021), which reported concentrations of plasticiser in soil samples ranging from 0.05 to 63.2 µg mL^{-1}. Additional plasticisers were chosen for a range of chemical structures and molecular weights. This allows for an assessment of whether plasticisers have an impact on plant physiology at the concentrations that are already prevalent in the environment and may be actively affecting plant growth, as well as whether the impacts of plasticisers are dose-dependent.

The efficacy of the root growth assay, and hence the validity of the observed effects of plasticisers, was confirmed using sodium chloride (50 mM NaCl) as a positive control, and 1% (v/v) ethanol was a negative control, the latter representing the highest solvent concentration used in the assays. As expected, 50 mM NaCl significantly inhibited primary root growth and number of secondary roots (Appendices 1 and 2). The 50 mM NaCl negative control showed that significant differences in root growth can be induced by growth in CM with the addition of a chemical that impedes root development. There was no impact of ethanol on either primary root length or the number of secondary roots (Appendices 1 and 2), confirming that effects observed above were driven by the plasticiser rather than the solvent.

Of the nine plasticisers tested, ANOVA analysis showed that BBP, DBS, DEHP, DINP, DMP and TOTM all affected root growth (Table 3). Post-hoc tests showed significant variance in BBP, DBS, and DMP primary root growth with different plasticiser concentrations. Despite the significant effect in the ANOVA, DEHP and DINP had no significant differences between plasticiser concentrations in post-hoc tests. For BBP, DBS and DMP, there was a clear dose-dependent affect (Figure 5). The impact of BBP started at 5 µg mL^{-1}, DBS started at 40 µg mL^{-1} and DMP started at 80 µg mL^{-1}. Whilst post-hoc tests showed that ATBC and TOTM treatments resulted in significant differences in primary root growth, no clear trend was observed, as the control was not significantly different to 80 µg mL^{-1} of each plasticiser. DOTP, and TCP had no significant differences between the control and different doses of plasticiser in neither the ANOVA nor post-hoc tests.

Table 3: ANOVA analysis comparing mean primary root length at different concentrations (0 – 80 µg mL^{-1}) of plasticiser for each different treatment. Significant P-values are shown in bold.

	ATBC	BBP	DBS	DEHP	DINP	DMP	DOTP	TCP	TOTM
F ratio	1.697	16.145	22.872	2.503	2.673	4.695	1.055	0.869	4.734
P value	0.139	**P < 0.001**	**P < 0.001**	**0.033**	**0.024**	**0.001**	0.388	0.504	**0.001**

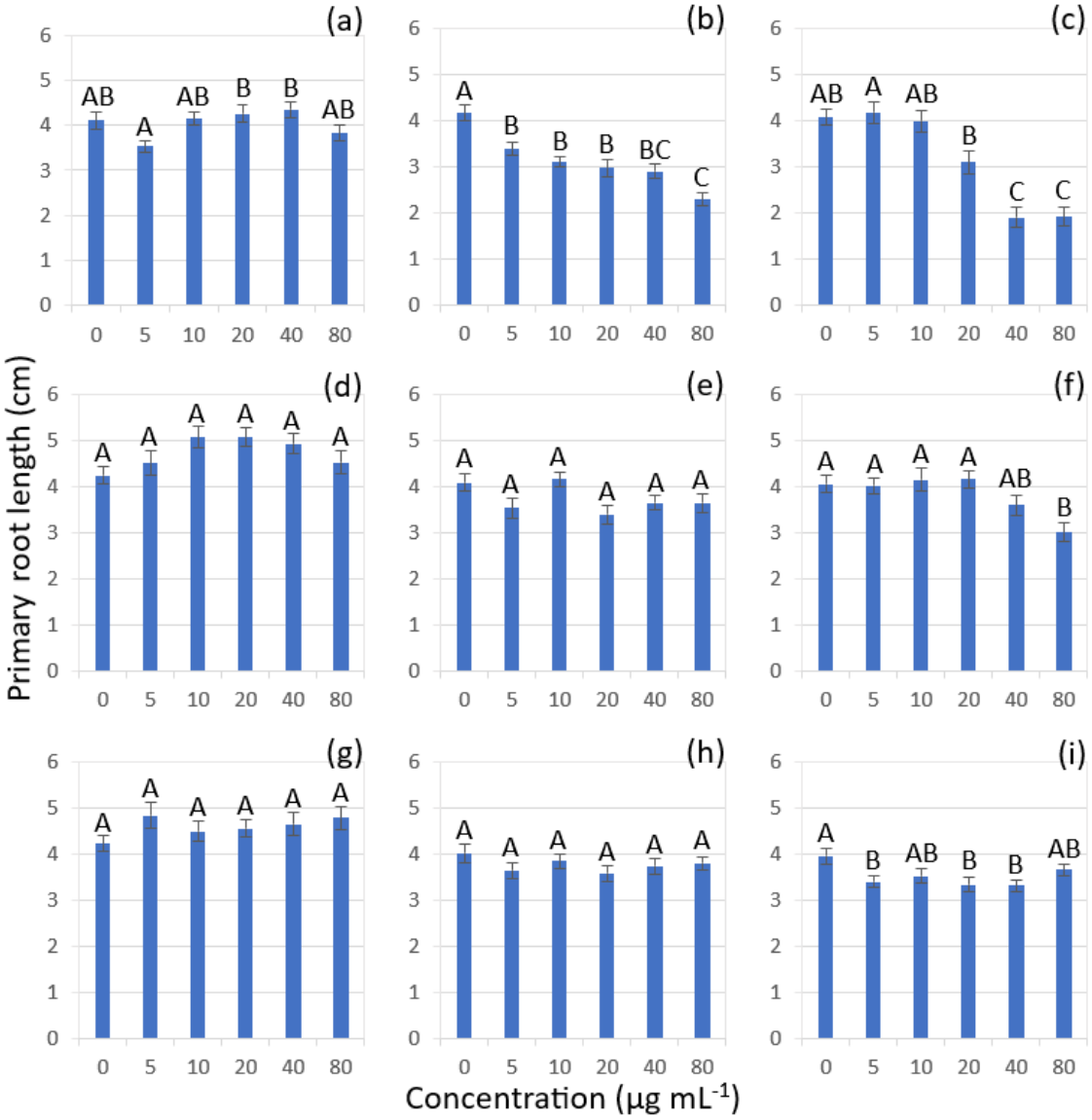


Figure 5: Mean primary root growth after 10 days of exposure to 0 - 80 µg mL^{-1} of plasticiser. Graphs represent exposure to ATBC (a), BBP (b), DBS (c), DEHP (d), DINP (e), DMP (f), DOTP (g), TCP (h), and TOTM (i). Results show mean ± SEM (n ≥ 20 per treatment), data with significant differences ($p < 0.05$) are denoted by different letters, as evaluated by post-hoc Tukey's test.

Plants will change their root architecture, including lateral root development in response to abiotic stresses by modulating hormone levels (Miransari and Smith, 2014). To assess the impacts of plasticisers on root architecture, secondary root number was recorded at the end of the 10-day growth period as a surrogate for their effects on root hormone levels in roots.

Similar trends to the effects of plasticisers on primary root growth were observed, with secondary root number being reduced by a number of plasticisers (Table 3, Figure 6).

ANOVA analysis showed significant effects of ATBC, BBP, DBS, DEHP, DMP, TCP and TOTM on secondary root number (Table 4). Post-hoc tests showed that BBP, DBS, and DMP had significant effects on secondary root number that were dose-dependent (Figure 6). The initial concentration at which the effects of plasticisers was observed differed between plasticisers; BBP had a significant effect from 5 µg mL^{-1}, DBS had a significant effect at 40 µg mL^{-1} and the effect of DMP started at 40 µg mL^{-1}. Although significant differences were observed between several concentrations for ATBC, DEHP, and DINP, the control was not significantly different from 80 µg mL^{-1}, nor were there dose dependencies showing an increased effect at higher concentrations. There were significant differences between the control treatment and different concentrations of TOTM and TCP for secondary root number, including 80 µg mL^{-1}, however no similar effects were observed on the total root length, and the effects of TCP and TOTM were not dose-dependent, therefore these chemicals were not considered for further experiments.

Table 4: ANOVA analysis comparing mean secondary root number at different concentrations (0 – 80 µg mL^{-1}) of plasticiser for each different treatment.

	ATBC	BBP	DBS	DEHP	DINP	DMP	DOTP	TCP	TOTM
F ratio	3.324	70.961	7.270	3.862	1.972	22.507	0.611	4.308	7.565
P value	**0.007**	**P < 0.001**	**P < 0.001**	**0.003**	0.087	**P < 0.001**	0.691	**0.001**	**P < 0.001**

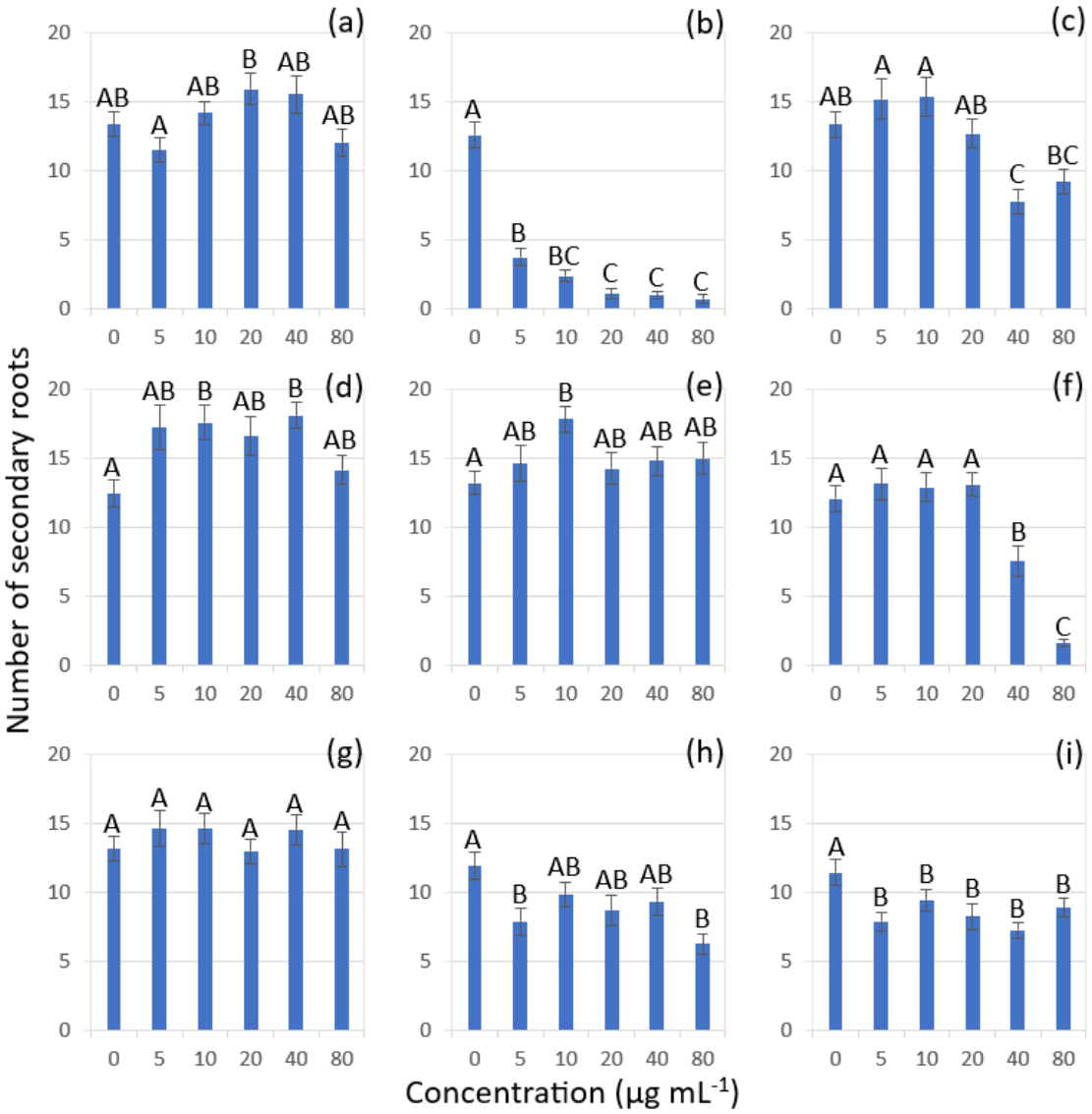


Figure 6: Mean number of secondary roots after 10 days of exposure to 0 - 80 µg mL^{-1} of plasticiser. Graphs represent exposure to ATBC (a), BBP (b), DBS (c), DEHP (d), DINP (e), DMP (f), DOTP (g), TCP (h), and TOTM (i). Results show mean ± SEM (n ≥ 20 per treatment), data with significant differences ($p < 0.05$) are denoted by different letters, as evaluated by post-hoc Tukey's test.

To ascertain whether the number of secondary roots was being impacted by the decreased primary root length, or this was a function of different hormones having an effect on root architecture, the root architecture ratio was calculated. This provided the number of secondary roots per unit of primary root length for each treatment and concentration. Only DBS and DMP had significant interactions between concentration and root architecture ratio (Table 5). DBS produced an increasing root architecture ratio with increasing plasticiser concentration, therefore at high concentrations the root architecture changed to produce

more secondary roots per unit of primary root length (Figure 7). DMP was observed to have the opposite effect, as with increasing concentrations of DMP there were fewer secondary roots per unit of primary root length. This trend was also shown for roots exposed to BBP, however this relationship was not linear and therefore did not fit a linear regression model.

Table 5: ANOVA analysis comparing mean root architecture ratio at different concentrations (0 – 80 µg mL^{-1}) of plasticiser for each different treatment. Linear regression R values and P values are shown.

