Phytohormonal and Physiological Responses of *Solanum lycopersicum* to Strong Soils

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Declaration

Except where references are made to other sources, I declare that the contents in this thesis are my own work and have not been previously submitted, in part or full, for the award of a higher degree elsewhere.

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December 2018

Publications arising from this thesis

Donaldson, S.M., Dodd, I.C., Whitmore, A.P., 2018. Soil bulk density impacts on root water potential and ABA export in drying soil. Acta Hortic. 1197, 15–22.

The original publication is available at:
https://doi.org/10.17660/ActaHortic.2018.1197.3

(Reproduced as Appendix 1)

Acknowledgements

I would like to express my deepest gratitude to my supervisor Professor Ian Dodd for his belief in my skills and for his patience, help, advice and the constructive criticism over the years. I would not be the person I am today without his guidance, and I would not have been able to overcome so much throughout my studies without him. Thank you also to my co-supervisor, Professor Andy Whitmore at Rothamsted Research, for useful discussions. I am grateful to the BBSRC for funding this project as part of the Food Security DTP, and thanks also to the Faculty of Science and Technology at Lancaster University, who also granted me additional funds in 2017 for international travel.

I am also thankful to Dr Richard Whalley for providing me the fantastic opportunity to work with the Soil Physics group at Rothamsted Research, Harpenden. Thanks also to staff at Rothamsted for their help and support during my time there, particularly Rhys Ashton and Yaoxiang Ge. Special thanks to my good friend Edward Green, who was a fantastic host during my time down south.

Thank you to Dr Alfonso Albacete (CEBAS-CSIC) for his invaluable help with phytohormonal analyses, an integral part of this thesis.

I would like to thank members of the Plant Physiology lab and wider community at Lancaster Environment Centre for all the useful discussions, practical help and friendship: Jaime Puertolas, Shane Rothwell, Annette Ryan, Maureen Harrison, Dawn Worrall, Geoffrey Holroyd, Elizabete Carmo-Silva, Dennis Touliatos, Xiaoqing Li, Richard Boyle, Lucas Gent, Hannah Wright, Laura Hodgkinson, Arnauld Thiry, Noorliana Mohd Zahn, Paul Cambre, Yan Li, Kirsty Forber, Joe Acton, Sam Jones, Esti Leibar, Bea Burak, Tom Williams, Rob Kempster, Pedro Castro Valdecantos, Thomas Monvain-Monval, Chris Blacklock, Aimee Brett, Katharina Huntenberg, Rachel Baxter, Hend Mandour, Vasilis Giannakopoulos and many more.

A special thank you to Dr Carlos de Ollas Valverde for his endless support and willingness to help, despite being thousands of miles away. A true friend and mentor.

Thank you to my best friends, Katie Fairweather, Jessie Davidson and Hattie Serocold, for always providing a listening ear, warm hugs and plenty of laughs. Thank you for all your encouraging words and advice. Thank you to my family, for your unconditional love and understanding. I am glad you love Lancaster as much as I do!

Finally, thank you to Max, for being there through all the late nights and early mornings, from pot-filling and harvests to writing-up. Thank you for your constant support and devotion, and for looking after me so well over the last four years. This thesis would not be here without you!

Abstract

Soil compaction occurs when external pressures (from heavy machinery or grazing animals) exerted on the soil surface increase soil bulk density, reducing porosity and aggregation. Nutrient, air and water holding capacities of the soil are reduced, and plant roots encounter increased mechanical resistance as they grow. Soil compaction also stunts shoot growth, with hydraulic and chemical signalling systems between below- and above-ground parts allowing the plant to adapt to this multi-stress environment. However, relatively few studies have characterised root-to-shoot signalling systems of plants with mechanically-impeded roots.

Tomato plants (*Solanum lycopersicum* cv. Ailsa Craig) were grown under low and high soil bulk densities, and allowed to dry the soil to investigate plant physiological responses. Compact soil stunted plant growth, decreased stomatal conductance of well-watered plants and decreased plant water status at higher soil water contents. Multi-hormone analyses of root xylem sap and foliar tissues revealed that high bulk density soils attenuated the soil drying-induced increase in xylem [ABA]. Moreover, high bulk density soil increased xylem jasmonic acid concentrations and decreased foliar bioactive gibberellins, which were correlated with reduced shoot growth.

Root drenches of bioactive gibberellic acid (GA_3) were then applied to determine its ability to improve tomato shoot growth in compact soil. GA_3 was transported from root to shoot tissues and significantly increased leaf expansion, but at the expense of plant water status. Further multi-hormone analyses indicated that GA_3 application increased foliar cytokinin (*trans*-Zeatin) levels and decreased xylem jasmonic acid concentrations.

Finally, to isolate soil strength from possible confounding effects of nutrient and water availability, tomato plants were grown in a sand culture system. A light foam block or 17 kg weight was placed upon the surface of the sand to increase substrate strength, while tanks were supplied with ample nutrients and water by capillary action. While GA_3 again rescued shoot growth, shoot and leaf water potentials were reduced. Furthermore, xylem jasmonic acid concentration consistently decreased in both sand- and soil-grown plants as soil strength increased, which was not attributed to any decrease in leaf water status.

Taken together, this thesis is the first to employ multi-hormone analyses on tissues and sap from plants growing in compact or strong soils. Novel roles for gibberellins and jasmonic acid in regulating plant growth when roots are mechanically impeded were discovered. GA_3 appears to promote shoot growth against water potential gradients. Further study of the physiological significance of xylem-transported jasmonic acid and its cross-talk with gibberellins seem necessary to help determine how plants respond to soil mechanical stresses.

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List of commonly-used abbreviations

ABA	Abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
BD	Bulk density
BL	Block
CK	Cytokinin
D	Drought (treatment)
DAT	Days after transplanting
DI	Deionised
DW	Dry weight
ET	Evapotranspiration
GA	Gibberellin
GCC	Global climate change
GHG	Greenhouse gas
g_s	Stomatal conductance
GSWC	Gravimetric soil water content
HESI	Heated electrospray ionisation
IAA	Indole-3-acetic acid
iP	Isopentenyladenine
IPT	Isopentyl transferase
JA	Jasmonic acid
JAZ	JASMONATE ZIM-domain
KNOX	Knotted1-like homeobox
LSD	Least significant differences
MACC	1-(malonylamino)-cyclopropan-1-carboxylic acid
MEP	Methylerythritol phosphate
MVA	Mevalonic acid
NCEDs	9-*cis*-epoxy-carotenoid dioxygenases
PIF	Phytochrome interacting factor
PIP	Plasma membrane intrinsic protein
REML	Restricted maximum likelihood
SA	Salicylic acid
SCF	Skp, Cullin, F-box containing complex
SOC	Soil organic carbon
SPAD	Spectral adsorbance
SWC	Soil water content
TIP	Tonoplast intrinsic protein
tZ	trans-Zeatin
U-HPLC-MS	Ultra-high performance liquid chromatography-mass spectrometry
VPD	Vapour pressure deficit
W	Watering
WD	Water-deficient (treatment)
WT	Wild-type

WW	Well-watered (treatment)
ZR	Zeatin riboside
Ψ_{leaf}	Leaf water potential
Ψ_{root}	Root water potential
Ψ_{shoot}	Shoot water potential

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Table 4-1: A) Nutrient solution recipe adapted from (Clark et al., 2002) to suit tomato growth. B) Macronutrient composition of adapted nutrient solution compared to half-strength Hoagland's solution 111

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Chapter 1 General introduction

1.1 Introduction

Huge advances in agricultural technology over the last 100 years led to unprecedented increases in food production, termed the "Green Revolution". Development of semi-dwarf varieties of rice and wheat and the creation of hybrid maize resulted in crops which produced higher yields, due to characteristics including improved fertiliser responsiveness, resistance to lodging and pest invasions (Godfray et al., 2010). Widespread mechanisation has improved efficiency of farming, reducing manpower required for cultivation and harvest. Development and deployment of synthetic nitrogen fertilisers and investment in irrigation infrastructure also greatly contributed to increased crop yields in the 20th century (Matson et al., 1997). Other management practices, such as short cropping rotations and monoculture cultivations also grew in popularity to keep pace with market demands. While global farmed land area increased 47-fold between 1700 and 1980 (Matson et al., 1997), yields of many staple crops have more than doubled since the 1960s alone (FAO, 2018; Figure 1-1A) without similar increases in cultivated land area over the same period (FAO, 2018; Figure 1-1B & C).