	ATBC	BBP	DBS	DEHP	DINP	DMP	DOTP	TCP	TOTM
R value	0.1005	0.5991	0.9385	0.1609	0.3179	0.9645	0.6495	0.7796	0.4488
P value	0.8498	0.2089	**0.0056**	0.7607	0.5392	**0.0019**	0.1628	0.0675	0.3720

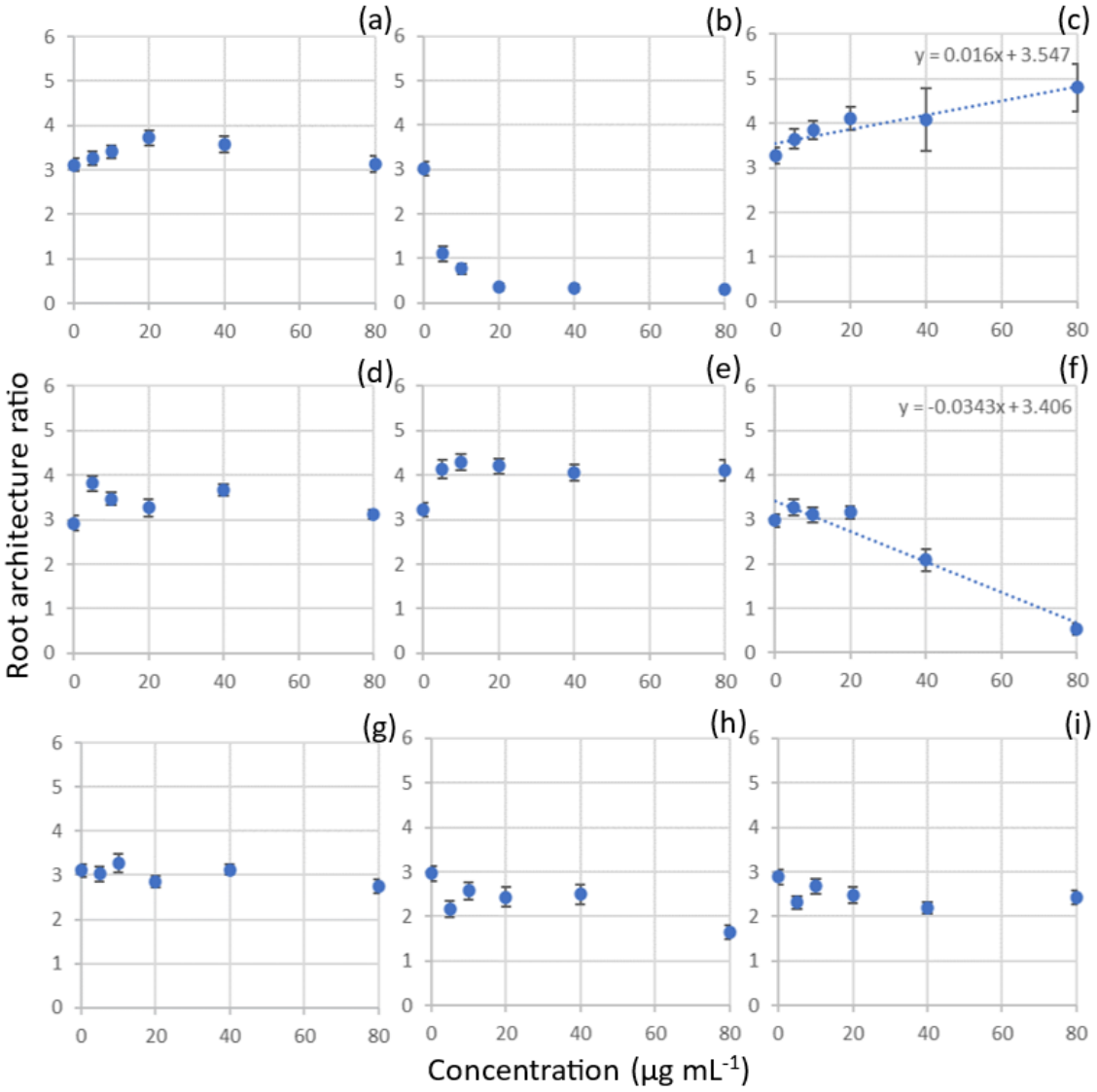


Figure 7: Root architecture ratio according to plasticiser treatment and concentration following 10 days of exposure to 0 - 80 µg mL^{-1} of plasticiser. Graphs represent exposure to ATBC (a), BBP (b), DBS (c), DEHP (d), DINP (e), DMP (f), DOTP (g), TCP (h), and TOTM (i). Results show mean ± SEM (n ≥ 20 per treatment), data with significant linear regression ($p < 0.05$) have a trendline fitted and trendline equation displayed.

To compare the inhibition of root length to the molecular weight of the plasticisers, primary root length inhibition at 80 µg mL^{-1} was calculated (Figure 8). This would imply whether molecular weight impacts the potential toxicity of plasticisers and therefore insinuate whether plasticisers need to be taken up by the plants to have an effect on root architecture. There is a clear divide between plasticisers with lower molecular weights and those with a higher weight in terms of root length inhibition.

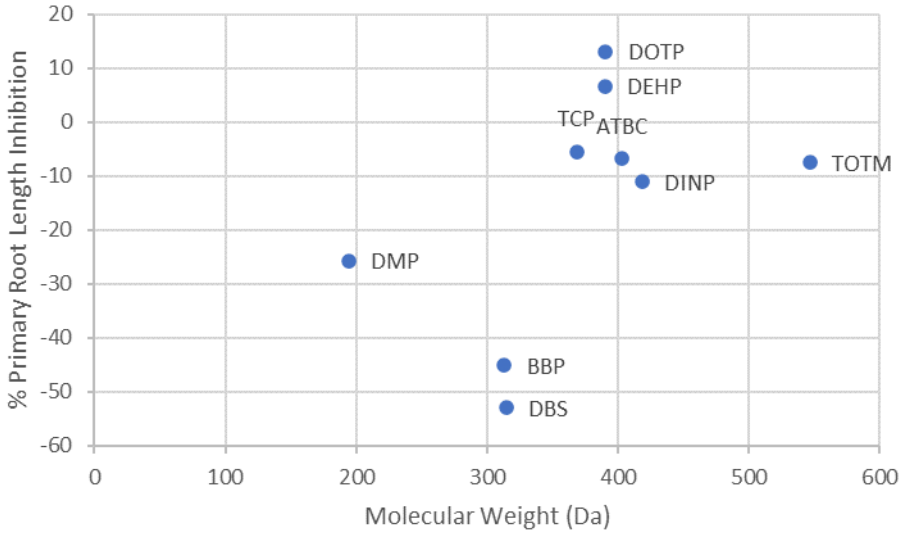


Figure 8: Primary root length inhibition at 80 µg mL^{-1} of each plasticiser treatment compared to the control. Results show mean percentage inhibition (n ≥ 20 per treatment).

Different hormones and physiological functions are active at different points during root growth (Jia, Giehl and von Wirén, 2022). Therefore, for the plasticisers shown to have the clearest impact on root growth and architecture (BBP, DBS and DMP), further analysis was completed to evaluate at which point in the growth period plasticisers have the most impact. This would imply the mechanism of plasticisers impeding root growth, as different hormones and physiological functions are active at different points in the growth period.

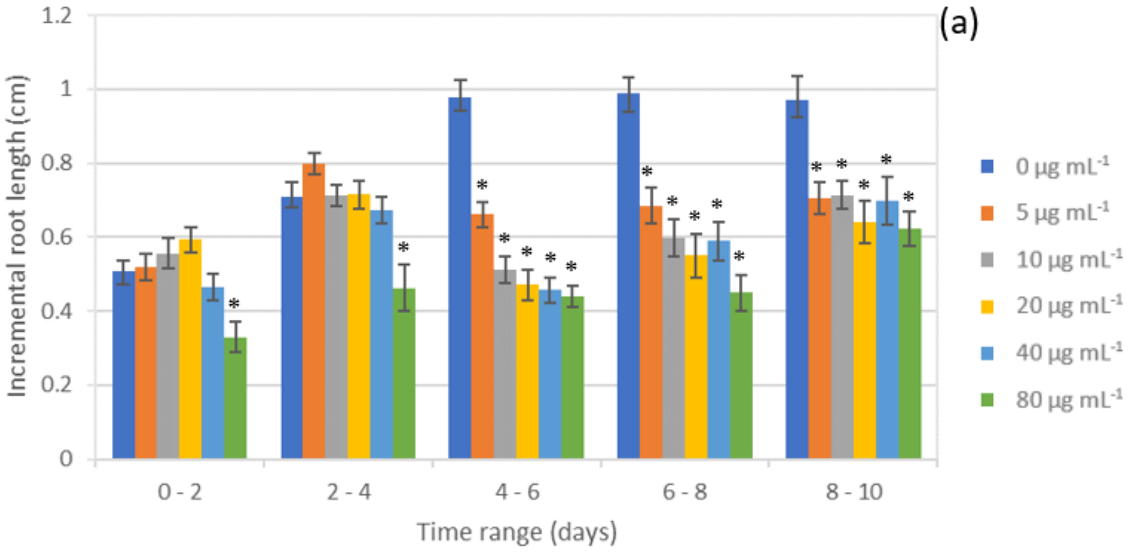
Measurements of root length were recorded every two days for a ten-day period and a repeated measures ANOVA used to investigate the effects of plasticisers over time. As expected from the data described above, root growth was significantly affected by both

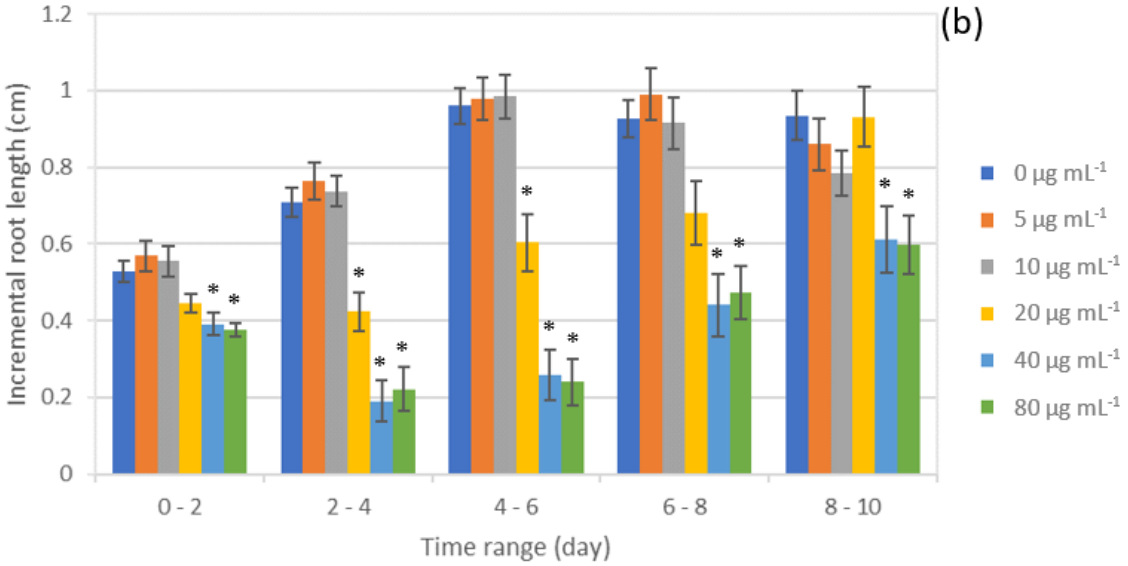
plasticiser concentration and time. Furthermore, there were significant interactions between time and concentration for all plasticisers, suggesting that the time at which treatments inhibit growth varies by concentration (Table 6).

Table 6: Outcomes of within-subject effects test for the interaction between time range and concentration. Greenhouse-Geisser statistics are shown, with degrees of freedom, P-values, and F-values shown.

Chemical	Degrees of Freedom	F-value	Significance
BBP	13.432	8.878	**P < 0.001**
DBS	12.737	7.863	**P < 0.001**
DMP	12.594	3.277	**P < 0.001**

Figure 9 confirms that the rate of growth over time differs by concentration, with higher concentrations generally leading to reduction in growth earlier in the growth period. Significant differences were observed in incremental root growth between the control and 80 µg mL^{-1} BBP for the entire growth period recorded, from days 0-2 to days 8-10. All concentrations of BBP had significant impacts on incremental root growth beginning from days 4-6 of the growth period. DBS also had significant impacts on root growth throughout the growth period, with both 40 µg mL^{-1} and 80 µg mL^{-1} having significant differences from days 0-2 to days 8-10. The effect of DBS was limited to 20, 40 and 80 µg mL^{-1}, with 5 and 10 µg mL^{-1} having no impact on incremental root growth on any time range recorded. DMP had fewer significant impacts on incremental root growth, as days 0-2, 2-4 and 8-10 had no significant differences between the control and any concentration of DMP. Days 4-6 and 6-8 were the only time ranges in which any concentration of DMP impeded root growth, and only 80 µg mL^{-1} had an impact on these days. This shows that the rate of growth over time differs by concentration, with higher concentrations generally leading to reduction in growth earlier in the growth period.