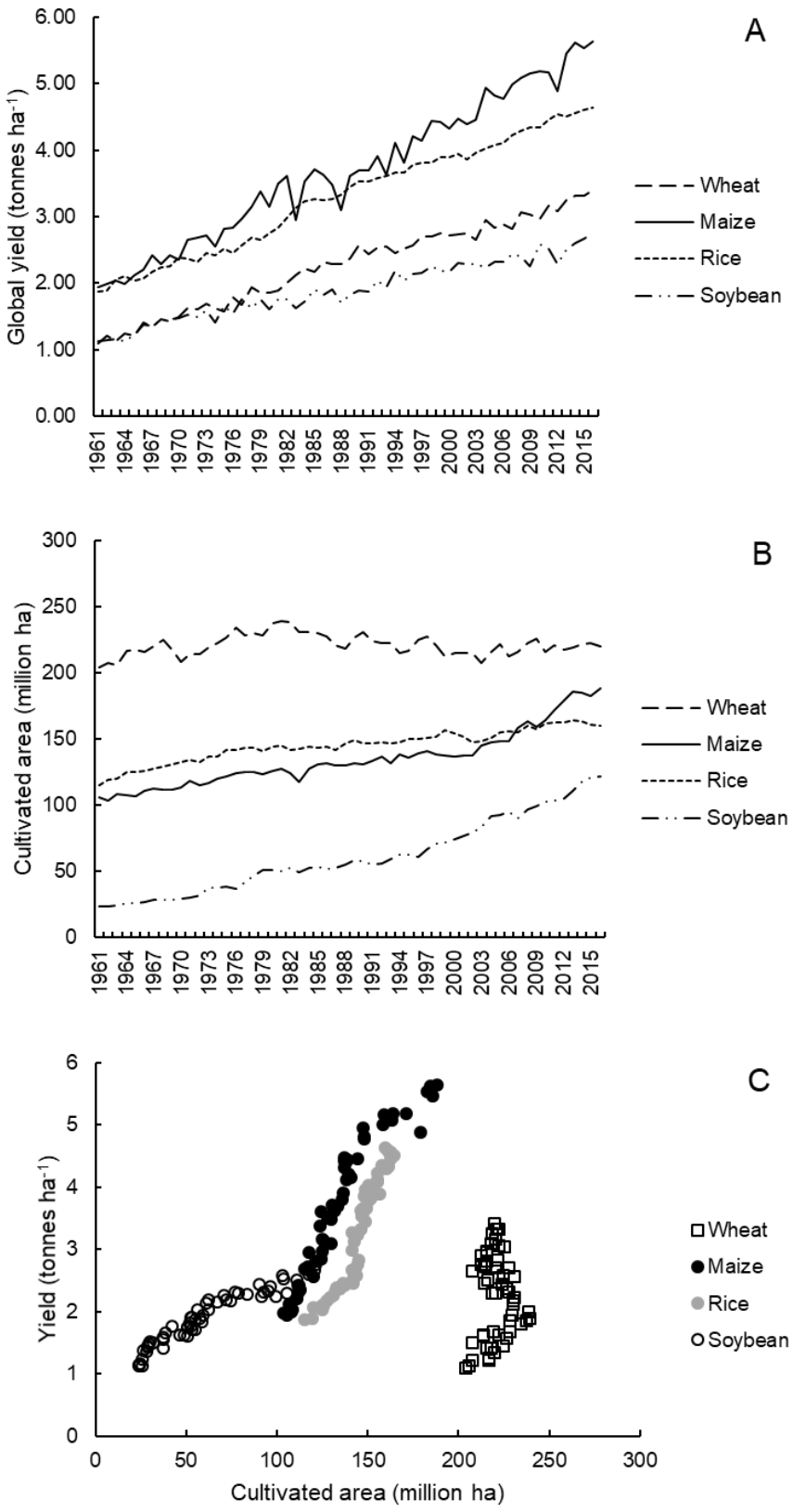


Figure 1-1: A) Global yield data and B) Area of cultivated land per crop, for four staple crops, 1961 - 2016. C) Relationships between cultivated area and yield from panels A and B. Yields of these staple crops have increased by up to 200% but without similar increases in farmed area, due to improved cultivation practices. Data: (FAO, 2018)

Despite such rapid improvement in crop yields, unequal distribution of food ensures that hunger is a pressing issue in many countries in both the developed and developing world. Much progress has been made to improve food security in recent years: the number of undernourished people fell from 900 million in 2000 to 815 million by 2016 (FAO, 2017). However, population projections suggest that the global population may reach as high as 9.8 billion by 2050 and 11.2 billion in 2100 (UN, 2017), putting severe pressure on agriculture to supply sufficient food. As average wealth rises worldwide, there will be increased demand for variety and luxury in food products. However, acquiring further land area for agriculture to feed the growing global population is set to become more expensive as space is required for other land uses such as residential and industrial, and as some regions are earmarked for conservation of biodiversity and ecosystem services (Godfray et al., 2010). Food production into the 21st century must further intensify to keep up with the demands of a growing population, but there is a pressing need to adapt to and mitigate the changing climate, and to preserve crucial ecosystem services.

Human activity is widely accepted to have directly driven observed global climate change (GCC). As industries, technologies and economies have developed, various aspects of modern lifestyles contribute to the emission of greenhouse gases (GHGs) into the atmosphere and to the degradation of the natural environment. Carbon emissions from agriculture alone have risen 13% in 10 years from 2001 to 2011 alone, to 5.3 billion tonnes (Tubiello et al., 2014). FAO estimates that by 2050, agricultural carbon dioxide emissions will increase by a further 50% under a business-as-usual scenario. Depending on emissions model used, global mean surface

temperature is predicted to increase by between 1.8 (IPCC SRES B1, lowest emissions scenario) and 4.0 °C (IPCC SRES A1) by 2100 (Schmidhuber and Tubiello, 2007). However, specific changes will not be ubiquitous worldwide – higher temperature increases are expected over land than sea (Wheeler and Braun, 2013). GCC presents a multitude of challenges for farming and food production in the 21st century. While a small degree of warming may benefit crop yields in temperate regions, the increased frequency of extreme weather events such as floods and drought pose threats to the stability of global food supplies (Schmidhuber and Tubiello, 2007). Although there may be a slight fertilisation effect from increased levels of carbon dioxide present in the atmosphere leading the improved crop yields, it is likely that such impacts have been overestimated (Wheeler and Braun, 2013). There may be opportunities to grow alternative crops which would not survive within these regions at present, but environmental conditions may become optimal for invasions of pest populations (Schmidhuber and Tubiello, 2007). Intensive agriculture has also contributed to widespread physical environmental degradation. Approximately 20% of global land area is cultivated (Follett, 2001), and conversion to agricultural land contributes to biodiversity and habitat loss for native plants and animals. Soil, the foundation of the environment, is at high risk of degradation and loss due to current agricultural practices.

1.2 Soil structure and degradation

Well-structured soils provide a wide range of invaluable ecosystem services such as flood mitigation and prevention, maintenance of floral and faunal biodiversity and as

sinks for atmospheric carbon in the form of organic matter (Defra, 2009). Soil structure may be defined as the arrangement, size and shape of soil aggregates and particles within soil layers (Bronick and Lal, 2005). Soil aggregate stability is an important influence on soil structure and depends on a variety of abiotic and biotic factors. For example, soil biological activity encourages aggregation: fungal hyphae enmesh soil particles together, and chemical exudates from plant roots aid in the binding of soil particles (Bronick and Lal, 2005). Climate also affects stability of aggregates through seasonal variations in freezing-thawing or drying-rewetting cycles, or by stimulating seasonal fluctuations in soil microbial activity (Annabi et al., 2011).

Soil structure is also influenced by the ratio of sand, silt or clay particles (soil texture), which in turn impacts soil water and nutrient holding capacities that sustain plants and animals in natural and agricultural ecosystems. Sandy soils have lower holding capacities due to large particle sizes and large pores: they are susceptible to nutrient leaching or soil drying (Plaster, 2014) and appropriate management strategies are required to alleviate these issues in agricultural settings. Clayey soils with smaller particle sizes hold more water in networks of smaller pores, but are prone to compaction, reducing soil aeration and ability to hold and transmit water and other resources to support plants and soil organisms (Plaster, 2014).

Cultivation of soils and subsequent poor agricultural management can disrupt soil structure and provision of ecosystem services. For example, ploughing of soil exposes stored carbon to oxidation, degrading organic matter, thus releasing greenhouse gases and reducing water and nutrient holding capacities of the soil (Follett, 2001).

Poor nutrient management can also have far-reaching consequences off-farm. Not only are mineral fertilisers costly to produce in terms of carbon (0.82 kg CO_2 equivalent per kg N produced – Follett, 2001), but inappropriate application can lead to leaching or run-off, resulting in pollution of groundwater, waterways and surrounding land (Graves et al., 2015).

Gregory et al., (2015) identified nine threats to UK soil systems (Table 1-1) which could impair the ability of soil to provide ecosystem services. Many of these issues relate to soil structure. For example, irrigated systems are vulnerable to compaction and erosion: continuous impact of water on the soil surface can compress air between soil pores, resulting in sudden pressure release and destabilising of aggregates (Rickson et al., 2015). Solidification of the remaining soil particles can lead to surface capping and increase the risk of surface runoff and erosion. Heavy machinery such as tractors and harvesters also exert physical pressure upon the soil, compacting it and disturbing soil structure (Batey, 2009). Livestock grazing may damage soil structure, as animals trample on the soil surface (Hamza and Anderson, 2005), while cultivation can decrease soil organic carbon storage. Therefore, it is important to select appropriate management strategies to maintain optimal agricultural soil structure to avoid environmental and economic costs, both on- and off-site.

Table 1-1: Nine threats to UK soils identified by Gregory et al. (2015).

Threat to soil	Description
Erosion	Loss of soil as a result of human activity beyond rates of natural soil formation
Compaction	Deformation of soil structure leading to increased soil density and reduced porosity
Sealing	Covering of soil by buildings or other development, that are slowly permeable to water
Contamination	Presence of a substance in the soil from anthropogenic sources
Salinisation	Increase in water-soluble salts by both natural processes and human activity
Brownfield development	Development of land previous used for other commercial or industrial purposes
Decline in organic matter	Accelerated decomposition of soil organic matter, overtaking rates of build-up
Landslides	Movement of soil, rock or debris down a slope
GCC	Long-term shift in global weather patterns and temperatures affects soil stability and habitat

1.3 Soil compaction

Soil compaction is a widespread form of soil structural degradation. Across Europe, between 32 and 36% of soils are highly susceptible to compaction due to factors

including soil texture and water content, organic matter content and land use (Jones *et* al., 2010), while 33 million hectares (4% of land area) is already affected by soil compaction to some degree (Soane and van Ouwerkerk, 1995). In England and Wales alone, 42% of agricultural land area is susceptible to compaction, leading to annual costs of £472 million for both on and off-site remediation (Graves et al., 2015).

Compaction occurs as pressure exerted on the soil surface reduces pore spaces between soil aggregates, limiting the space available for plant roots to grow unimpeded by increasing soil strength and resistance. Plant growth slows as roots must move or break the soil aggregates to obtain water and nutrients, and potential for gas exchange between the soil and atmosphere decreases (Stirzaker et al., 1996; Hakansson, 2005; Batey, 2009).

The breakdown of pore networks and aggregation changes both soil structure and its relationship between soil matric potential (Ψ_m) and bulk soil water content. This soil moisture release characteristic describes the potential energy required for water to be extracted from the soil matrix, for a given soil water content. Smaller soil pores in a compact soil hold water more tightly, producing a more negative matric potential at the same soil water content (Figure 1-2). Even if compact soil is relatively wet, plants may find it more difficult to extract water from the soil matrix.

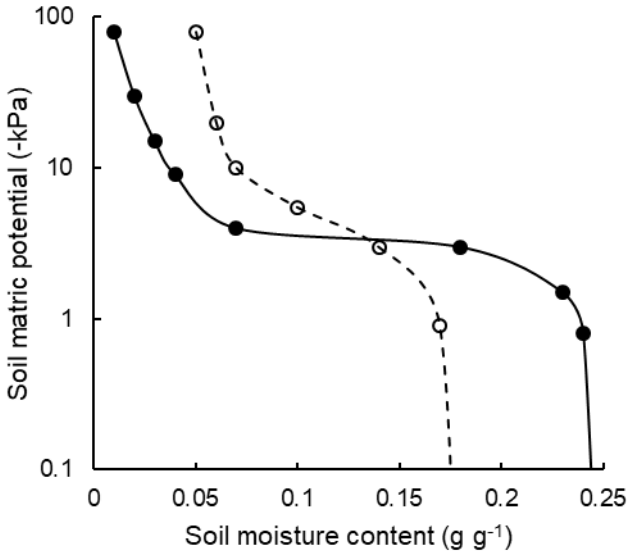


Figure 1-2: Soil moisture release characteristic for a soil packed at low (solid circles) and high (hollow circles) dry bulk densities. At lower moisture contents, matric potential of compact soil is more negative, requiring more energy to extract water from the soil matrix (redrawn from Gupta et al., 1989).