(a)
Incremental root length (cm)
1.2
1
0.8
0.6
0.4
0.2
0
0 - 2
2 - 4
4 - 6
6 - 8
8 - 10
Time range (days)
0 µg mL-1
5 µg mL-1
10 µg mL-1
20 µg mL-1
40 µg mL-1
80 µg mL-1


(b)
Incremental root length (cm)
1.2
1
0.8
0.6
0.4
0.2
0
0 - 2
2 - 4
4 - 6
6 - 8
8 - 10
Time range (day)
0 µg mL-1
5 µg mL-1
10 µg mL-1
20 µg mL-1
40 µg mL-1
80 µg mL-1

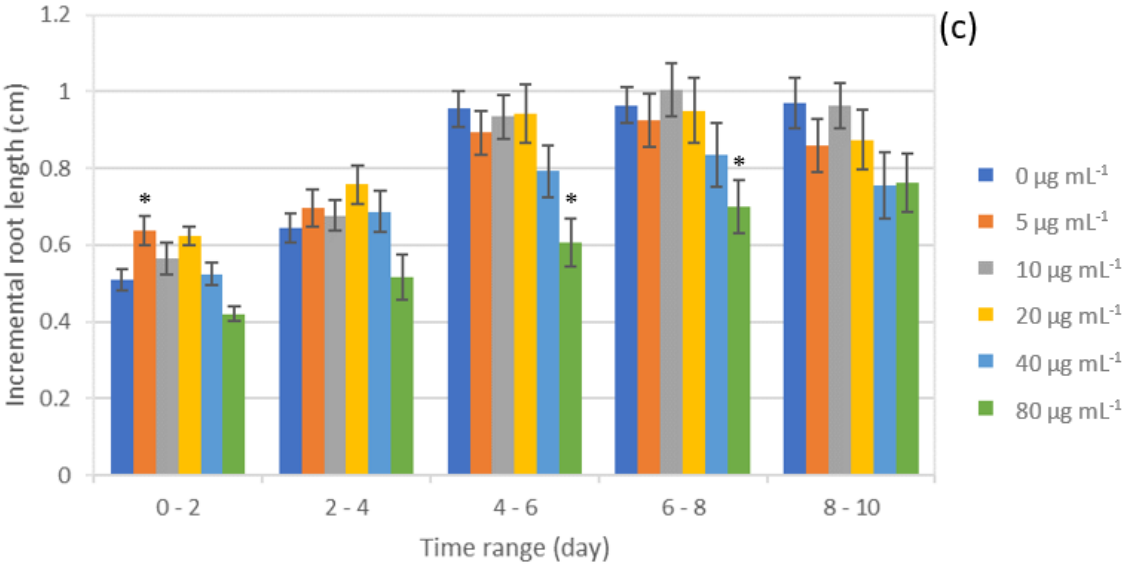

(c)
Incremental root length (cm)
1.2
1
0.8
0.6
0.4
0.2
0
0 - 2
2 - 4
4 - 6
6 - 8
8 - 10
Time range (day)
0 µg mL-1
5 µg mL-1
10 µg mL-1
20 µg mL-1
40 µg mL-1
80 µg mL-1

Figure 9: Mean incremental root length for up to 10 days of exposure to either 0 µg mL^{-1} (CM), or different concentrations of plasticiser (5 - 80 µg mL^{-1}). Graphs represent exposure to BBP (a), DBS (b), and DMP (c). Results show mean ± SEM (n ≥ 20 per treatment). Compared to the control for day range and treatment concentration, * significant to $p < 0.05$, as evaluated by post-hoc Tukey's test.

3.2. Acute Addition of Plasticisers Affects Ca^{2+} Signalling.

The root growth assay illustrated the long-term or chronic effects of plasticisers on seedlings. To investigate possible mechanisms underlying this response, dynamics of intracellular calcium (Ca^{2+}) were monitored, a ubiquitous second messenger. Since Ca^{2+} signalling is implicated in regulating root growth (X. P. Zhang *et al*., 2020), the impact of acute exposure to plasticisers on Ca^{2+} signalling was examined in *Arabidopsis thaliana* seedlings constitutively expressing apoaequorin. Seedlings were grown in CM then exposed to two different concentrations of plasticiser. These were 80 µg mL^{-1} of plasticiser, the highest concentration of plasticiser which had significant impacts on root growth for BBP, DBS and DMP to examine whether concentrations equivalent to those present in soils can impact Ca^{2+} signalling, and 800 µg mL^{-1}, as an acute concentration to examine whether short-term treatment with high levels of plasticisers impact Ca^{2+} signalling. $[Ca^{2+}]_{cyt}$ was quantified as the total increase over a 1-hour period, the peak value after plasticiser addition to evaluate the instantaneous response, $[Ca^{2+}]_{cyt}$ at the mid-point in the time series to show whether there was a return to baseline after treatment addition, and mean increase in $[Ca^{2+}]_{cyt}$ across the time period after test chemical addition for a representation of the change in signalling over time, including the peak value.

ANOVA analyses showed that there were significant effects of treatment on total Ca^{2+} release and the increase after addition (Table 6). Concentration affected all measures except from total Ca^{2+} which was marginally non-significant and there was a significant interaction for all measures. The $[Ca^{2+}]$ traces suggested that compared to the 1% (v/v) ethanol control, there was no effect on Ca^{2+} signalling within the time period recorded for any treatment other than for plants exposed to 800 µg mL^{-1} DMP (Figures 10 and 11). This was shown by the presence of a peak, as well as significantly higher total $[Ca^{2+}]_{cyt}$ (Figure 12), first phase peak, mid-point and mean after addition for 800 µg mL^{-1} DMP addition (Figure 13). The small 'spikes' of $[Ca^{2+}]_{cyt}$ observed at 0 seconds for treatments other than 800 µg mL^{-1} DMP when 0.5 mL of solution was added is the injection artifact from briefly exposing the photometer to light to inject the solution into the test tube. This can also be from touch response from the needle and solution

contacting the plant, causing Ca^{2+} release which is not reflective of a reaction to the test chemical (Knight *et al.*, 1991).

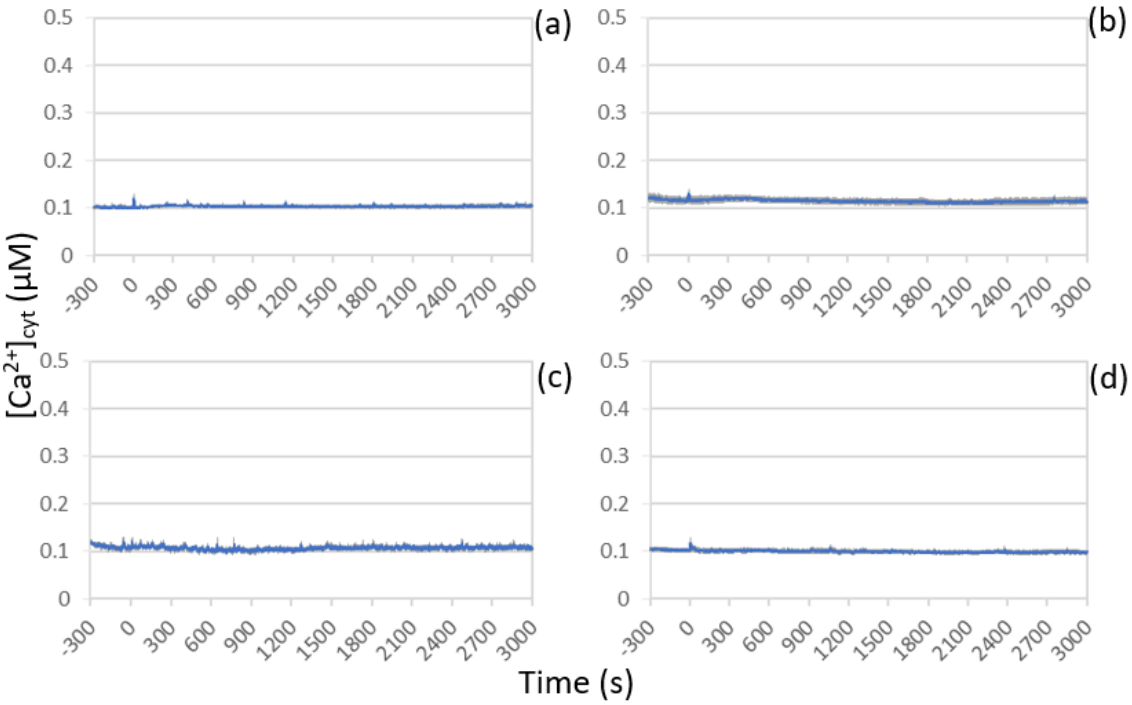


Figure 10: Stimulus-induced changes in $[Ca^{2+}]_{cyt}$ in response to 80 µg mL^{-1} plasticiser or RO water. Plants were grown on CM. Graphs represent addition at 0 seconds with 0.5 mL RO water (a), BBP (b), DBS (c) and DMP (d) at 0 seconds. Results show mean ± SEM (n = 5 per treatment).

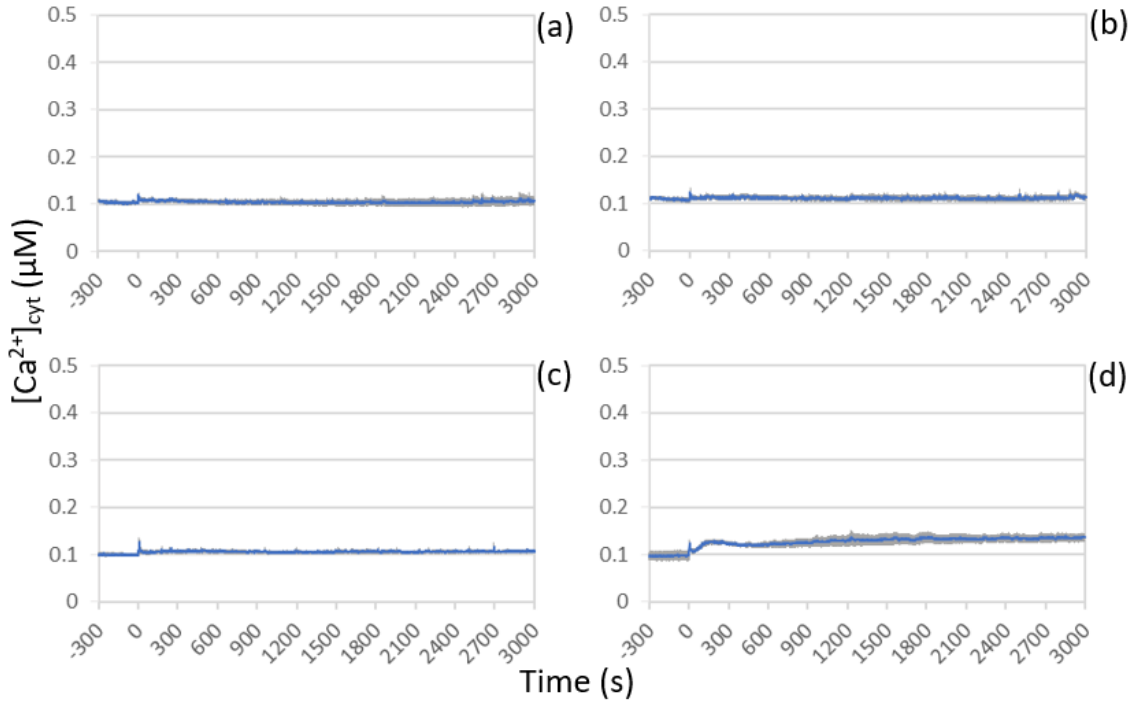


Figure 11: Stimulus-induced changes in $[Ca^{2+}]_{cyt}$ in response to 800 µg mL^{-1} plasticiser, and 1% (v/v) ethanol. Plants were grown on CM. Graphs represent addition at 0 seconds with 0.5 mL 1% (v/v) ethanol (a), BBP (b), DBS (c) and DMP (d) at 0 seconds. Results show mean ± SEM (n = 5 per treatment).

Table 6: Outcomes of between-subjects effects tests for the plasticiser treatment (control, BBP, DBS or DMP) and concentration (80 or 800 µg mL^{-1}), as well as the interaction between the two variables. ANOVA statistics are shown, with P-values and F-values shown.

		Total $[Ca^{2+}]$	First phase peak	Mid-point	Increase after addition
Treatment	F-value	2.878	0.213	1.791	4.763
	P-value	**0.035**	0.930	0.150	**3.08×10^{-3}**
Concentration	F-value	4.042	22.556	3.136	8.423
	P-value	**0.051**	**P < 0.001**	0.084	**6.00×10^{-3}**
Interaction	F-value	5.547	6.843	4.288	9.543
	P-value	**0.001**	**P < 0.001**	**5.57×10^{-3}**	**P < 0.001**

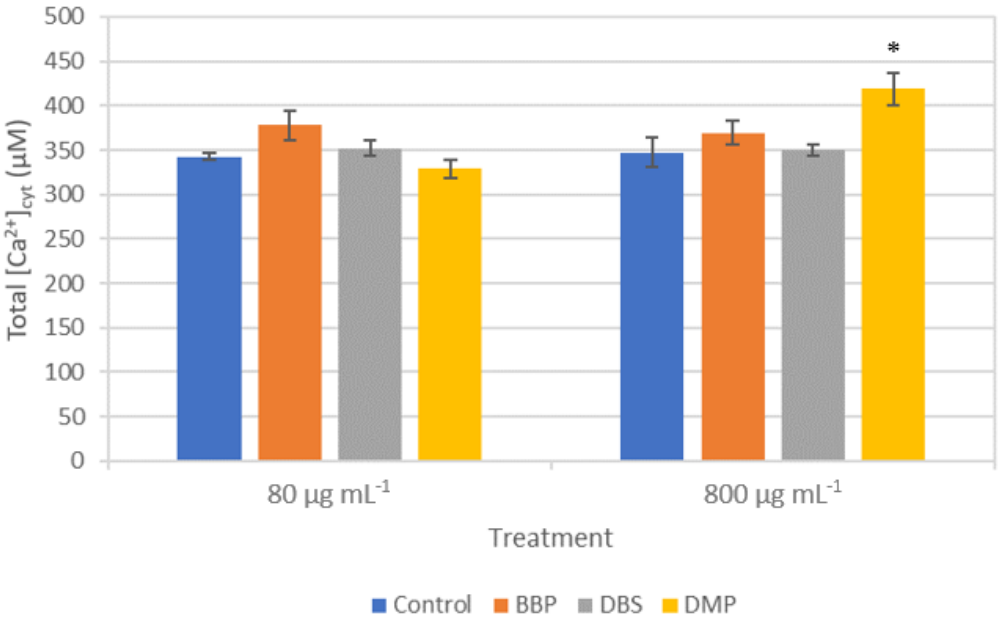


Figure 12: Total increase in $[Ca^{2+}]_{cyt}$ in response to 80 and 800 µg mL^{-1} BBP, DBS and DMP. Plants were grown on CM, control treatments were 0.5 mL RO water, and 0.5 mL 1% (v/v) ethanol, respectively. Results show mean ± SEM (n = 5 per treatment). Compared to the control for each variable and treatment concentration * significant to $p < 0.05$, as evaluated by post-hoc Tukey's test.