Poor soil aeration can also inhibit plant growth via multiple mechanisms. Lack of soil oxygen disrupts proper root function, affecting the production of chemicals and hormones associated with root growth (Hakansson, 2005). Decreased gas exchange within the soil also impairs microbial communities which may work symbiotically with plants, thereby potentially limiting their ability to acquire necessary resources (Hakansson, 2005). Taken together, soil compaction creates a multi-stress environment for both plants and fauna residing in the soil by reducing availability of crucial resources for growth.

As many growers look to switch to conservation tillage methods such as no or reduced tillage to reduce inputs and mitigate the threats listed in Table 1-1, bulk density and penetration resistance of the upper layers may increase over several growing seasons (Soane et al., 2012; Martínez et al., 2016). While conservation

tillage offers a variety of economic and ecosystem benefits including reduced energy inputs and improved SOC storage, further work is required understand its impacts on soil quality and consequent effects on crop yields, as results may vary depending on local climatic conditions (Soane et al., 2012).

1.4 Plant growth responses to soil compaction

Tillage techniques have evolved over thousands of years to prepare the soil before sowing seeds, aiming to loosen upper layers of soil (reducing bulk density), remove crop residues and incorporate fertilisers (Daigh and DeJong, 2018). Tillage may aid seedling establishment by protecting seeds from adverse weather or predation, ensure appropriate sowing depth and root penetration of soil (FAO, 2003). However, there is evidence that with increasing mechanisation of agriculture, tillage is leading to compaction of subsoil layers (Hamza and Anderson, 2005; Knight et al., 2012).

Compaction exposes plants to a range of soil stresses including anoxia, water and nutrient deficiencies and increased soil strength. Plants grown in compact soil are often stunted, with slow emergence and diminished shoot/leaf elongation, but a wide range of responses reported in the literature indicates that growth reduction is often specific to soil type and plant species. High bulk density soil (1.7 g cm^{-3}) reduced barley leaf area by 24-30% compared to 1.1 g cm^{-3} soil (Mulholland, Black, et al., 1996). Increasing bulk density from 1.12 to 1.42 g cm^{-3} decreased mature leaf area of wheat by 35%, by decreasing both cellular length and width (Beemster and Masle, 1996). When tobacco was grown in a range of soil bulk densities, growth responses were highly dependent upon the co-occurrence of particular stresses:

when not exposed to water deficit, tobacco growth increased with bulk density until 1.4 g cm^{-3} (Alameda et al., 2012). However, mechanical stress and water deficit in combination resulted in growth declining linearly with increasing bulk density. Clearly, shoot responses to soil compaction are highly dependent on stress combinations, with distinct inter and intra-species variation.

Root growth is also restricted by soil compaction. Roots may become concentrated in the upper layers of the soil, growing horizontally as porosity decreases at depth (Lipiec et al., 1991). For a variety of plant species grown in compact soil, a significant positive correlation ($r = 0.78$) between root diameter and root length suggests that root thickening facilitates soil exploration (Materechera et al., 1991). The shape of the root tip (the ratio between radius and length) influences root elongation in strong soil, with smaller ratios improving elongation (Colombi et al., 2017). Cotton increased its ability to penetrate soil in response to soil compaction, when mechanical stress and anaerobic conditions were present (Iijima et al., 2007). Understanding growth responses of both the root and shoot to soil compaction will help select genotypes and traits better suited to growing in compact soils (Colombi et al., 2018).

Increasing soil strength is one of the primary factors constraining plant growth in compact soil and may occur before the onset of other abiotic stresses such as drought (Lipiec et al., 1991; Bengough et al., 2011; Valentine et al., 2012). Penetrometer pressures of 0.5-1.0 MPa are often experienced by plants in soil and can significantly reduce growth rates. Depending on species, root elongation may cease at penetrometer pressures between 0.8-5 MPa (Bengough and Mullins,

1990a). In perennial ryegrass (*Lolium perenne* L.), increasing soil strength from 0.25 MPa to 2.30 MPa reduced total root length by 75% and delayed formation of nodal roots, while reducing mature leaf area by 35%, and area of younger leaves by approximately 62% (Cook et al., 1996). Taken together, the co-ordinated reduction in above and below-ground growth in response to stresses present in the rootzone suggests a form of root-to-shoot communication of soil conditions.

While water and nutrient limitation may explain plant growth responses to soil compaction, growth can be inhibited even when these resources are non-limiting, indicating a specific soil strength response. Leaf area of young wheat seedlings growing in compact soil was reduced by 66%, even when soil water availability and aeration were not limiting (Masle and Passioura, 1987). Decreased growth rates of sunflower leaves also occurred without changes in leaf water status (Andrade et al., 1993). Many workers have manipulated soil strength using columns of incompressible sand loaded with weights (or a foam block as a control) upon the surface, ensuring nutrients or water are not limiting by standing tubes in tanks of nutrient solution, while producing soil strengths comparable to or exceeding field conditions. In this system, the growth of wheat, rice, carrot and onion was reduced (Whalley et al., 1999; Clark et al., 2002; Whalley et al., 2006). Thus, there is substantial evidence for the action of a feed-forward chemical signal originating from the site of stress, the roots, regulating shoot growth.

1.5 Plant signalling of soil conditions

Plants are sessile organisms and must avoid, adapt to or tolerate a range of abiotic and biotic stresses to survive. Plants utilise a range of endogenous signals to alter growth and metabolism in response to suboptimal conditions.

Changes in availability of resources, such as water or nutrients, may act as signals of stress and alter growth rate. Plants are a key component of the pathway of water movement from soil to the atmosphere (soil-plant-atmosphere continuum), and gradients of water potential throughout the continuum drive transpiration (Christmann et al., 2013). Hydraulic signalling communicates changes in water potential gradients throughout the plant. While the molecular mechanisms remain unclear, plants are known to sense changes in tissue turgor or solute concentration, or changes in mechanical forces exerted upon cell walls and plasma membranes due to varying cell volume (Christmann et al., 2013). Plants regulate their water status by controlling water uptake, transport and release by adjusting tissue water potentials, hydraulic conductance and stomatal aperture. These hydraulic changes may also trigger biosynthesis of chemical signals to elicit stress responses in plants.

Like animals, plants produce a range of organic substances to alter physiological processes – hormones. However, unlike animal hormones, synthesis of phytohormones is not necessarily localised within a certain tissue, cell or organelle, and phytohormones can act in the tissues in which they are synthesised (Davies, 2010). Furthermore, phytohormones may be transported from the site of synthesis to act upon developmental or physiological processes in distant tissues, such as in the communication of rootzone stresses to the shoot. Increasingly, a range of

phytohormones are recognised as being involved in many developmental and regulatory processes in plants.

1.6 Abscisic acid

One of the most well-studied plant hormones is abscisic acid (ABA), derived from carotenoid pigments. ABA has been implicated in a wide range of abiotic stress responses, notably water deficit, temperature and salinity. ABA concentrations in tissues are regulated by rates of biosynthesis and catabolism and transport between roots and shoots (Jiang and Hartung, 2008).

ABA is synthesised from the isoprenoid precursors via the methylerythritol phosphate (MEP) pathway in the chloroplast or mevalonic acid (MVA) pathway in the cytosol (Schwartz and Zeevaart, 2010). Condensation reactions lead to the production of phytoene, the first step towards carotenoid production. 9-*cis*-epoxy-carotenoid dioxygenases (NCEDs) play a crucial role in biosynthesis, cleaving carotenoid 9-*cis*-neoxanthin to form xanthoxin, which moves out of the chloroplast and is converted to ABA in the cytoplasm.

Expression levels of NCED mRNA and proteins in leaves increase quickly in response to tissue dehydration (within 0.5 hours; Figure 1-3), and after a slight lag ABA accumulates over 8 hours post-dehydration (Qin and Zeevaart, 1999). Similar responses were found in roots, with lower absolute ABA concentrations as the pool of carotenoids present in root cells is smaller.

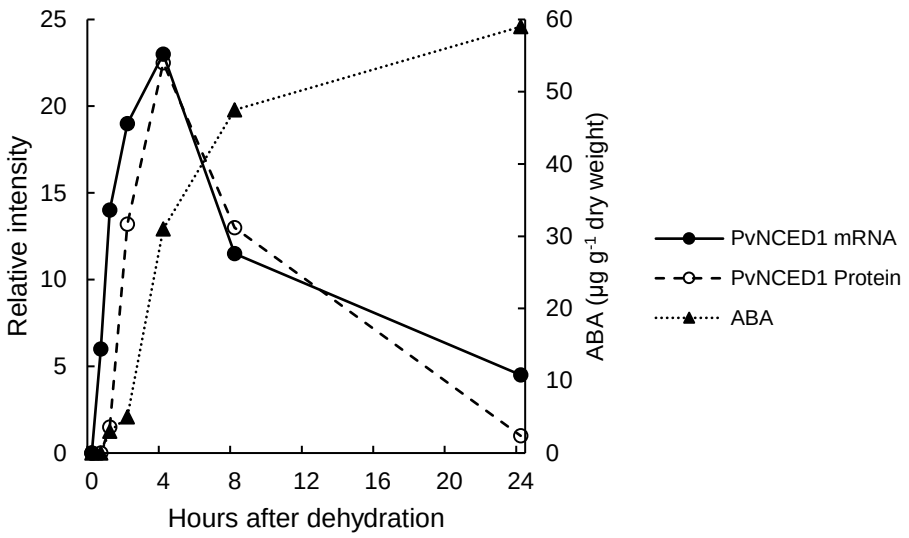


Figure 1-3: Dehydration elicits rapid increase in NCED1 mRNA and protein levels in *Phaseolus vulgaris* leaves. After a slight lag, ABA concentrations begin to rise and continue to increase 24 hours after onset of stress, while enzyme levels return close to control levels (Redrawn from Qin and Zeevaart, 1999).

Plants regulate leaf gas exchange with the atmosphere by modifying stomatal aperture. Stomatal closure may be triggered by decreasing guard cell turgidity or promoted by ABA synthesised in response abiotic stress conditions. The classical model of ABA signalling under drought conditions begins with loss of turgor in roots exposed to drying soil, triggering ABA biosynthesis (Zhang et al., 1987). ABA is then transported through the vascular system to aerial portions of the plant, inducing stomatal closure in the leaves (Wilkinson and Davies, 2002). Stomatal conductance and leaf expansion decreased in drying soil, even when shoot water status was maintained on the verge of full turgor by root pressurisation (Gollan et al., 1986), indicating the action of a root-sourced signal.

More recent work offers an alternative model of ABA signalling (McAdam, Manzi, et al., 2016; Lacombe and Achard, 2016). Biosynthesis of ABA may begin rapidly at the site of action, the leaf, in response to tissue dehydration (Qin and Zeevaart, 1999).