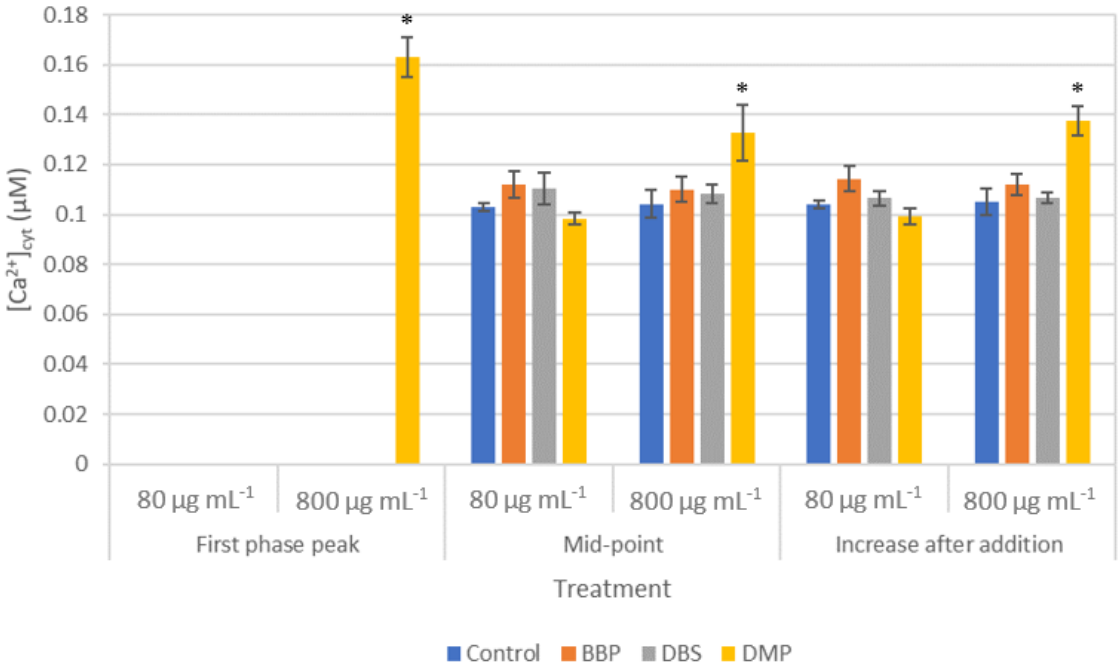


Figure 13: Changes in $[Ca^{2+}]_{cyt}$ in response to 80 and 800 µg mL^{-1} BBP, DBS and DMP. Plants were grown on CM, control treatments were 0.5 mL RO water, and 0.5 mL 1% (v/v) ethanol, respectively. The first phase peak $[Ca^{2+}]_{cyt}$, mid-point (1500 seconds) and the mean $[Ca^{2+}]_{cyt}$ increase following treatment addition are shown. Results show mean ± SEM (n = 5 per treatment). Compared to the control for each variable and treatment concentration, * significant to $p < 0.05$, as evaluated by post-hoc Tukey's test.

3.3. Chronic Plasticiser Treatment Alters Ca^{2+} Responses.

The uptake and accumulation of plasticisers in plants has been observed to accelerate the generation of ROS (Kumari and Kaur, 2020), raising the likelihood of plasticisers interfering with ROS-Ca^{2+} signalling or causing ROS stress in plants, both of which could affect root growth resulting in the effects observed in the root growth assay (Figures 5-9). H_2O_2 treatments have been shown to elicit changes in Ca^{2+} signalling by inducing stress and activating ROS signalling pathways (Niu and Liao, 2016). In this experiment, this mechanism was utilised to show how stress response can be altered when *Arabidopsis thaliana* is chronically exposed to plasticisers that have impact plant growth and development. This can show if plants are primed for H_2O_2 stress, as plants may be constitutively activating ROS signalling pathways due to plasticiser exposure and more readily respond to H_2O_2 addition. This may be responsible for the impeded growth observed in the root assay, due to division of resources being oriented towards stress responses rather than growth. This experiment showed a significant effect of both H_2O_2 concentration and plasticiser treatment on Ca^{2+} release.

Figures 14-18 show the effects of 1 mM, 10 mM or 100 mM H_2O_2, respectively, on $[Ca^{2+}]_{cyt}$ in seedlings grown on media containing 80 µg mL^{-1} BBP, DBS and DMP. ANOVA analysis showed significant effects of H_2O_2 concentration on Ca^{2+} signalling (Table 7). Post-hoc tests showed different plasticiser treatments did not affect the total, first phase peak, mid-point and mean $[Ca^{2+}]_{cyt}$ induced by 1mM H_2O_2 Ca^{2+} treatment (Figures 17 and 18). The shapes of the $[Ca^{2+}]_{cyt}$ signatures in figure 12 were also similar, therefore 1 mM H_2O_2 elicited no significant differences in plant response. 10 mM H_2O_2 induced significant responses when comparing the control plants to those treated with plasticiser. There were significantly higher releases of total Ca^{2+} for both BBP and DMP, a higher mid-point $[Ca^{2+}]_{cyt}$ for DMP and higher mean increases in $[Ca^{2+}]_{cyt}$ after addition for BBP and DMP. DBS had a significantly higher peak $[Ca^{2+}]_{cyt}$ after 10 mM H_2O_2 addition. DMP conversely showed a lower peak $[Ca^{2+}]_{cyt}$, however this was not significant. Figure 15 also shows that there may have been a second phase peak for BBP treatment, however this was not present in the control or other treatments, and the individual responses did not consistently have a second peak, therefore this was not further investigated. Addition of 100 mM H_2O_2 showed that plants grown in BBP have a significantly higher total $[Ca^{2+}]_{cyt}$ release, mid-point and increase after addition compared to control plants. BBP and DMP treatments also showed significantly lower first phase peaks than the control. There were no significant differences between the control treatment and DBS when exposed to 100 mM H_2O_2, this is consistent with the mean traces shown in Figure 16 as the $[Ca^{2+}]_{cyt}$ responses for control and DBS treatments had the same shape.

Considering the effects of H_2O_2 by plasticiser treatment, compared to control plants, those grown in media containing 80 µg mL^{-1} BBP had insignificant differences in total $[Ca^{2+}]$ when exposed to 1mM H_2O_2, and significantly higher responses when exposed to 10mM and 100mM concentrations of H_2O_2. At 100mM H_2O_2 there was also a significantly lower first phase peak $[Ca^{2+}]_{cyt}$ for BBP treated plants, but a higher $[Ca^{2+}]_{cyt}$ at the mid-point and the mean after addition. There was also a higher mean $[Ca^{2+}]_{cyt}$ following addition when exposed to 10 mM H_2O_2. Growth in media with 80 µg mL^{-1} DBS had no significant impact on total $[Ca^{2+}]_{cyt}$ compared to control plants when exposed to 1mM, 10 mM and 100mM concentrations of H_2O_2. There was a significantly higher first phase peak $[Ca^{2+}]_{cyt}$ with 10 mM H_2O_2, however there were no other significant differences to control treated plants for the $[Ca^{2+}]_{cyt}$ at the mid-point, or mean increase after addition for any concentration of H_2O_2. DMP treatment caused a significantly higher total $[Ca^{2+}]_{cyt}$ with 10 mM H_2O_2 compared to control treatment, but not at 1 mM or 100 mM H_2O_2. 10 mM exposure also caused increased $[Ca^{2+}]_{cyt}$ at the mid-point and for

the mean increase after addition. There was a significantly lower peak $[Ca^{2+}]_{cyt}$ at 100 mM H_2O_2, but not at other concentrations of H_2O_2.

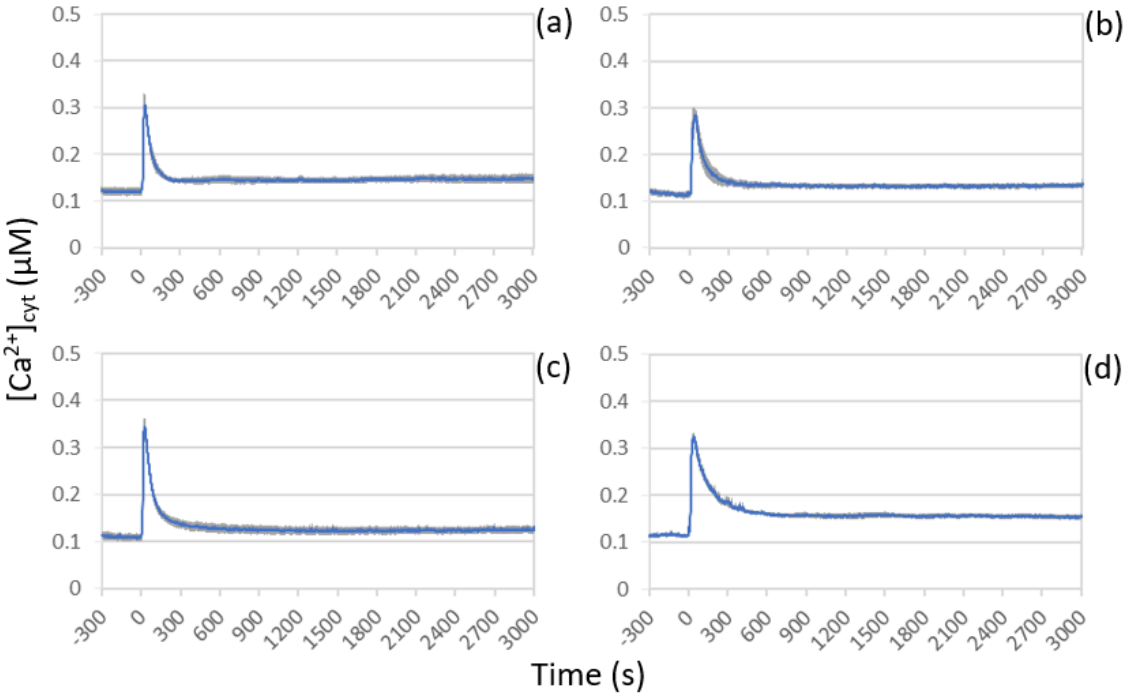


Figure 14: Stimulus-induced changes in $[Ca^{2+}]_{cyt}$ in response to 0.5 mL 1 mM H_2O_2 added at 0 seconds to seedlings grown on CM (a), or on media containing 80 µg mL^{-1} BBP (b), DBS (c) and DMP (d). Results show mean ± SEM (n = 5 per treatment).

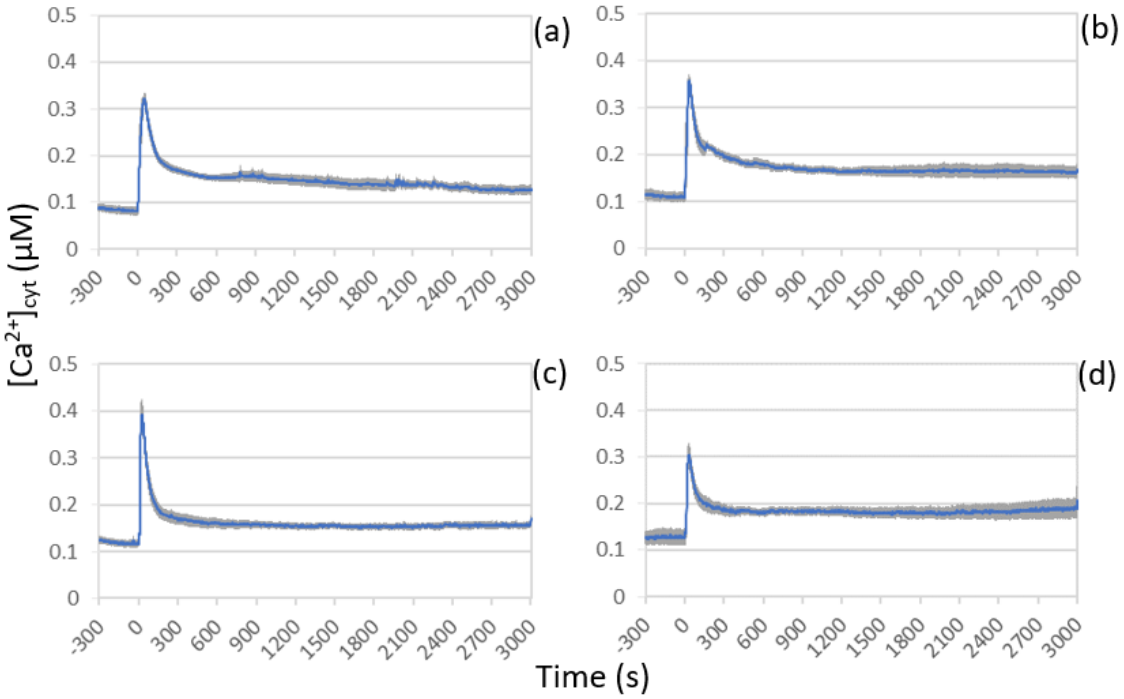


Figure 15: Stimulus-induced changes in $[Ca^{2+}]_{cyt}$ in response to 0.5 mL 10 mM H_2O_2 added at 0 seconds to seedlings grown on CM (a), or on media containing 80 µg mL^{-1} BBP (b), DBS (c) and DMP (d). Results show mean ± SEM (n = 5 per treatment).

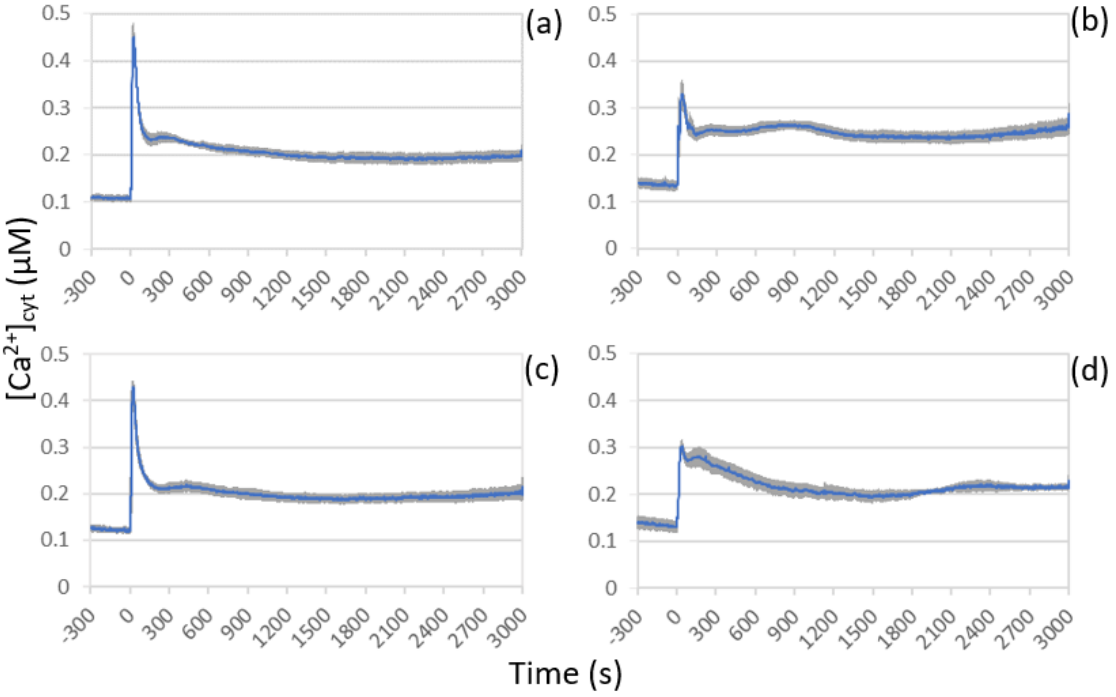


Figure 16: Stimulus-induced changes in $[Ca^{2+}]_{cyt}$ in response to 0.5 mL 100 mM H_2O_2 added at 0 seconds to seedlings grown on CM (a), or on media containing 80 µg mL^{-1} BBP (b), DBS (c) and DMP (d). Results show mean ± SEM (n = 5 per treatment).

Table 7: Outcomes of between-subjects effects tests for the plasticiser treatment (growth in CM or media with 80 µg mL^{-1} BBP, DBS or DMP) and concentration of H_2O_2 (1, 10 and 100 mM), as well as the interaction between the two variables. ANOVA statistics are shown, with P-values and F-values shown.

		Total $[Ca^{2+}]$	First phase peak	Mid-point	Increase after addition
Treatment	F-value	8.887	7.988	6.361	8.818
	P-value	**P < 0.001**	**P < 0.001**	**1.02×10^{-3}**	**P < 0.001**
H_2O_2 concentration	F-value	107.596	14.135	66.883	114.257
	P-value	**P < 0.001**	**P < 0.001**	**P < 0.001**	**P < 0.001**
Interaction	F-value	4.255	5.178	4.489	4.352
	P-value	**1.63×10^{-3}**	**P < 0.001**	**1.10×10^{-3}**	**1.39×10^{-3}**

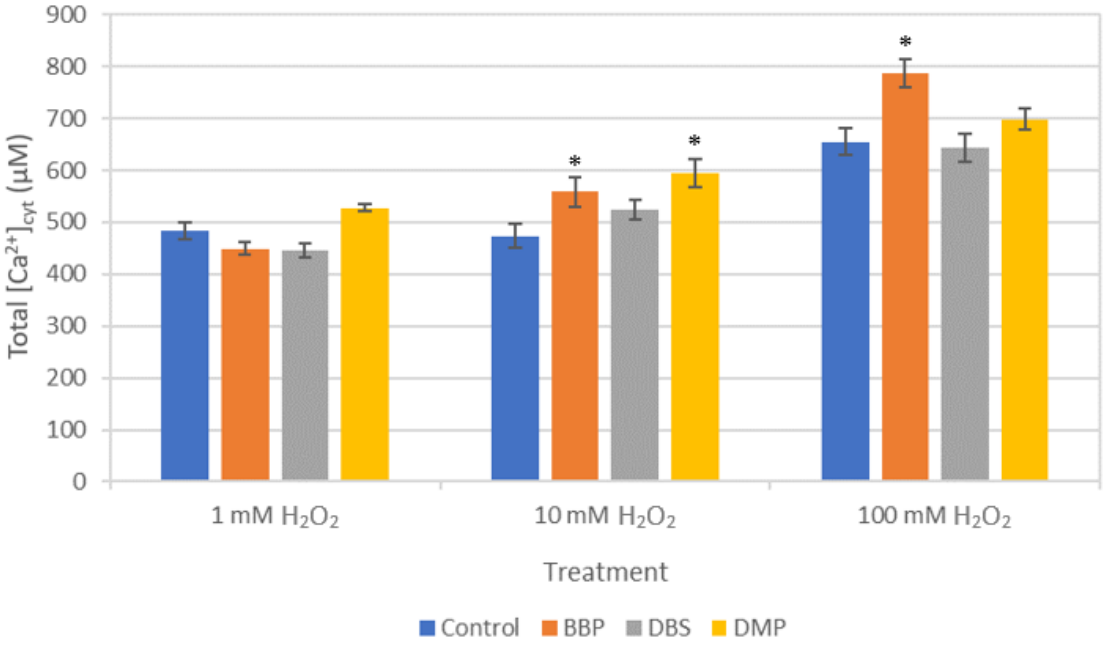


Figure 17: Total release of $[Ca^{2+}]_{cyt}$ (µM) in response to 0.5 mL 1 mM, 10 mM or 100 mM H_2O_2 addition, to seedlings grown on CM, or media containing 80 µg mL^{-1} of BBP, DBS or DMP. Results show mean ± SEM (n = 5 per treatment). Compared to the control for each variable and H_2O_2 concentration, * significant to $p < 0.05$, as evaluated by post-hoc Tukey's test.

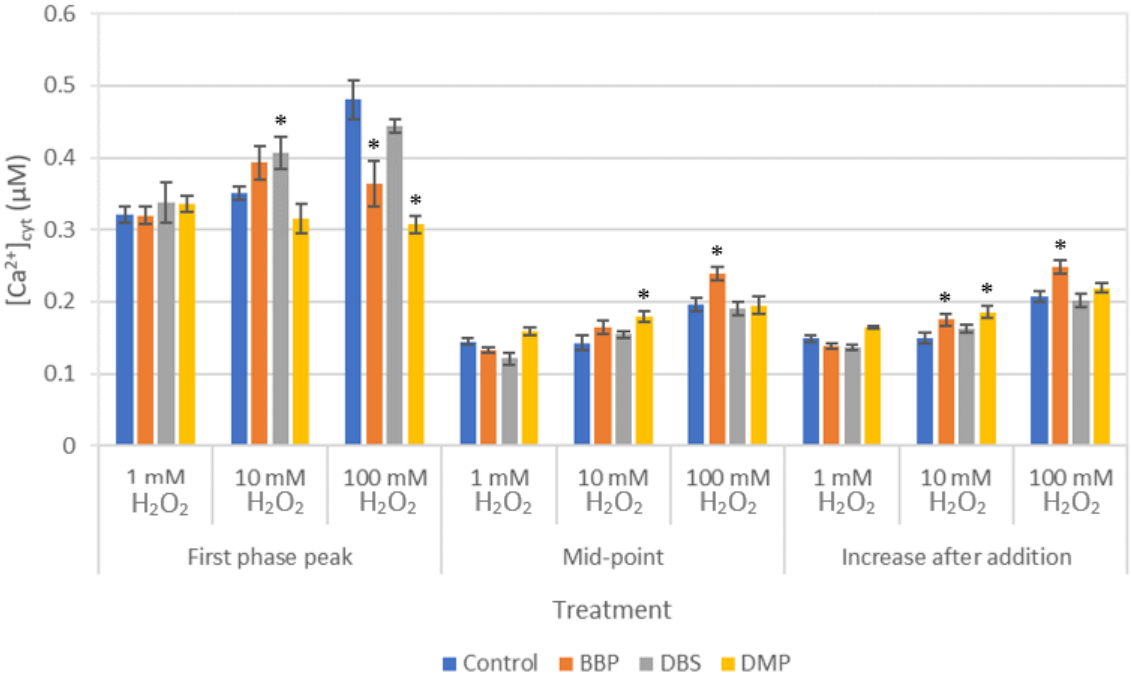


Figure 18: Changes in $[Ca^{2+}]_{cyt}$ (µM) in response to 0.5 mL 1 mM, 10 mM or 100 mM H_2O_2 addition, to seedlings grown on CM, or media containing 80 µg mL^{-1} of BBP, DBS or DMP. The first phase peak $[Ca^{2+}]_{cyt}$, mid-point (1500 seconds) and the mean $[Ca^{2+}]_{cyt}$ increase following

H_2O_2 addition are shown. Results show mean ± SEM (n = 5 per treatment). Compared to the control for each variable and H_2O_2 concentration, * significant to $p < 0.05$, as evaluated by post-hoc Tukey's Test.

For DMP treatment, the different doses of H_2O_2 revealed a downwards trend in the first phase peak $[Ca^{2+}]_{cyt}$ with increasing H_2O_2 concentration (Figure 18). This trend was opposite to the control, which had increasing first phase peak $[Ca^{2+}]_{cyt}$ in response to increasing concentrations of H_2O_2. As shown in Table 8, 100 mM H_2O_2 addition also had a significantly higher peak than 10 mM H_2O_2 for the control treatment, whereas DMP treatment induced no significant difference between the two concentrations of H_2O_2. DBS and BBP also showed slightly different relationships between the first phase peaks for 10 and 100 mM H_2O_2 addition, as there were insignificant differences between the two concentrations for BBP (P = 0.486), and DBS (P = 0.195). However, BBP and DBS treatments did not share the downwards trend with increasing H_2O_2 concentration that DMP treatment illustrated, and were more similar to that of the control.

Table 8: A matrix showing significant differences between first phase peaks $[Ca^{2+}]_{cyt}$ according to control (blue) or DMP (red) treatment for different concentrations of H_2O_2 (1, 10 and 100 mM). P-values are shown, as determined by post-hoc Tukey's Test.

	1 mM	10 mM	100 mM
1 mM		0.306	**P < 0.001**
10 mM	**0.012**		**P < 0.001**
100 mM	0.063	0.486	

When comparing the first and second phase peak $[Ca^{2+}]_{cyt}$ for seedlings grown on CM and on media containing 80 µg mL^{-1} DMP, the first phase peak was significantly lower for plants grown on DMP, however the second phase was not significantly different to that of the control (Figure 19). The relationship between the first and second phase peak $[Ca^{2+}]_{cyt}$ for the control did have a significant difference, however DMP treatment showed no difference between the first and second phase peak $[Ca^{2+}]_{cyt}$. Whilst the first phase peak $[Ca^{2+}]_{cyt}$ for BBP was significantly lower than the control, the second phase peak was not significantly different to the control and the relationship between the two peaks for BBP treatment was the same as that of the control. No significant differences were shown for the first and second phase peak $[Ca^{2+}]_{cyt}$ compared to the control, or the relationship between the two peaks themselves for plants grown in DBS.

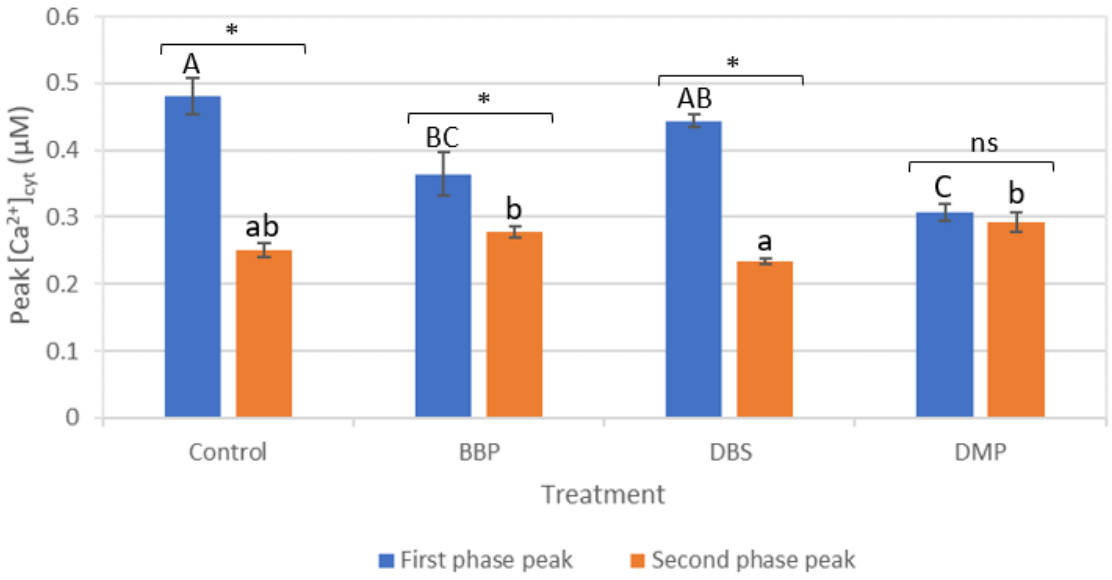


Figure 19: Mean first phase peak $[Ca^{2+}]_{cyt}$ (from Figure 16), and second phase peaks (µM) in response to 100 mM H_2O_2, to seedlings grown on CM, or media containing 80 µg mL^{-1} of BBP, DBS or DMP. Results show mean ± SEM (n = 5 per treatment). Upper case letters denote first peak significant differences, lower case letters denote second peak significant differences determined by post-hoc Tukey's Test. Between treatment pairs, * significant to $p < 0.05$, ns not significant as evaluated by paired T-test.