High atmospheric vapour pressure deficit (VPD) stimulated the entire ABA biosynthetic pathway in guard cells of wild-type (WT) Arabidopsis plants. Furthermore, restoration of ABA biosynthesis in the guard cells of ABA-deficient *aba3-1 Arabidopsis* mutants, prevented wilting in response to high VPD (Bauer et al., 2013). Reciprocal grafting experiments of WT and ABA-deficient plants demonstrated that shoot-derived ABA restores ABA status of ABA-deficient rootstocks similar to wild-type self-grafts in both *Solanum lycopersicum* (tomato) and *Pisum sativum* (pea), highlighting the basipetal movement of foliar-derived ABA, which was confirmed using deuterium-labelled ABA (McAdam, Brodribb, et al., 2016). Foliar-derived ABA from WT scions promoted root biomass of ABA-deficient rootstocks, with similar root:shoot ratios as WT self-grafts. However, plants were not subjected to abiotic stresses in this experiment and the relative contributions of root or shoot derived ABA in response to adverse environmental conditions was not tested. The relative importance of leaf *versus* root-synthesised ABA remains a hot topic of debate, but clearly ABA has a critical role in the regulation of stomatal aperture in response to abiotic stresses (Assmann, 2010).

1.7 ABA and other signalling candidates of soil compaction

Since multiple stresses can occur in compact soil, it is not surprising that ABA has been implicated in compaction stress signalling. When wild-type and ABA-deficient barley were grown in soils of a range of bulk densities, foliar [ABA] rose as bulk density increased, and xylem sap ABA concentrations increased significantly under compacted soils in the first 6 days after initial seedling emergence (Mulholland,

Black, et al., 1996). Compacting soil (to a strength 50% greater than controls) also increased xylem ABA concentration of maize 8-10-fold relative to plants grown under control conditions, but this effect diminished with time (Hartung et al., 1994). However, whether ABA was produced solely in response to mechanical resistance is unclear, as reductions in leaf water status (stomatal conductance, water potential and turgor) were observed in these studies, indicating limiting soil water status. In contrast, no changes in root tissue [ABA] were observed in maize roots subjected to mechanical impedance, when water was not limiting (Moss et al., 1988).

Instead, ABA may play a role in maintaining plant growth in compacted soils, as opposed to inhibiting leaf gas exchange. When 100 nM ABA was supplied to WT and ABA-deficient barley (*Az34*) plants growing in compact (1.6 g cm^{-3}) soil, leaf expansion rates of *Az34* were restored to a similar level as WT barley receiving the same treatment. In contrast, this concentration of ABA had little effect on the expansion rates of wild-type leaves (Mulholland, Taylor, et al., 1996). Exogenous ABA applications to ABA-deficient *notabilis* tomatoes growing in a split-root system (half compact, half loose) rescued leaf expansion similar to plants growing in uniformly loose soil (Hussain et al., 2000). Foliar ABA concentrations of *notabilis* were increased similarly to WT plants growing in split pot or uniformly compact soil. By 16 days after emergence, stomatal conductance had halved in *notabilis*, to levels similar to WT plants. Therefore, it is possible that ABA maintains leaf expansion by regulating stomatal aperture under compaction stress conditions, improving water use efficiency and assimilation.

However, ABA may only be able to maintain leaf expansion in compact soil until a critical point. ABA application to WT plants growing in split pot conditions (as previously described) also partly restored shoot growth, but not to the same extent as in *notabilis*, potentially as stomatal conductance was already lower in WT plants (Hussain et al., 2000). When grown at 1.7 g cm^{-3}, both WT and *Az34* barley exhibited 40-62% smaller leaves compared to control plants grown at 1.4 g cm^{-3} (Mulholland, Black, et al., 1996). However, WT barley grown at 1.6 g cm^{-3} were only 6% smaller than controls, while ABA-deficient *Az34* plants were 22% smaller, suggesting that ABA accumulation maintains leaf expansion at intermediate soil densities. The critical bulk density at which responses to ABA become overridden is likely to vary, as root penetration ability differs between species, and the relationship between soil bulk density and strength depend on specific soil characteristics.

Furthermore, transient increases in xylem sap [ABA] have been shown by several workers (Hartung et al., 1994; Mulholland, Black, et al., 1996; Hussain et al., 2000), and not present when measured later in the growing season, despite reductions in growth and yields (Whalley et al., 2006). Taken together, while ABA may have some role in root-to-shoot communication of compact soil conditions, no consistent response has been shown across experiments or throughout the growing period. It is likely that other signals are also involved in regulating plant growth in compact soil, especially when soil water availability is not limiting.

Ethylene is a gaseous hormone synthesised in all higher plants from its precursor methionine, and the intermediate 1-aminocyclopropane-1-carboxylic acid (ACC) may be transported both acro- and basipetally to act in distant tissues from the site of

synthesis (Pech et al., 2010). Ethylene regulates many growth and developmental processes in plants, including germination, root hair development, abscission and fruit ripening (Nehring and Ecker, 2010).

With roles in plant responses to abiotic stresses (hypoxia (Else and Jackson, 1998) and drought (Sobeih et al., 2004)) and wound responses (O'Donnell et al., 1996), ethylene seems to be an important candidate for soil compaction signalling. Production of the gaseous hormone ethylene increased when roots met a mechanical barrier, and developmental responses to strong substrates are similar to ethylene treatment, including root swelling and thickening, and reduced root and leaf elongation (Masle, 2002). Ethylene evolution from maize roots was stimulated when external pressure was applied to the whole plant, also causing foliar ACC accumulation (Sarquis et al., 1991). However, this response has not been consistently observed in response to high strength: application of ethylene inhibitors 2,5-norbornadiene or aminoethoxyvinylglycine reduced endogenous ethylene to below control levels in maize growing with impeded roots, but did not rescue root growth (Moss et al., 1988). Furthermore, increased root ethylene production lagged 22-24h behind changes in root morphology when maize plants were grown in strong substrate, suggesting that ethylene is not the primary regulator of root growth in strong or compact soils. Ethylene evolution from the roots of Eucalyptus seedlings decreased 6-fold when grown in compact soil, but root elongation was reduced by 44% by compaction (Benigno et al., 2012). The biosynthesis and metabolism of ethylene in response to compact and/or strong soils may depend on system or type

of mechanical resistance, including impacts on duration, severity, and exposure of particular parts of the rootzone (Masle, 2002).

Further evidence of a complex role for ethylene in regulating leaf expansion in compact soils comes from work by Hussain et al. (2000, 1999). When WT and $ACO1_{AS}$ (with low stress-ethylene synthesis) tomatoes were grown in vertical split-pots of low and high bulk density soil, growth of only WT plants was inhibited. Foliar ethylene production and xylem [ABA] were negatively correlated, suggesting that antagonism between the two phytohormones regulates leaf expansion in heterogeneously structured soil. However, similar reductions in leaf expansion were observed in the two genotypes when grown in uniformly- compact soil, despite up to 5-fold increase in foliar ethylene evolution in WT (Hussain et al., 2000). Thus, like ABA, it is possible that ethylene may regulate plant growth in soil of "sub-critical" bulk density, above which other signals take precedence.

Taken together, the inconsistent results from ABA and ethylene-centric studies (Figure 1-4) paint a complex picture of root-to-shoot signalling of compaction stress. It is possible that changes in soil structure due to compaction, or using artificial growth media (e.g. ballotini) essentially render many studies incomparable, as workers are unintentionally varying multiple stresses simultaneously, and to different degrees. On the other hand, plant growth is stunted in compact soils regardless of whether changes in ABA or ethylene are observed, perhaps indicating roles for other phytohormonal signals. Studies of alternative signalling candidates would help to elucidate soil compaction signalling further, as it is likely that ABA or ethylene are not the only signals involved.

It seems necessary to establish experimental systems in compact soil, where exact methodologies are recorded such that they may be repeated. However, isolating soil strength from soil compaction to study whole plant physiological responses are necessary in order to better our understanding of soil compaction signalling, as there is already much literature on responses to other soil compaction components (water deficit, anoxia). Soil strength is a vital component of not only soil compaction, but also soil drying (Whalley et al., 2005; Valentine et al., 2012), but since it is difficult to manipulate in the field without altering other soil conditions, it is rarely studied in isolation. Better understanding of physiological responses to soil strength are vital to better understand how plants respond to soil compaction stress.

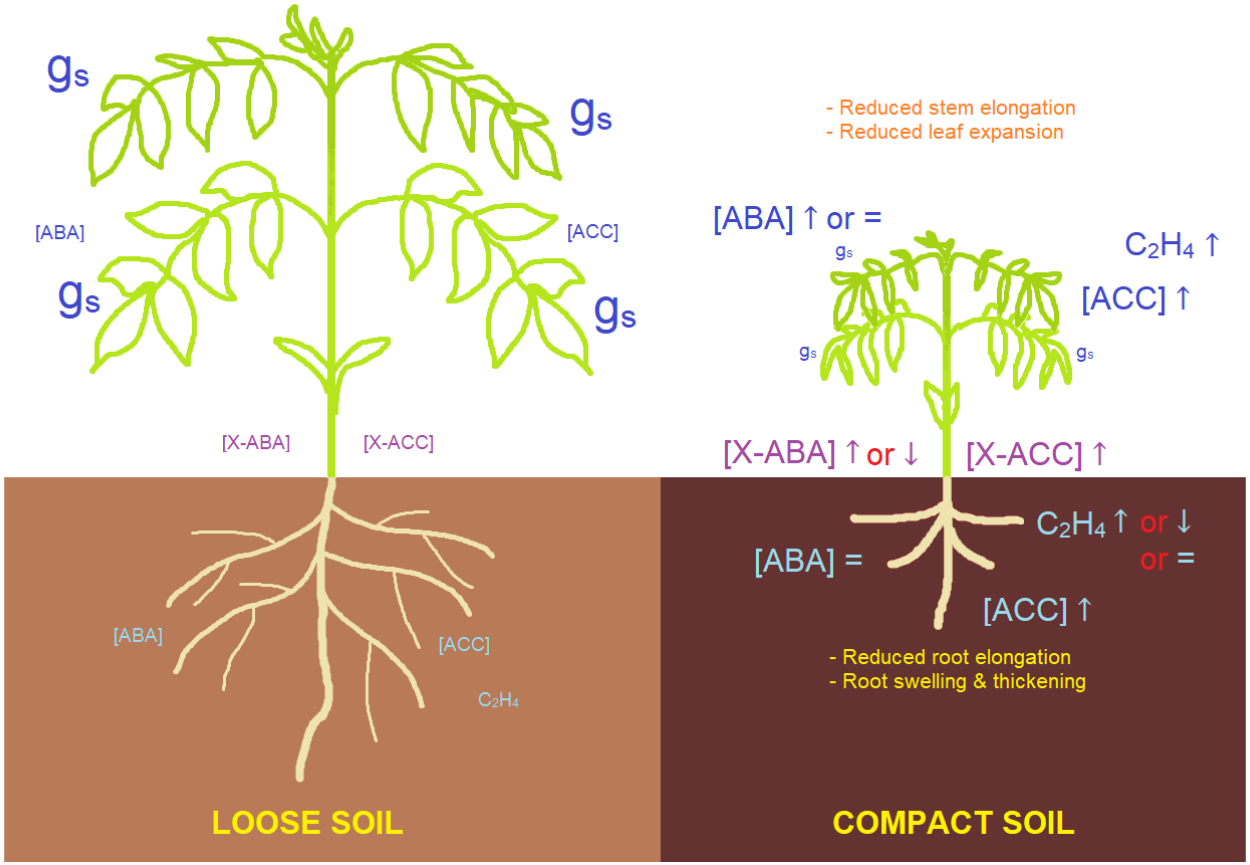


Figure 1-4: Summary of growth and physiological responses of plants to compact soil; much information about phytohormonal responses reported in the literature are conflicting or inconsistent, as discussed in Section 1.7.

1.8 Thesis structure

This thesis aimed to investigate the effects of soil compaction on growth and physiology of *S. lycopersicum* cv. Ailsa Craig. Tomato is an appropriate model species due to its high economic importance worldwide and availability of a wide range of genetic material and phytohormonal mutants for future research. Early experiments also showed increased sensitivity of shoot growth to increased soil bulk density relative to another model species, *Helianthus annuus* (sunflower; Figure 1-5). Sunflower leaf area was reduced by 26% in high bulk density soil, while tomato total leaf area was reduced by 78%.

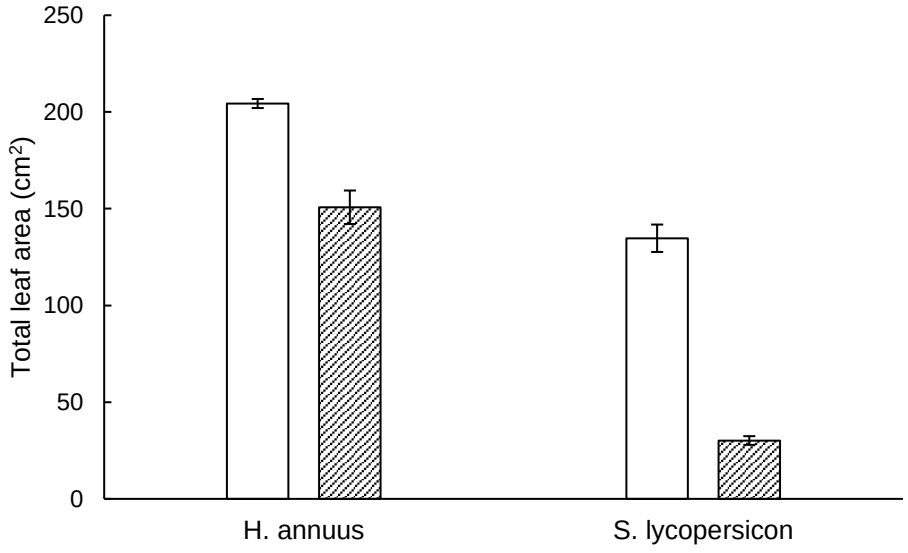


Figure 1-5: Total leaf area of *Helianthus annuus* (sunflower) and *Solanum lycopersicum* (tomato) grown under low (white bars) and high (shaded bars) bulk density conditions. Bars are means ± S.E.

In Chapter 2, *S. lycopersicum* was grown at a range of bulk densities of a sandy loam soil to establish growth responses. Subsequently, low (1.4 g cm^{-3}) and high (1.7 g cm^{-3}) bulk densities were selected, and plants were exposed to a gradual drying cycle to assess physiological responses to a multi-stress environment. For the

first time, root xylem sap was collected from plants grown in compacted soil at transpirational flow rates (unlike the literature reviewed above), and multi-hormonal analyses were conducted on sap and foliar tissues to establish phytohormonal profiles of plants under well-watered and water-stressed conditions, and further elucidate the responses previously discussed.

Since plants grown in high bulk density soil under well-watered conditions had lower levels of bioactive gibberellins (GAs) in expanding leaves, Chapter 3 applied GA_3 to plants as a soil drench (the site of stress) to determine whether it could improve shoot growth. While leaf expansion was rescued, GA_3 application affected plant water and phytohormonal status in both low and high bulk density soils, notably increasing levels of xylem and foliar cytokinins (CKs), and decreasing xylem jasmonic acid (JA) concentrations.

To isolate mechanical resistance stress imposed by strong soils from possible water or nutrient limitations, Chapter 4 grew plants in a sand culture system (as described in Coelho Filho et al., 2013; Jin et al., 2015) to determine whether soil compaction and GA application responses were consistent.

Chapter 2 Growth and physiological responses of tomato (*Solanum lycopersicum*) to compaction of a sandy loam soil

2.1 Introduction

The negative impacts of soil compaction on shoot growth are well known. Compacted soil decreased wheat leaf expansion rates (Masle and Passioura, 1987) and sunflower maximum leaf size (Andrade et al., 1993) without changes in leaf water potential, suggesting the action of a phytohormonal or chemical signal regulating plant growth in compacted soil, before any water deficit becomes limiting. The phytohormone abscisic acid (ABA) is best known as a regulator of stomatal aperture in response to changes in soil water status and has also been implicated in regulating growth under a range of abiotic stress responses, including soil compaction. Xylem sap ABA concentrations in barley grown in high bulk density soils increased significantly at early growth stages (6 days after emergence), but decreased to control levels by 18 days – stomatal conductance at 18 days remained low as foliar ABA concentrations increased (Mulholland, Black, et al., 1996). In contrast, maize grown on compacted soil while receiving irrigation did not exhibit reduced g_s or increased ABA until soil water reserves were almost depleted (Tardieu, Zhang, et al., 1992). Similarly, Whalley *et al*. (2006) observed reduced stomatal conductance in field-grown wheat in compact soil, but without changes in leaf ABA concentration. Inconsistent responses in [ABA] to soil compaction suggests that ABA is not the primary signal regulating plant growth in compact soil.

Soil penetration resistance increases with increasing bulk density (Colombi et al., 2018). However, soil compaction changes the soil water release characteristic (See Figure 1-2), and may alter the relationship between soil moisture content and plant ABA biosynthesis (Dodd et al., 2010), increasing ABA biosynthesis at higher bulk soil water contents. Additionally, roots may become restricted to the upper layers of soil, reducing access to water stored in the bulk soil profile, thus reducing plant water status (Tardieu, Bruckler, et al., 1992; Grzesiak et al., 2013). Thus, it is unclear if ABA is produced in response to increased mechanical impedance and/or decreases in response to local root (rather than bulk soil) water availability. Better understanding the relationship between soil water content and endogenous ABA levels in plants growing in differentially-structured (compact vs. uncompact) soils would improve models of crop growth in response to soil physical conditions. Root water potential (Ψ_{root}), a measure of root water status, is a parameter generally inaccessible in the field, but its measurement potentially offers greater understanding of rootzone water status, particularly under heterogeneous soil conditions, as bulk Ψ_{root} encompasses the conditions of the entire rootzone (Whitmore and Whalley, 2009; Dodd et al., 2010).

However, ABA may not be the primary signal communicating soil compaction stress from the rootzone to the growing shoot. Indeed, root ABA biosynthesis requires sufficient oxygen availability (Milborrow, 2001), which should be lower in compacted (than well-drained) soils due to loss of pore space. Thus several workers have explored the role of ethylene (a well-known signal of hypoxia) as a signal of soil compaction. When different tomato genotypes with different ethylene and ABA

biosynthesis were grown in soils of varying compaction stress, it was suggested that an antagonistic relationship between ABA and ethylene regulated shoot growth (Hussain et al., 1999; 2000), as reviewed in Chapter 1 (see p. 32). While there is some evidence for a role of ethylene in compaction stress signalling, there may be other significant phytohormonal signals regulating plant growth in compact soil. Many workers investigating abiotic stress responses often consider the roles of one or two hormones in isolation. In recent years, major developments in analytical methods using liquid or gas chromatography in combination with mass spectrometry now allow multiple phytohormones to be detected in a single sample (Šimura et al., 2018). Multi-hormone analyses therefore allow the functions of and interactions between many hormones to be investigated simultaneously, and have been employed for study of abiotic stress responses such as salinity (Albacete et al., 2008), but have yet to be used for soil compaction stress.

This chapter aims to determine the shoot growth response of tomato (*Solanum lycopersicum* cv. Ailsa Craig) to increasing bulk density and investigate the physiological responses of tomato to combined compaction and soil drying stress. The relationship between soil water content and physiological responses to compaction in a sandy loam soil is investigated. In Experiment 2.1, tomato plants were grown at a range of bulk densities to determine leaf expansion rate and final biomass after 3 weeks. It was hypothesised that as soil bulk density of the Norfolk

sandy loam soil used here increased, growth rates and final biomass of tomato plants would decrease.

The relationships between bulk soil water content, soil bulk density, plant water status and phytohormone profiles were investigated in Experiment 2.2. Tomatoes were grown at contrasting bulk densities under well-watered conditions, then water was withheld. At harvest, root xylem sap was collected at transpirational flow rates and leaf tissues collected from expanded and expanding leaves for multi-hormonal analyses according to Albacete et al. (2008); this study is the first to do so for soil compaction signalling. Furthermore, the use of the Scholander Pressure Chamber to pressurise whole root systems allows measurement of root water potential (Ψ_{root}), which is generally inaccessible in the field.

2.2 Materials and Methods

Method development

Sandy loam topsoil was purchased from Bailey's of Norfolk (Hevingham, Norfolk, U.K.). Textural and nutrient analyses were conducted (Table 2-1). This topsoil is low in available ammonium, magnesium and potassium (ADHB, 2017), which is easily leached in soils of high sand content.

Table 2-1: Physical and chemical properties of the sandy loam topsoil.

Parameter	Method	Values
Organic matter content	Loss on Ignition	2.35 % ± 0.01
Soil texture (< 2 mm)	Sedimentation test	Sand: 71% Silt: 26% Clay: 3%
Phosphorus	Olsen P (Olsen, 1954)	27.68 ppm ± 0.19
Plant available nitrogen	2M KCl extraction + Autoanalyzer	**NO_3:** 17.02 ppm ± 0.17 **NH_4:** 1.04 ppm ± 0.04
Cations	Ammonium acetate extraction, Atomic absorption spectrophotometry (Mg, Ca) Flame photometry (K)	**Mg:** 35.03 ppm ± 2.72 **Ca:** 2414 ppm ± 46 **K:** 75.50 ppm ± 0.93

A 3-tonne capacity arbor press (PK3000, Jack Sealey Ltd., Bury St. Edmunds, U.K.) was modified by fitting a metal disc (diameter: 6.2 cm) to the base of the piston in order to exert pressure onto the soil surface (Figure 2-1). A torsion wrench (0 – 70 N m) was attached to a nut on the handle to vary and control the force used. The

consistency of force was confirmed by placing a 10 kN load cell beneath the piston (Figure 2-2), which showed that categorical levels of force could be achieved.