3.4. Plasticiser Treatment Changes Auxin Distribution.

There is crosstalk between ROS and auxin signalling in the regulation of root growth and development (Mangano *et al*., 2017). Therefore, the effects of chronic plasticiser treatment on auxin signalling were investigated to help understand the plasticiser-induced changes in root architecture observed in the root assays (Figures 20 and 21). An *Arabidopsis thaliana* DR5::GFP reporter line was used to assess the levels and distribution of auxin across the primary roots and secondary root nodes. Fluorescence was observed by epifluorescence microscopy in both the primary root caps and secondary root nodes (Figure 20), however this appeared to differ between the control and chronically treated plants in both the intensity of fluorescence for each location measured, as well as the distribution of auxin when comparing the primary cap to the secondary nodes. Fluorescence was quantified using Fiji, recording the auxin-dependent fluorescence relative to the autofluorescence of the surrounding plant tissue. ANOVA analysis showed that the growth media treatment had a significant impact on auxin-dependent fluorescence for both primary roots ($F = 8.831$, **$P < 0.001$**) and secondary root nodes ($F = 4.666$, $P = 0.010$).

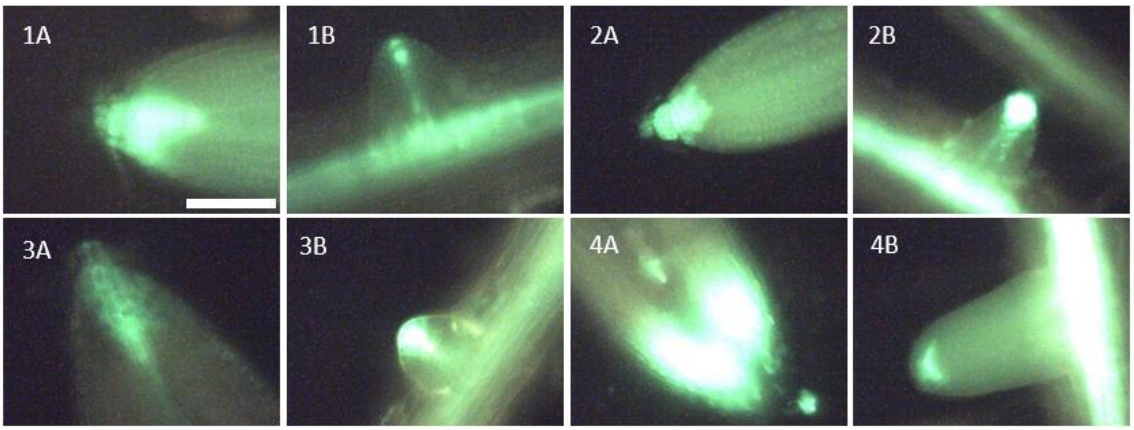


Figure 20: DR5::GFP fluorescence in the primary root caps (A) and secondary root nodes (B) of 10-day old seedlings grown on CM (1), or media containing 80 µg mL^{-1} BBP (2), DBS (3), or DMP (4). Images were taken using GX Capture at 2584 x 1936 resolution, with maximum gain, 1 S, 220 mS and 260 µS exposure, and low frame speed. Scale bar = 100 µM.

Plants grown on CM had significantly higher GFP fluorescence in their primary root cap compared to secondary root node (Figure 21). BBP and DBS treatments caused insignificant differences between secondary nodes and the primary root cap. The secondary node fluorescence was also not significantly different to the control primary root cap. However, BBP and DBS elicited significantly higher fluorescence for the secondary root node than the control secondary root node. DMP caused a significant increase in primary root cap GFP abundance which was significantly higher than the DMP secondary node fluorescence. The secondary root node for DMP treated plants was not statistically different from that of DBS and BBP, as well as the control primary root cap, but was significantly different from the control secondary root node. The primary root cap and secondary root node for DMP treatment were significantly different to one another; this relationship is similar to that of the control. These results indicate a role of auxin in modulating changes in plant root architecture induced by plasticiser treatment due to the accumulation of auxin in secondary roots across plasticiser treatments.

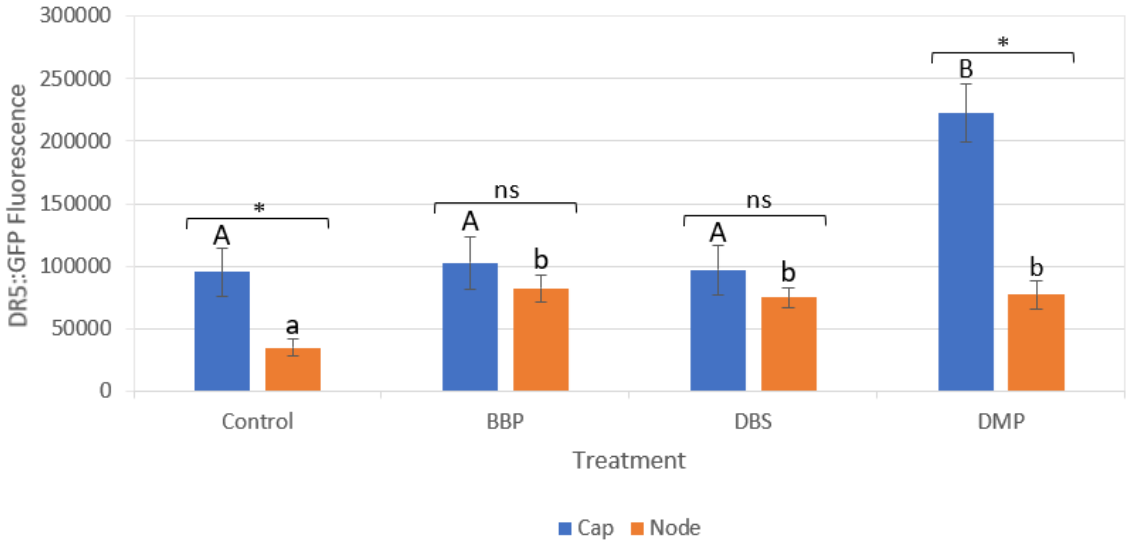


Figure 21: Mean DR5::GFP fluorescence in the primary root caps and secondary root nodes of seedlings grown on CM, or media containing 80 µg mL^{-1} of BBP, DBS or DMP. Results show mean ± SEM (n = 7 per treatment for primary root cap, n = 14 per treatment for secondary root nodes). Subsets of statistically similar data are denoted by letters. Upper case letters denote primary root cap differences, lower case letters denote secondary root node significant differences determined by post-hoc Tukey's Test. Between treatment pairs, * significant to $p < 0.05$, ns not significant as evaluated by paired T-test.

Chapter 4: Discussion.

4.1. Plasticisers at Environmentally Relevant Concentrations Have Marked Effects on Root Growth.

This study shows that at levels of plasticiser previously reported in soils (0.05 – 63.2 µg mL^{-1}; Billings *et al*., 2021), BBP, DBS and DMP had significant effects on primary and/or secondary root growth compared to the control treatment (see Figures 5-9). In contrast, ATBC, DEHP, DINP, DOTP, TCP and TOTM, had no significant, consistent negative impact on primary root growth or secondary root growth (see Figures 5 and 6). The effects of BBP, DBS and DMP were dose-dependent, with increasing effects on primary and secondary root architecture with increasing concentrations. Importantly, no significant effect of 1% (v/v) ethanol (the maximum concentration of solvent present in treatments) on root growth was observed whilst 50 mM NaCl treatment, used as a positive control (Zhu *et al*., 1997; Liu *et al*., 2015), had a significant impact on primary root length and secondary root formation (Appendices 1 and 2), indicating the observed effects were due to the plasticiser exposure rather than other confounding variables.

For BBP, 5 µg mL^{-1} had a significant impact on both total primary root growth and secondary root length. This is consistent with other studies using BBP, which show that it has negative impacts on barley physiology (Kumari and Kaur, 2019). BBP has also been shown to be toxic towards model organisms other than Arabidopsis, as it affects morphology, survival, and fertility of *Daphnia magna* when they were chronically exposed for 21 days (Li *et al*., 2021), and repeated exposure to 500 µg mL^{-1} BBP every two days enhanced tumour progression in mice (Tsai *et al*., 2014). Whilst this concentration is not readily available through environmental contamination, bioaccumulation could expose organisms to this level of BBP (Hu *et al*., 2005) and other plasticisers within their tissues which could cause the effects observed at acute concentrations in other studies (Staples *et al*., 1997; Wilkinson *et al*., 2018).

DBS had a significant impact on total primary root growth and secondary root length at 40 µg mL^{-1}. There have been no studies of the toxicity of this plasticiser in other model organisms, consequently the effects of DBS on development and growth are unknown. Despite this, DBS is commonly used in food packaging and facemasks in commercial production (Vimalkumar *et al*., 2022), therefore exposure to DBS for consumers can be high and with possible profound consequences.

DMP had a significant impact on total primary root growth at 80 µg mL^{-1}, and secondary root growth at 40 µg mL^{-1}. This is consistent with the results of Zhang *et al*. (2015), who showed that concentrations of DMP including and above 30 mg L^{-1} inhibited root growth of cucumber seedlings. DMP has otherwise been shown to have no impact on duckweed at 3 or 30 µg mL^{-1}, however it does inhibit photosynthesis at 600 mg L^{-1} (Pietrini *et al*., 2022). DMP is also toxic towards mammalian systems, the mechanism for which relates to DMP inducing oxidative stress and release of iron from haemoglobin (Li, Chi and Li, 2019). This in turn reduces the capacity for red blood cells to produce an immune response by altering membrane surface receptors (Chi *et al*., 2021).

The mean concentrations of plasticisers present in soils has been reported to be 0.05 to 63.2 mg kg^{-1}, and detection frequencies for plasticisers in soil samples ranges from 33 to 100% (Billings *et al*., 2021). Therefore, the concentrations of plasticisers used in the present study (5 - 80 µg mL^{-1}) suggest that the changes in root growth observed are likely to be representative of the real environmental consequences of plasticiser presence in the environment. Not only have these concentrations been observed in the environment, but the half-life of some plasticisers is extended in anaerobic conditions observed in soils by approximately 8 times, including that of DMP (Shanker *et al*., 1985). This indicates that microbial breakdown is an important factor in the degradation of plasticisers (Shanker *et al*., 1985). This means that plasticisers will have increased accumulation in the soil, as they are in their active state for a longer period, as well as enhanced bioaccumulation due to the longevity of plasticisers increasing.

Despite ATBC, DEHP, DINP, DOTP, TCP and TOTM not having observable impacts on root architecture in this study, a number of these plasticisers have impacts on other systems. ATBC has been reported to have an LD_{50} above 400 mg kg^{-1} in rats, with the cause of death being circulatory collapse (Johnson, 2002). However, this high concentration is unlikely to be found in the environment or accumulated from repeated exposure. In contrast, intestinal Cytochrome P450 3A4 has been shown to be induced by 4.02 µg mL^{-1} ATBC (Takeshita *et al*., 2011), a much lower concentration which could be readily available in the environment.

DEHP has a wide range of adverse effects in animals, frequently acting as an endocrine disruptor. Reduced testosterone levels have been observed in rats following in utero exposure to DEHP by decreasing adrenal tissue mass in rats (Martinez-Arguelles *et al*., 2011), and causing demasculinisation during perinatal exposure in rats via testosterone reduction (Gray *et al*., 2000). Zebrafish larvae treated with the LC_{50} (54.02 mg L^{-1}) for DEHP have altered gene

expression and physiological malformities (Boran and Terzi, 2019). In human thyroid cells, 10 µM DEHP induces cAMP secretion without affecting thyroid specific gene expression and had slower metabolism than its analogues, di-ethyl phthalate, and di-n-butyl phthalate (Hansen *et al*., 2016). Demasculinisation has also been reported in rats in response to BBP, DINP, and DOTP, and although the influence of DINP was less severe, exposure still affected testosterone levels (Gray *et al*., 2000). In rats, DINP also causes dose-dependent increases in carcinoma and adenoma, as well as increased incidences of carcinoma and adenoma (Moore, 1998). DINP does not have an LC_{50}, as an acute concentration of 500 ppm does not induce death in more than 50% of zebrafish embryos after 72 hours of exposure (Chen *et al*., 2014). Exposure to DINP also induces enhanced oestrogenic activity in zebrafish (Xu *et al*., 2020). With DINP exposure in children averaging 5.7 µg kg^{-1} per day and ranging up to 94.3 µg kg^{-1} per day (Babich *et al*., 2004), the effects of DINP on human health could be just as severe as those in model organisms. DOTP is widely considered to be a green alternative to other plasticisers that have harmful impacts on the environment. DOTP has been shown to have no significant effect on sexual differentiation in rats (Gray *et al*., 2000) so is not regarded as an endocrine disruptor and causes little to no dermal sensitisation or irritation (David *et al*., 2003). It has also been reported to have low toxicity (Barber and Topping, 1995; Wirnitzer *et al*., 2011). TCP is a mixture of isomers, many of which are considered to be toxic. Despite the root growth assay showing no significant impact of TCP, it causes delayed neurotoxicity by inhibiting acetylcholine esterase, and prolonged exposure can cause damage spanning the nervous system (Hausherr *et al*., 2017). TOTM does not have an LD_{50} and is considered to be non-toxic, only showing slight dermal irritation in animal studies, but not in human studies (David *et al*., 2003).