Figure 2-1: 3-tonne Arbor press modified to allow a torsion wrench to fit near the handle. A metal disc of appropriate diameter is attached to the piston to allow force to be exerted on surface of soil.

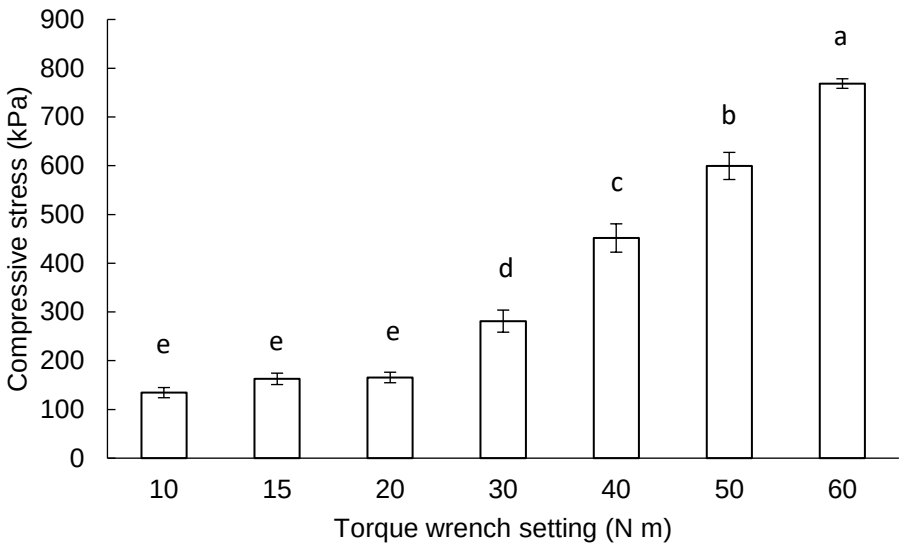


Figure 2-2: Compressive stress exerted by arbor press at different settings of the torsion wrench, when operated by the author. Bars are means ± SE of 10 replicates; data analysed by one-way ANOVA, with significant ($p < 0.05$) differences between levels indicated by different letters according to post-hoc Tukey's HSD.

Experiment 2.1: Tomato growth response to increasing soil bulk density

Plant growth conditions

Sandy loam soil (Bailey's of Norfolk, Hevingham, Norfolk, U.K.), sieved to 10 mm, was wetted to drained capacity (approximately 0.20 g g^{-1} gravimetric soil water content (GSWC)) in 10 L pots, before being air-dried to a range of SWCs: approximately 0.01, 0.05 and 0.165 g g^{-1}. Black PVC pots (diameter 6.4 cm, height ~24 cm, volume 0.77 L) were used as they were designed to fit exactly within a tall Scholander Pressure Chamber (Soil Moisture Equipment Corp., Santa Barbara, CA, USA). Pots were filled in 3 cm layers, and each was compacted using the arbor press set at a particular torsion wrench setting (See Table 2-2 for combinations of soil water content and torsion wrench setting). The surface of the compacted soil was scarified before the next layer was added to reduce boundary effects. The upper surface of the filled pots

were covered in aluminium foil to reduce evaporation, and pots were then placed in trays of tap water, immersing the bottom 3 cm, and allowed to re-wet to drainage capacity by capillary action (48 hours). Pots of highest initial water content (0.165 g g^{-1}) did not absorb much water during this time period and were instead rewatered to their initial weight.

Table 2-2: Range of soil bulk density levels produced under different combinations of soil water content and torsion wrench settings. Bulk density data are means ± SE of 6 replicates, letters denote mean discrimination (post-hoc LSD $p < 0.05$)

Treatment	**Soil water content at filling (g g^{-1})**	**Torsion wrench setting (N m)**	**Soil bulk density (g cm^{-3})**
1	0.01	10	1.40 ± 0.01^{d}
2	0.01	70	1.51 ± 0.01^{c}
3	0.05	40	1.63 ± 0.01^{b}
4	0.165	10	1.78 ± 0.01^{a}

Tomato (*Solanum lycopersicum* Mill. cv. Ailsa Craig) seeds were surface-sterilised for 5 minutes in 10% sodium hypochlorite solution and left to germinate in sealed petri dishes on filter paper soaked in deionised water. Petri dishes were left in the dark at 21°C until most radicles were at least 2-3 mm long (~ 72 hours). Holes (3, each 2 cm deep) were made at the soil surface of each pot using a small stick, and a single germinated seed was carefully placed in each hole. The holes were then covered with a small amount of soil and moistened with tap water. Aluminium foil was replaced over the top of the pots until seedlings emerged. After 10 days of growth in controlled environment room (day temp. 24°C, night temp. 19°C, photoperiod 07:00 – 19:00), tomatoes were etiolated in the dark until stems were at least 5 cm from the

top of the pot and thinned to 1 individual per pot. Pots were weighed each day to calculate bulk soil water content and were kept well-watered by watering to drained capacity (Treatments 1 – 3) or 0.165 g g^{-1} (Treatment 4) using tap water. 14 days after transplanting (DAT), plants were watered with 50% Hoagland's Solution every 2-3 days. Cotyledons were removed at around 24 DAT for ease of root xylem sap sampling.

Measurements

Pots were weighed each day to estimate bulk SWC and water losses to evapotranspiration (ET). From Days 17 to 21, length and breadth of each leaf of each plant was measured with a ruler to estimate leaf area and leaf expansion rates.

Plants were harvested at approximately 28 DAT. Stomatal conductance (g_s: AP4 Porometer, Delta-T Devices, Cambridge, U.K.) and leaf water potential (Ψ_{leaf}: C-52 Thermocouple Psychrometer, Wescor) of the newest expanding leaf were measured, before the leaf was excised, frozen in liquid nitrogen and stored at -20°C for hormonal analysis.

Total leaf area was measured using a leaf area meter (Li-3100 Leaf Area Meter, Li-Cor inc., Lincoln, Nebraska, USA), fresh leaf and shoot weight recorded. Soil cores were removed from pots and stored at -20°C for three days before root sampling could begin. All tissues were dried at 80°C for at least 72 hours before being weighed to obtain biomass.

Statistical analyses

Data was analysed using one-way ANOVA for categorical data and significant differences between treatments were identified using post-hoc LSD ($p < 0.05$).

Experiment 2.2: Combined effects of soil compaction and water deficit on growth and physiology of tomato

Soil preparation and plant growth conditions

Treatments 1 and 4 (1.4 and 1.74 g cm^{-3}; Table 2-2) were selected to ensure contrasting bulk densities, significant differences in plant growth rates and final biomass. Soil was prepared and pots filled as detailed in Experiment 2.1. All pots were placed in trays of water and the lower portion immersed to allow re-wetting, but as before, the compact treatment exhibited poor infiltration and was instead top-watered to water content at filling (approximately 0.165 g g^{-1}). Thirty replicates of each soil treatment were prepared, to allow 10 well-watered (WW) and 20 droughted (D) plants.

Tomato plants were grown as detailed in Experiment 2.1. Water was withheld from 20 pots of each treatment from Day 19 (after transplanting) onwards, to generate well-watered (WW) and drought (D) treatments. Pot weight was recorded daily to monitor changes in bulk SWC and evapotranspiration (ET). From Day 21, 2 x WW and 3 x D plants grown in each soil treatment were harvested daily.

Plant measurements

Length and breadth of each leaf on each plant was measured daily and multiplied by a constant to estimate total leaf area (Figure 2-3).

$$Leaf\ area = 0.29\ (length * breadth) + 1.24$$

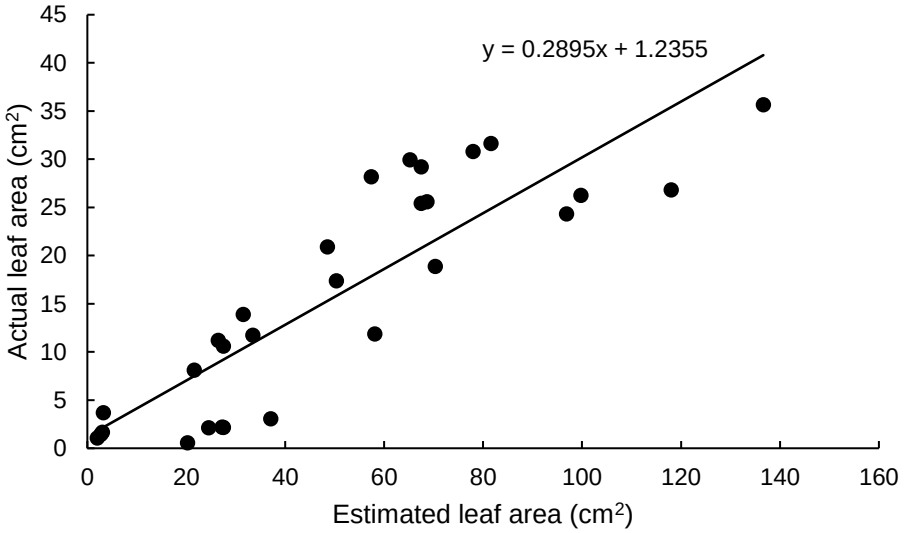


Figure 2-3: Relationship between leaf area estimated using length and breadth measurements against actual leaf area measured using Li-cor Leaf Area Meter. The equation y = 0.29x + 1.24 was applied to measurements. Each symbol is an individual leaf.

At harvest, measurements were carried out as in Experiment 2.1, and water status measurements were also carried out on expanding leaves. The distal leaflets from an expanding leaf were also excised, flash-frozen and stored at -20°C for phytohormonal analyses. The pots were weighed one hour prior and again at harvest. Sap flow rate due to transpiration was calculated using the equation:

$$Flow\ rate\ (mL\ min^{-1}) = \frac{Sap\ Volume\ (mL)}{Time\ elapsed\ (\text{mins})}$$

The plants were de-topped immediately below the first leaves (counting from base of shoot, ignoring cotyledons) and the pot transferred to a tall Scholander pressure vessel (Soilmoisture Equipment Corp., Santa Barbara, CA, USA) to measure root water potential (Ψ_{root}). Pressure in the chamber was increased in 0.04 MPa increments until sap bubbled to the cut surface of the stem. When sap spontaneously exuded from the cut stem, a Ψ_{root} value of 0 was recorded. Pressure was further increased by similar increments and xylem sap collected for 30 s to 1 minute at each step to accurately determine flow rate. When transpirational flow rate was matched, pressure was maintained to collect at least 100 µL of xylem sap. Sap was stored in 1.5 mL Eppendorf tubes, frozen in liquid nitrogen and stored at -20°C for further analysis.

Nitrogen analyses

Total N and C percentages of whole shoot tissues from were measured by elemental analyser (vario EL III; Elementar UK Ltd., Cheadle, UK.). Shoot tissue was dried at 80°C for at least 48 h and ground to a fine powder by ball mill. Powdered tissue (15 ± 1 mg) was weighed into tin cups and folded. Samples were combusted at 800°C to determine N and C content.

Multi-hormone analysis

Phytohormones including cytokinins (*trans*-zeatin, tZ, zeatin riboside, ZR, and isopentenyl adenine, iP), gibberellic acids (GA1, 3, and 4), indole-3-acetic acid (IAA),

ABA, salicylic acid (SA), jasmonic acid (JA), and 1-aminocyclopropane-1-carboxylic acid (ACC) were analysed in leaf tissues and xylem sap according to a protocol modified from Albacete et al. (2008).

Freeze-dried leaf material (0.01 g DW) was extracted overnight at -20^{o}C using a methanol/water/formic acid solution (15/4/1 by volume, pH 2.5). Solids were then separated by centrifugation (20, 000 *g*) for 15 mins, and extracted again for 30 mins in an additional 5 mL of the extraction mixture. The pooled supernatants were filtered through a Sep-Pak Plus C18 cartridge (SepPak Plus, Waters, USA) to remove interfering lipids and plant pigments, and evaporated at 40^{o}C under a vacuum until samples were near dryness or all solvents were removed. Any remaining residue was dissolved in 1 mL methanol/water (20/80, v/v) in an ultrasonic bath. Samples were filtered through 13 mm diameter Millex filters with 0.22 µm pore diameter nylon membrane (Millipore, Bedford, MA, USA).

Filtered extracts and xylem sap samples (10 µL) were injected into a U-HPLC-MS system comprising an Accela Series U-HPLC (ThermoFisher Scientific, Waltham, MA, USA) using a heated electrospray ionisation (HESI) interface. Xcalibur software version 2.2 (ThermoFisher Scientific, Waltham, MA, USA) was used to obtain mass spectra. Calibration curves were constructed to quantify each plant hormone (1, 10, 50, 100 µg L^{-1}) and 10 µg L^{-1} deuterated internal standards were corrected for.

Statistical analyses

Group-level differences between growth, physiological and phytohormonal parameters were analysed using three-way ANOVA, accounting for the main factors of block (harvest day or analysis batch), bulk density and watering treatments. Bulk soil water content at harvest was used to separate the plants into well-watered and drought treatments, as plants from which water had been recently withheld did not exhibit water deficit responses. Phytohormonal analyses of foliar tissues were carried out in two batches: data were transformed into proportions of means (in order to reduce variance due to equipment maintenance) and are presented as relative hormone concentrations.

Pearson's Correlation was used to examine correlations between plant water status, hormone concentrations and soil water content. Multiple regression models were fitted to examine relationships between ABA, plant and soil water status and the changes between bulk density treatments. Full interaction models were fitted, and terms sequentially dropped when their removal did not significantly impact the model fit (drop1(), F-test, $p > 0.05$; R Core Team, 2018). Where necessary, the response variable was log-transformed to improve normality of model residuals.

2.3 Results

Experiment 2.1: Plant growth and physiology in response to increasing soil bulk density

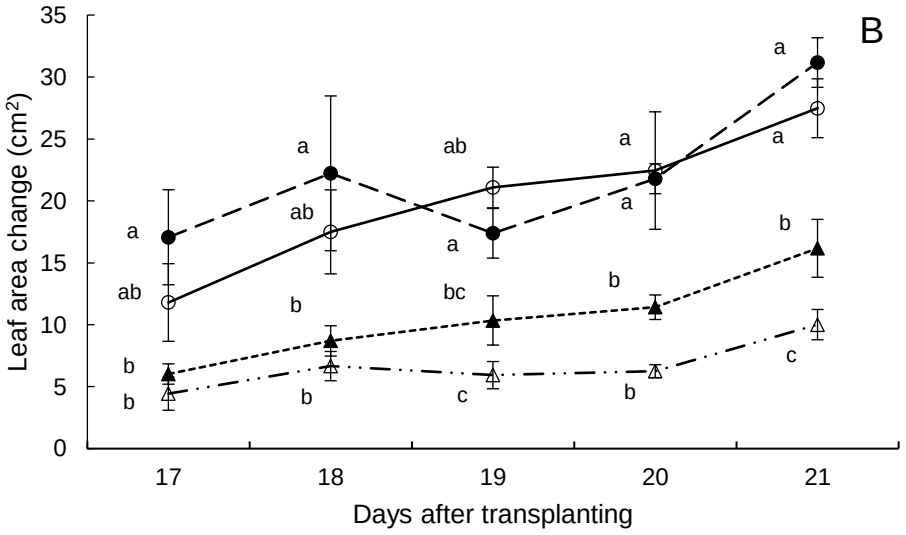


Figure 2-4: A) Differences in shoot size between 3 of the 4 soil compaction treatments are visually apparent. Pot height is 24 cm B) Daily leaf area increase (0.29(L*B) + 1.24) of tomato plants grown at 4 levels of soil bulk density (black circles: Treatment 1 (1.4 g cm^{-3}); white circles: Treatment 2 (1.5 g cm^{-3}); black triangles: Treatment 3 (1.6 g cm^{-3}); white triangles: Treatment 4 (1.74 g cm^{-3}). Symbols are means ± S.E. of 5-6 replicates. Different letters denote significant differences between means (post hoc LSD $p < 0.05$) on each day.

Daily increase in leaf area was always greatest in low bulk density Treatments 1 and 2 (Figure 2-4B). Leaf area change was consistently significantly higher in Treatment 1 compared to Treatments 3 and 4 on all days measured (post hoc LSD $p < 0.05$).

Table 2-3: Mean growth and physiology parameters (± S.E.) of tomato plants grown at each bulk density. Significant differences between treatment means were identified with post-hoc LSD ($p < .05$) and are indicated with superscript letters.

Treatment	**1 (1.4 g cm^{-3})**	**2 (1.5 g cm^{-3})**	**3 (1.6 g cm^{-3})**	**4 (1.78 g cm^{-3})**
Stomatal conductance (mmol m^{-2} s^{-1})	844 ± 200^{a}	928 ± 126^{a}	893 ± 239^{a}	981 ± 126^{a}
Leaf water potential (MPa)	-0.78 ± 0.03^{a}	-0.71 ± 0.02^{a}	-0.81 ± 0.03^{a}	-0.81 ± 0.04^{a}
Total leaf area (cm^{2})	347 ± 8^{a}	335 ± 20^{a}	215 ± 11^{b}	98 ± 5^{c}
Total dry biomass (g)	1.96 ± 0.10^{a}	1.70 ± 0.11^{b}	1.08 ± 0.09^{c}	0.66 ± 0.05^{d}
Root:Shoot Ratio	0.27 ± 0.02^{ab}	0.24 ± 0.02^{a}	0.29 ± 0.03^{ab}	0.35 ± 0.05^{b}
Specific leaf area (cm^{2} g^{-1})	255 ± 13^{a}	272 ± 7^{a}	297 ± 39^{a}	228 ± 24^{a}

No significant treatment differences in stomatal conductance or leaf water potential were detected (Table 2-3). Leaf area and biomass decreased as bulk density of soil increased. Root to shoot ratio in Treatment 4 was significantly (46%) higher than Treatment 2. Increasing bulk density had no significant impact on specific leaf area.

Experiment 2.2: Combined effects of soil compaction and water deficit on growth and physiology of tomato

Differences in soil bulk density treatments were consistently implemented in Experiments 1 and 2 using the Arbor press system (Figure 2-5).

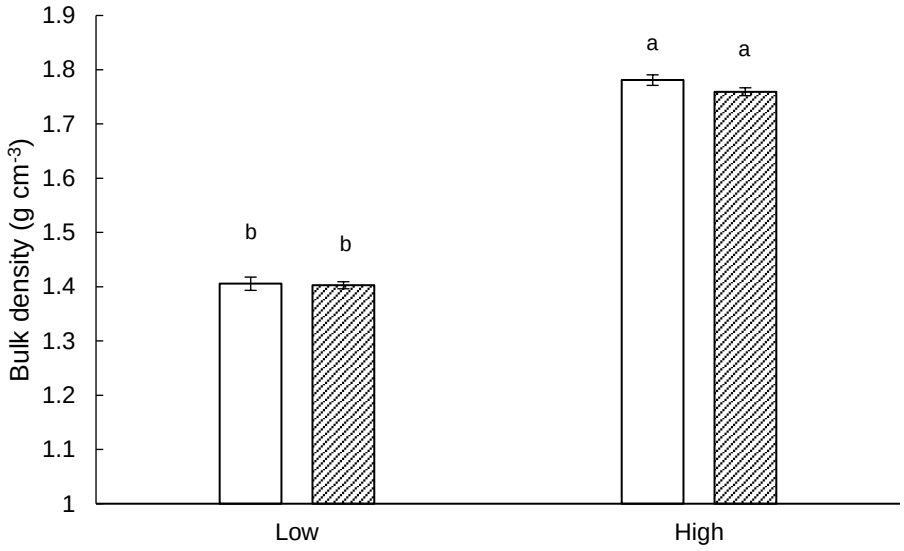


Figure 2-5: Soil bulk density in Experiments 1 and 2. Bars are means ± S.E. of 6 (Exp. 2.1 – hollow bars) and 30 (Exp. 2.2 – shaded bars) replicates. Different letters denote significant differences between means (post hoc LSD $p < 0.05$).