4.2. Molecular Weight Affects Plasticiser Uptake.

Plants show limited uptake of chemicals above 390 Da (Lamshoeft *et al*., 2018). This might explain the differential effects of the groups of plasticisers observed in the present study with BBP, DBS and DMP having a significant effect on root growth whilst the effects of ATBC, DEHP, DINP, DOTP, TCP and TOTM are not significant. Although there was no marked difference in the chemical structures between plasticisers that had significant impacts on root growth and those that did not, evaluated by presence of a benzene ring, chemical formulae and group (see Table 2), BBP, DBS, and DMP all have molecular masses below 315 Da, which might be responsible for the different effects observed between plasticisers in the same chemical group with similar chemical structures. TCP also has a molecular mass below 390 Da. Whilst TCP had no significant impact on primary root growth, significant differences were observed between

control and TCP-treated plants in terms of secondary root growth. However, this was deemed not sufficiently significant to continue with TCP for further experiments since the effects of TCP were not consistent. Nevertheless, it would explain the marginal effects of TCP observed in the root assay. Though this could not be statistically evaluated in Figure 8 due to the number of viable plants varying between plates which prevented direct comparison, the contrast between the primary root length inhibition for plasticisers that have the capacity for molecular uptake in plant roots and those that cannot can be inferred. DMP, DBS and BBP show clear separation from the other plasticisers in terms of their influence on primary root length at 80 μg mL^{-1}, all of which can be transported into plant roots due to their low molecular weights.

4.3. Uptake of Chronic Plasticiser Concentrations Alters Ca^{2+} Signalling.

Calcium (Ca^{2+}) is a ubiquitous second messenger in plants that plays key role in the signalling network regulating root morphogenesis (X. P. Zhang *et al*., 2020). Therefore, the effects of plasticisers which had a significant effect on root growth, BBP, DMP and DBS, on $[Ca^{2+}]_{cyt}$ was tested. As in the root growth studies, there was no significant effect of 1% (v/v) ethanol (the maximum concentration of solvent present in treatments) or RO water (as a control) on $[Ca^{2+}]_{cyt}$, confirming that any differences in $[Ca^{2+}]_{cyt}$ observed were due to the plasticiser treatments. Addition of 80 μg mL^{-1} of the chosen plasticisers had no significant impact on $[Ca^{2+}]_{cyt}$ (Figure 10). This suggests that although chronic exposure to this concentration of plasticiser is sufficient to inhibit root growth, acute exposure to plasticisers at this concentration is insufficient to cause changes in $[Ca^{2+}]_{cyt}$, and therefore unlikely to evoke a physiological responses in terms of root growth. Conversely, acute exposure to salt stress results in a change in $[Ca^{2+}]_{cyt}$ (Köster *et al*., 2019) due to an immediate osmotic stress (Xiao and Zhou, 2023). Plasticisers are long chain hydrocarbons with higher molecular weights, therefore plants sensing concentrations that had a significant impact on growth as a chronic treatment would be less likely in a short period of time because plants would not be adapted for perceiving this kind of stress or have the ability to rapidly uptake high molecular weight plasticiser molecules at low concentrations (Lamshoeft *et al*., 2018). This means that plasticiser perception may be delayed, which is consistent with the root assay as treatment with different concentrations of BBP, DMP and DBS had significant impacts more frequently later in the growth period compared to days 0-2 (Figure 9). For example, when treated with BBP, at days 0-2, only 80 μg mL^{-1} had a significant impact on root growth, whereas after days 4-6, all concentrations had a significant impact on incremental primary root growth. It is therefore possible that recording $[Ca^{2+}]_{cyt}$, for a longer period may have revealed an increase in Ca^{2+} signalling upon prolonged exposure to plasticiser. Whilst this has proved technically feasible

when studying circadian Ca^{2+} signals (Love *et al*., 2004), the immersion of the 10-day-old *Arabidopsis thaliana* seedling used in the current study in a plasticisers solution in the dark would likely have had profound effects on the plants independent of the plasticiser.

Addition of 800 µg mL^{-1} of the chosen plasticiser only resulted a significant increase in the total $[Ca^{2+}]_{cyt}$ with DMP treatment which gave a peak following plasticiser addition which was absent in the control or other plasticiser treatments. 800 µg mL^{-1} DMP addition caused a significantly higher mean $[Ca^{2+}]_{cyt}$ after addition and mid-point with 800 µg mL^{-1} DMP. As in the response to NaCl (Köster *et al*., 2019), this is indicative of a stress response, and the elevated mid-point shows a sustained response following 800 µg mL^{-1} DMP addition. Within the time period of recording, the $[Ca^{2+}]_{cyt}$ did not return to the baseline before DMP addition, suggesting the plant may be experiencing chronic stress. Nevertheless, the $[Ca^{2+}]_{cyt}$ response was still significantly lower than the response when plants were exposed to 1 mM H_2O_2 treatment, whilst the temporal dynamics of the responses were both different with the DMP-induced increase in $[Ca^{2+}]_{cyt}$ taking longer to peak than in plants treated with 1 mM H_2O_2.

The 80 and 800 µg mL^{-1} DBS and BBP additions did not cause significant differences in $[Ca^{2+}]_{cyt}$, therefore acute concentrations of these plasticisers were not sufficient to cause plant stress within the time period of recording. This may indicate that the effects of DBS and BBP on root growth and architecture are related to prolonged signalling mechanisms such as phytohormones instead of instantaneous signal transducing molecules like Ca^{2+} which provide short term responses to stress (Bradford and Trewavas, 1994). This is supported by Figure 9, which illustrates that BBP had significant impacts on root growth below 80 µg mL^{-1} after 4-6 days for BBP, therefore the effects of lower concentrations of BBP are delayed and may require additional uptake for plants to respond, or that long-term signalling mechanisms are required to modulate responses to BBP toxicity.

4.4. Chronic Plasticiser Exposure Impacts ROS Signalling.

Ca^{2+} and H_2O_2 have been implicated in mediating adventitious root formation (Liao *et al*., 2012), and H_2O_2 exposure at high concentrations is a form of ROS stress which induces changes in cellular signalling in plants, including Ca^{2+} signalling (Short *et al*., 2012). This makes responses to H_2O_2 an interesting factor to explore in relation to chronic plasticiser treatment, because it shows how signalling under chronic stress is altered following plasticiser treatment. This also has implications of the mechanisms behind how plasticiser exposure causes changes in root growth, as the Ca^{2+} responses are specific to the type of stress and dependent on pre-exposure to other types of stress treatments (Kiegle *et al*., 2000; Evans *et al*., 2005).

Chronic exposure to BBP, DBS and DMP had differential effects on ROS-induced changes in $[Ca^{2+}]_{cyt}$. Compared to control plants, BBP had no significant effect on the total change in $[Ca^{2+}]_{cyt}$ in response to 1mM H_2O_2, whereas this was significantly higher in response to 10mM and 100mM H_2O_2. At 100mM H_2O_2, BBP treated plants also had a significantly lower peak $[Ca^{2+}]_{cyt}$. In contrast, DBS treated plants had no significant differences in total $[Ca^{2+}]_{cyt}$ to control plants when exposed to 1mM, 10mM and 100mM H_2O_2, other than a significantly higher peak $[Ca^{2+}]_{cyt}$ for 10mM H_2O_2. DMP however resulted in significantly higher total $[Ca^{2+}]_{cyt}$ at 10 and 100 mM H_2O_2, but a lower peak at 100 mM H_2O_2. This may indicate chronic stress from plasticiser treatment since the plant is producing a higher sustained $[Ca^{2+}]_{cyt}$ signal over time rather than having a high instantaneous response and returning to a low resting value. This is also supported by insignificant differences between the first and second peaks for DMP treatment with 100 mM H_2O_2 addition. The relationship between the low peak and high total $[Ca^{2+}]_{cyt}$ could also show that plants grown in DMP have diminished capacity to have acute responses, which could be due to cellular damage caused by DMP presence throughout the growth period which impairs the plant's ability to respond to stimuli.

The higher total $[Ca^{2+}]_{cyt}$ observed in DMP-treated seedlings may be the result of chronic exposure to DMP increasing ROS in in cells prior to the addition of H_2O_2 (Zhang *et al*, 2015). In which case, plants may already be primed making them more sensitive to H_2O_2 and readily increase $[Ca^{2+}]_{cyt}$ in response to external H_2O_2 exposure which increases their response compared to control plants (Choi *et al*., 2017). DMP treated plants also had lower peak $[Ca^{2+}]_{cyt}$ compared to control plants for 10mM and 100mM H_2O_2 (Figure 18). This suggests that due to their constitutive activation of ROS response pathways, they have a lower peak but elevated signal over time and have a higher resting signal following the peak. This is supported by DMP treated plants having a longer time for the Ca^{2+} concentration to return to a resting level following 100mM H_2O_2 exposure compared to control.

With increasing concentrations of H_2O_2, the control treatment had increasing first phase $[Ca^{2+}]_{cyt}$ peaks. However, the opposite trend was observed with plants grown in 80 µg mL^{-1} DMP as this treatment induced decreasing first phase $[Ca^{2+}]_{cyt}$ peaks when the H_2O_2 concentration increased (Figure 19). This indicates a diminished ability of the plants to respond to H_2O_2 at higher concentrations, and may be because of a reduced ability to rapidly upregulate $[Ca^{2+}]_{cyt}$ in response to stress. This is consistent with the CM control having a significantly lower second phase $[Ca^{2+}]_{cyt}$ peak compared to the first phase with 100 mM H_2O_2 exposure, whereas the peaks for DMP treatment were not significantly different.

Interestingly, 50 mM NaCl was found to have a significant impact on root growth, and produce similar changes in root architecture to the 80 µg mL^{-1} BBP, DBS and DMP treatments. *Arabidopsis thaliana* grown in the presence of 40 mM NaCl has also been shown to induce a lower peak $[Ca^{2+}]_{cyt}$ response to 4 mM H_2O_2, which was hypothesised to be caused by reduced sensitivity to H_2O_2 as an adaption to salinity stress (Liu *et al*., 2018). This being the case, in the present study a decreased sensitivity to H_2O_2 addition would also be predicted to result in a lower total $[Ca^{2+}]_{cyt}$ than the control with increasing H_2O_2, as a result of the chronic plasticiser exposure preventing plants from responding to the increase in concentration. This was not found to be the case, as the plasticiser treatments caused similar, if not higher total $[Ca^{2+}]_{cyt}$ with increasing H_2O_2 concentrations (Figure 17). However, treatment with different concentrations of H_2O_2 revealed that it may not be the result of a decreased sensitivity, because the total $[Ca^{2+}]_{cyt}$ was not significantly lower with DMP treatment for increasing concentrations of H_2O_2, despite the decreasing first phase $[Ca^{2+}]_{cyt}$ peaks. Instead, the capacity of plants to respond to Ca^{2+}-mobilizing stimuli may be affected resulting in changes to the temporal dynamics of an elevated $[Ca^{2+}]_{cyt}$. This mechanism may be indicative of subcritical chronic stress as plants that have been stressed by plasticisers may already have cellular damage, and therefore must mitigate additional cellular damage from $[Ca^{2+}]_{cyt}$ flux at levels associated with programmed cell death upon H_2O_2 exposure (Ren *et al*., 2021).

Plasticisers have been observed to increase H_2O_2 presence in plant cells (Zhang *et al*., 2015). Therefore, chronic exposure of plants plasticisers in growth media could prime seedling to respond to subsequent exposure to H_2O_2 in the cuvette (Choi *et al*., 2017). Previously, *Arabidopsis thaliana* has been shown to respond differently to repeat exposure of both O_3 and H_2O_2, with repeated 10 mM H_2O_2 exposure generating a much lower response on the second exposure (Evans *et al*., 2005).

This study showed decreased first phase peaks in response to 100 mM H_2O_2 when plants were grown in the presence of plasticiser, which could indicate that plasticiser exposure induces a similar mechanism to H_2O_2 priming as observed in Evans *et al*., 2005, however there was also an elevated Ca^{2+} response over time. This evidence could support either H_2O_2 priming caused by plasticisers, or a different mechanism for the Ca^{2+} response. It has been observed that exposure to 10 mM H_2O_2 following O_3 exposure has a biphasic response and a peak similar to that of plants with no prior O_3 treatment (Evans *et al*., 2005). Therefore, plants can distinguish between ROS stress and have different Ca^{2+} transients as a consequence, showing that plants respond differently in terms of Ca^{2+} signalling to plasticiser and H_2O_2 stresses. The Ca^{2+} responses observed following plasticiser exposure did have a biphasic response, which could

suggest that chronic plasticiser exposure also did not produce enough H_2O_2 for plants to be considered primed with H_2O_2, and the mechanism for their impact on growth and Ca^{2+} signalling may be due to other physiological changes induced by plasticisers. 100 µg mL^{-1} DMP exposure at 7 days caused H_2O_2 concentrations to rise from below 0.08 mM to 0.15-0.18 mM (Zhang *et al*., 2015), when concentrations below 0.3 mM H_2O_2 have been shown to not induce significant influx of ions (Wang *et al*., 2018). Alternatively, the small increase in H_2O_2 from chronic DMP treatment may be sufficient to sensitise the plants to H_2O_2 treatment in vitro and accentuate the Ca^{2+} response over time, as the instantaneous treatment that caused dampened Ca^{2+} responses to H_2O_2 was 10 mM H_2O_2 which, in vitro, would cause significant plant death, as plant growth rate is inhibited by concentrations above 1 mM H_2O_2 (Claeys *et al*., 2014).

4.5. Auxin Upregulation, H_2O_2 and Polar Transport.

Auxin is at the centre of changing root architecture, interacting with multiple other hormones and signalling molecules to modulate stress responses in terms of root growth (Benková and Hejátko, 2009). Auxin is also linked with Ca^{2+} elevations (Leitão *et al*., 2019), therefore the experiment investigating Ca^{2+} responses to plasticiser and H_2O_2 exposure could indicate potential interactions with auxin signalling mechanisms. Auxin mediates root growth by increasing H_2O_2 accumulation at high auxin concentrations, which inhibits root growth (Ivanchenko *et al*., 2013). The capacity of plants to regulate their root architecture in response to stress can thus be inferred from their Ca^{2+} responses to H_2O_2, in combination with their auxin levels in the root tips, as plants with increased auxin signalling may have a reduced instantaneous response to H_2O_2 exposure, because H_2O_2 is already active at the root tip impeding growth and pre-exposing plants to ROS stress (Evans *et al*., 2005).

DBS and BBP exposure had no significant effect on auxin in the primary root cap, but significantly increased the presence of auxin in secondary root nodes. This may indicate that the plants are focussing their growth to lateral root length instead of quantity, shown by the reduced number of roots per unit of primary root length observed for both BBP and DMP in the root assay, and statistically similar auxin presence plasticiser treated secondary nodes, and the control primary root caps. This is a common trait illustrated by plants when exposed to increased salinity, as lateral root density is increased in attempt to grow to a niche that does not induce salinity stress (Arif *et al*., 2019). The Ca^{2+} transients also support this, as Ca^{2+} signalling increases polar transport of auxin away from the roots (Zhang *et al*., 2020b), and additions of acute concentrations of DBS and BBP had no effect on Ca^{2+} signalling which would allow auxin to accumulate in root tips. Alternatively, plants had a higher root architecture ratio

with increasing DBS concentrations which could be caused by the elevated auxin concentrations in the secondary roots, or as a function of another hormone being upregulated as well as auxin. BR or GA_3 could be responsible for this change in root architecture, as they has been observed to regulate lateral root proliferation in tandem with auxin (Gou *et al*., 2010; Cai *et al*., 2022; Zhang *et al*., 2022).

DMP exposure significantly increased auxin signalling in both the root cap and secondary root nodes. This may indicate decreased transport of auxin away from the root tip to facilitate primary root growth, or it could be a consequence of cellular damage causing auxin accumulation, as observed in wounds to plant tissue (Canher *et al*., 2020). Auxin follows a concentration dependency curve, meaning that high concentrations of auxin can reduce root growth. The presence of elevated auxin in the root caps illustrated by DMP treated plants may be because of increased auxin accumulation in the root tip that inhibits root growth. Accumulation of auxin may be due to the proteins that facilitate polar transport being damaged or their production being limited. Zhang *et al*, 2015 showed that DMP exposure elevated H_2O_2 presence, whilst Zwiewka *et al*., 2019 showed that oxidative stress induced by H_2O_2 decreases PIN protein abundance which would be responsible for reduced auxin polar transport and increased accumulation when plants are exposed to DMP. This would cause the primary root growth to be impeded, as the elevated auxin concentration in the root tip would reduce root growth by interactions with cytokinin that slows root growth (Růzička *et al*., 2009). There was a lower peak Ca^{2+} for plants exposed to DMP, this paired with the increased auxin in the primary and secondary root tips also reflects less polar transport of auxin away from the root tips because Ca^{2+} is required to facilitate active transport. There was a significantly higher total Ca^{2+} concentration over time, however this could be indicative of cell death (Ren *et al*., 2021). Auxin is also implicated in mediating programmed cell death, so high auxin levels could be due to cell death at the root tip (Kacprzyk *et al*., 2022).

The H_2O_2 concentration within cells exposed to DMP ranges from 0.15 - 0.18 mM, where normal cellular levels stay below 0.08 mM H_2O_2 (Zhang *et al*., 2015), however impediment of PIN2 protein activity occurrs at 1 mM H_2O_2 (Zwiewka *et al*., 2019). Concentrations below 1 mM H_2O_2 were not investigated for impacts on PIN2 proteins, therefore the mechanism behind plasticiser-induced changes in auxin signalling could be due to H_2O_2 elevations at concentrations that have not been explored. Increased DMP also causes distortion in the chloroplast which impeded their metabolic productivity (Zhang *et al*., 2015). Auxin is synthesised by the precursor tryptophan in chloroplasts (Rapparini *et al*., 1999), therefore the chloroplast damage caused by DMP would suggest decreased auxin transport leading to auxin

accumulation instead of increased auxin production, since excess auxin production would not be possible to provide the increase in fluorescence shown in Figures 20 and 21. Figure 22 summarises the findings and interconnectivity between plasticisers, plant hormone signalling and root growth.

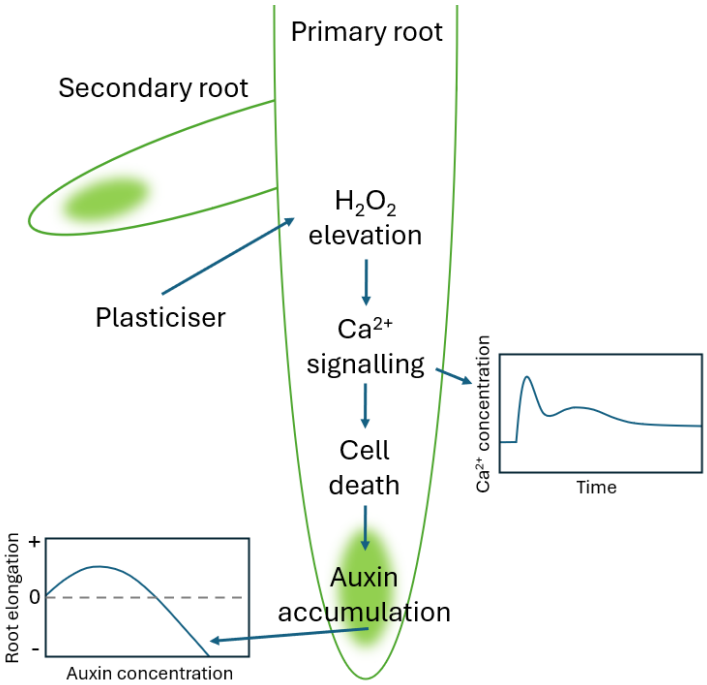


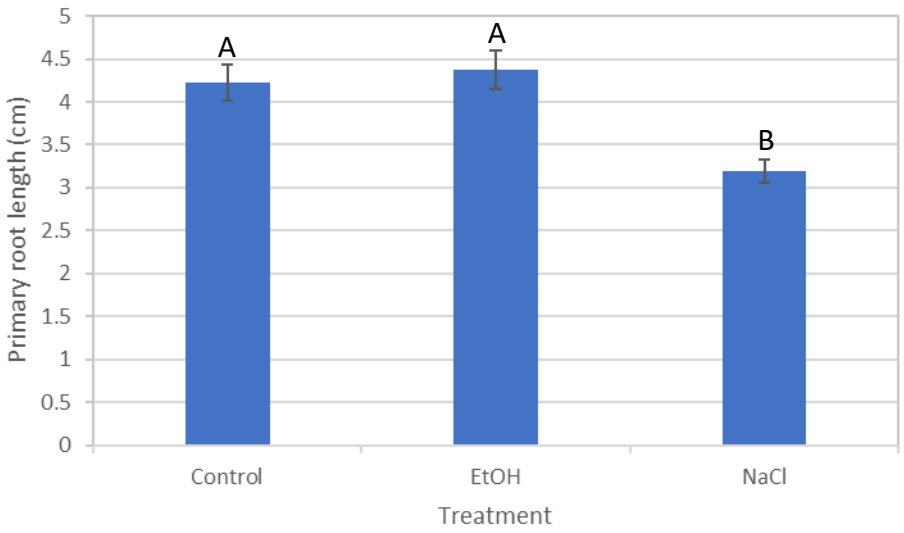
Figure 22: Summary of the connectivity of biochemical processes explored in this study. Plasticisers cause increases in H_2O_2 (Zhang *et al*., 2015), which activate ROS signalling pathways facilitated by Ca^{2+} transients (Niu and Liao, 2016). This causes ROS scavenging, however continued elevated H_2O_2 within cells can cause cell death and auxin accumulation as PIN protein activity is impeded (Zwiewka *et al*., 2019). Cell death also results in the accumulation of auxin at the root tip (Canher *et al*., 2020). The increase in auxin concentration at the root tip causes root elongation to be inhibited due to the inhibitory effect of auxin on root growth at high concentrations (Scott, 1972; Walter *et al*., 2020; Edelmann, 2022). There may be other plant hormones interacting to produce this change in root architecture phenotype, however the relationships between other hormones and plasticisers are yet to be characterised.

Chapter 5: Conclusion

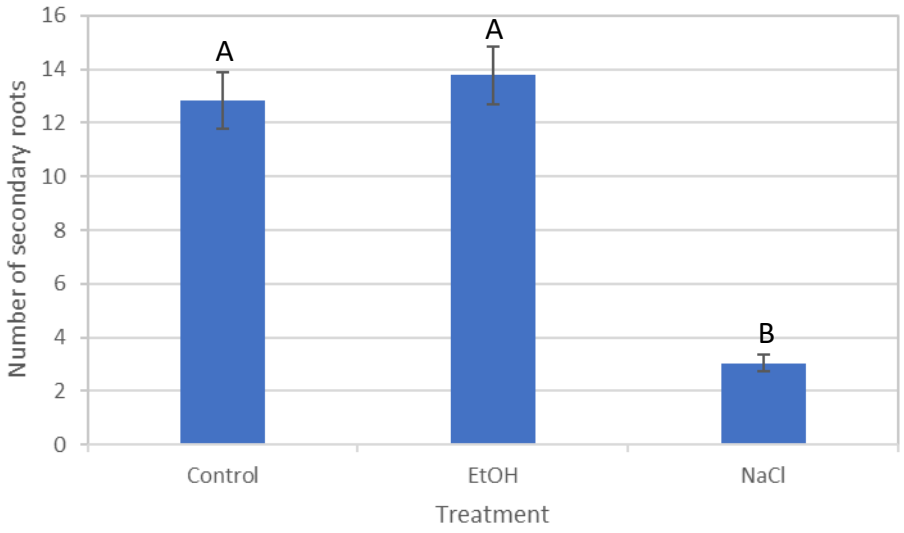
This study aimed to characterise the impacts of a selection of plasticisers on root architecture and plant signalling, as well as explore how these changes in plant growth occur through plant signalling mechanisms. It was found that BBP, DBS and DMP all impede root growth and alter root architecture at 80 μg mL^{-1}, shown by reduced primary root lengths and number of secondary roots. These plasticisers in particular were selected to study their effect on root growth over the others initially investigated in the study because they have molecular weights below 390 Da, which plants can readily uptake from soil (Lamshoeft *et al*., 2018). Once taken up, plasticisers therefore have the potential to disturb physiological activities within the plants through the disruption of stress signalling networks and to cause systemic damage. Chronic exposure to 80 μg mL^{-1} of plasticiser caused accentuated Ca^{2+} signalling over time and attenuated instantaneous responses to H_2O_2 treatment. Additionally, fluorescent imaging revealed that auxin was accumulated at secondary root nodes with chronic BBP, DBS, and DMP exposure, as well as in primary root caps with DMP treatment. This suggests that plasticiser treatment reduces the transport of auxin away from the root tip, resulting in the elevated auxin concentration to inhibit growth in a dose-dependent manner. This is consistent with reports that DMP treatments cause increased levels of H_2O_2 in cells (Zhang *et al*., 2015), since elevated H_2O_2 concentrations in roots can impede PIN2 protein production which actively transports auxin from the root tip to allow for root growth (Zwiewka *et al*., 2019). However, given the present study was performed on *Arabidopsis thaliana* grown on agar supplemented with plasticisers for a relatively short timescale providing the chronic exposure, this may not be representative of the interactions with plasticisers in soils. Therefore, additional soil-based studies will be required to confirm the impacts on root growth in the same time span observed in this study to confirm plasticiser toxicity in the environment at the concentrations already prevalent in soils. Confocal microscopy using different fluorescent reporter lines could help distinguish whether cells were damaged by plasticisers which caused auxin accumulation, or if there was overproduction of auxin which produced the phenotype with shorter roots as well as other hormone interactions. Importantly, these results implicate DBS as a potentially harmful plasticiser, which has previously been categorised as safe for use as an alternative to more prominent, toxic plasticisers. Moreover, it provides insight into the mechanisms behind how plasticisers induce physiological changes in plants and could lead to future studies investigating how plasticisers translocate into and interact with plant cells to produce the changes in signalling observed in this study, as well as longer-term studies on different

organisms to assess the ongoing threat of plasticisers in the environment, as they continue to accumulate and persist.

Chapter 6: Appendices.



Appendix 1: Mean primary root growth after 10 days of exposure to CM, 1% (v/v) ethanol, or 50 mM NaCl. Results show mean ± SEM, data with significant differences ($p < 0.05$) are denoted by different letters ($n \geq 20$ per treatment), as evaluated by post-hoc Tukey's test.



Appendix 2: Mean number of secondary roots after 10 days of exposure to CM, 1% (v/v) ethanol, or 50 mM NaCl. Results show mean ± SEM, data with significant differences ($p < 0.05$) are denoted by different letters ($n \geq 20$ per treatment), as evaluated by post-hoc Tukey's test.

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