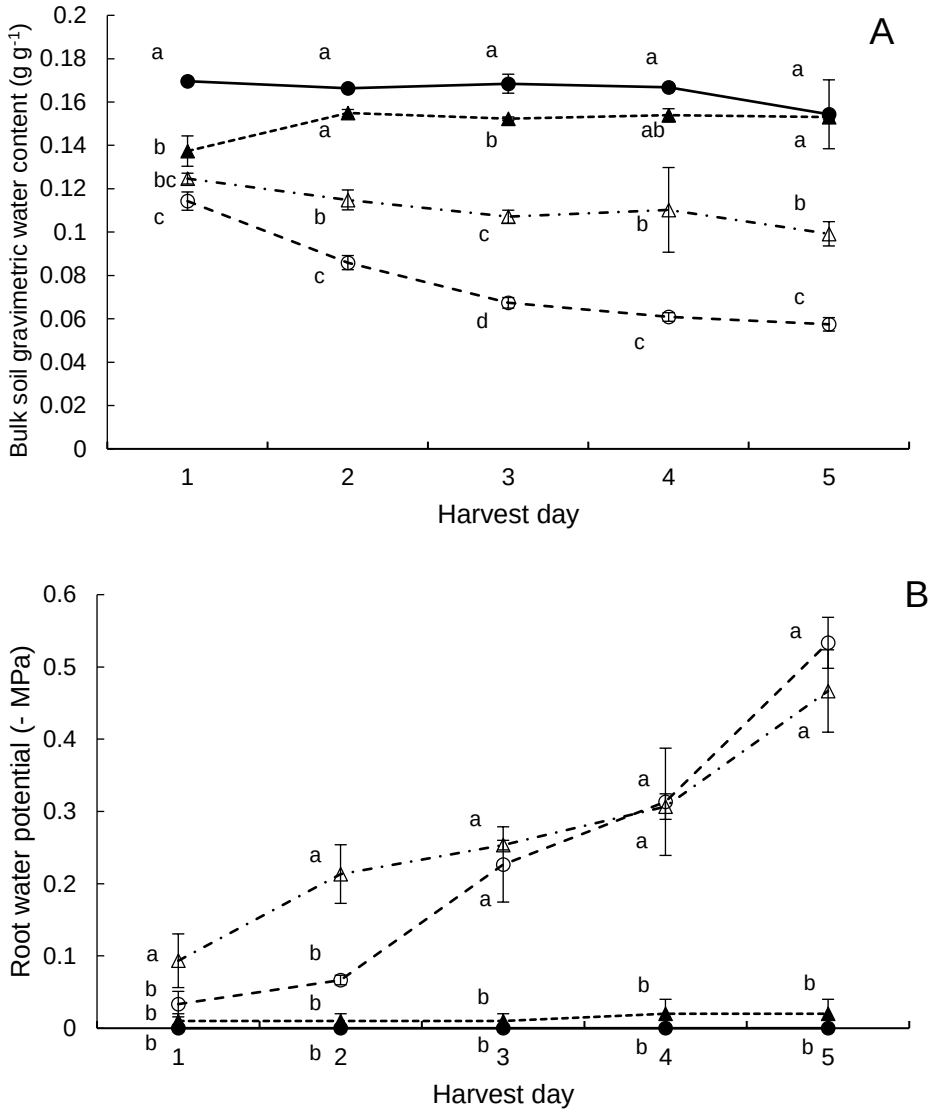


Figure 2-6: Changes in bulk soil gravimetric water content (A) and Ψ_{root} (B) during the experiment. Circles denote plants grown in 1.4 g cm^{-3} soil (low bulk density), and triangles correspond to 1.74 g cm^{-3} (high bulk density). Filled circles & triangles: well-watered; hollow circles and triangles: drought treatment. Symbols are means of 2-3 replicates. Error bars ± S.E of 2 (WW) or 3 (D) replicates. Different letters denote significant differences between means (post hoc LSD $p < 0.05$).

Although bulk soil water content slightly differed between well-watered plants grown at high and low bulk density (Figure 2-6A), no significant differences in (expanded) Ψ_{leaf} or Ψ_{root} (Table 2-4) were observed, suggesting that plants could

readily access sufficient water. Withholding water for 7 days decreased bulk soil water content by 71% and 41% at low and high bulk density respectively. Soil drying induced similar Ψ_{root} declines at both bulk densities over the 5-day harvest period (Figure 2-6B)

Table 2-4: Mean growth and physiology parameters of tomato plants grown at low (1.4 g cm^{-3}) and high (1.74 g cm^{-3}) bulk densities in either well watered (WW) or drying (D) soil. Significant effects of bulk density treatment are denoted with superscript letters ($p < .05$). No significant effect of watering treatment was observed on growth parameters.

Bulk density	**Low**		**High**	
Watering	**WW**	**D**	**WW**	**D**
Leaf area (cm^2)	74 ± 3^a	73 ± 2^a	32 ± 3^b	29 ± 2^b
Biomass (g)	0.47 ± 0.05^a	0.39 ± 0.05^a	0.18 ± 0.05^b	0.17 ± 0.05^b
Root:Shoot Ratio	0.27 ± 0.04	0.22 ± 0.04	0.28 ± 0.04	0.26 ± 0.04
Specific leaf area ($cm^2\ g^{-1}$)	314 ± 15^a	331 ± 11^a	245 ± 15^b	257 ± 11^b
Shoot N (%)	3.6 ± 0.18^a	3.7 ± 0.14^a	2.3 ± 0.16^b	2.6 ± 0.13^b
Leaf water potential	-0.83 ± 0.07^a	-	-0.86 ± 0.1^a	-
Root water potential	n.d.	-	-0.014 ± 0.01	-

Watering treatment had no significant impact on plant growth parameters (Table 2-4). Plants in compacted soil were significantly smaller than those grown in loose soil. Lower specific leaf area and shoot nitrogen (%) was observed in plants growing at high bulk density, suggesting nutrient limitation.

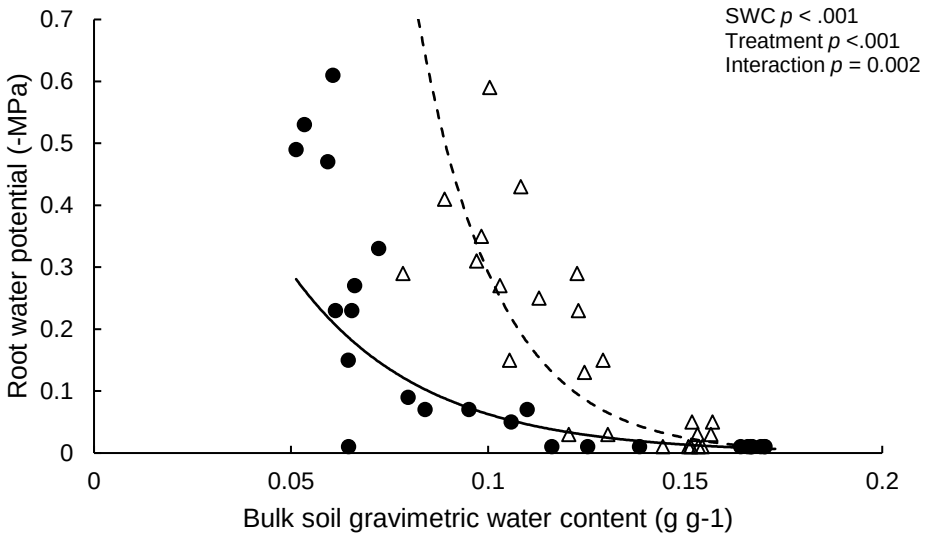


Figure 2-7: Ψ_{root} decreases with decreasing bulk gravimetric soil water content (SWC). Filled circles denote low bulk density (1.4 g cm^{-3}), hollow triangles correspond to high bulk density treatment (1.74 g cm^{-3}). Each symbol is an individual plant, with trendlines fitted where relationships are significant ($p < 0.05$), and p values reported for SWC, bulk density and their interaction.

Although Ψ_{root} was similar in well watered plants grown at the two bulk densities, soil drying decreased Ψ_{root}. At the same bulk soil water content, Ψ_{root} of plants in the high bulk density treatment was significantly lower than plants in low bulk density soil (Figure 2-7) as indicated by a significant compaction x soil water content interaction.

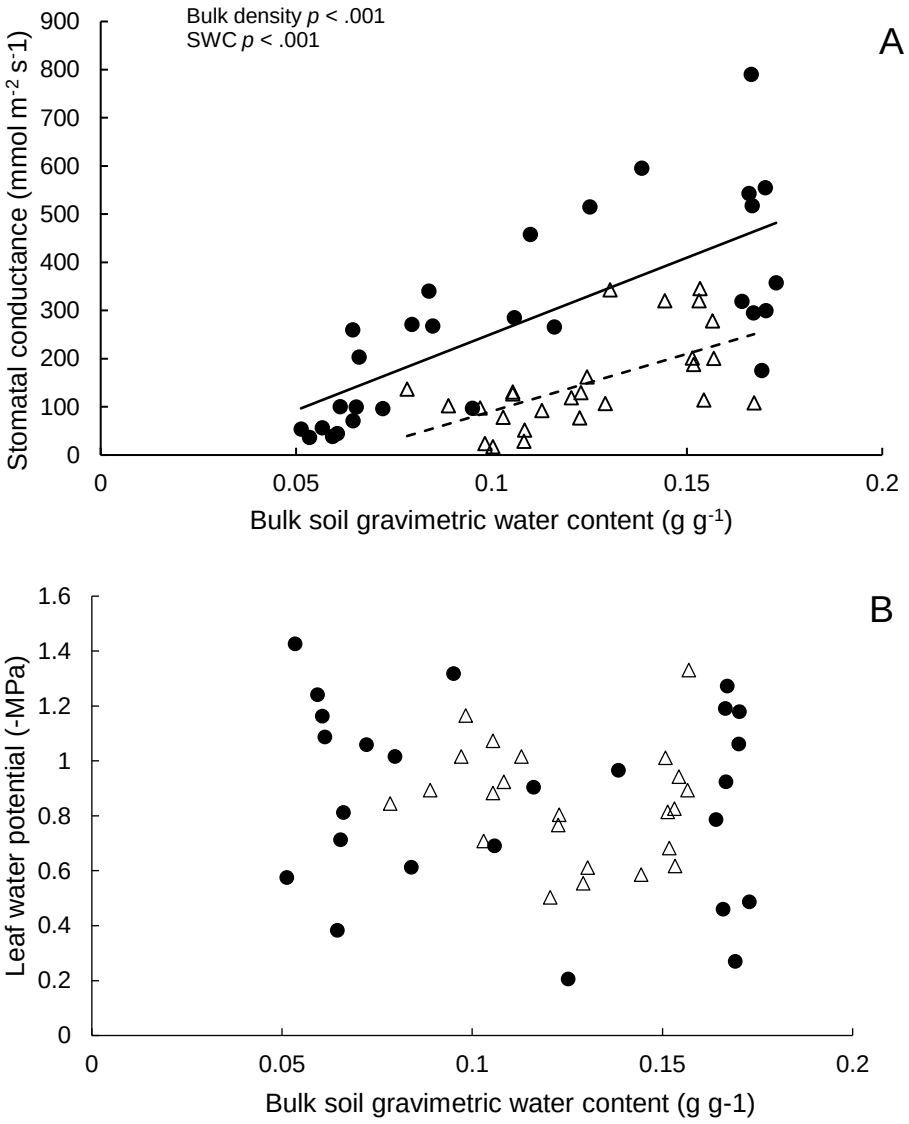


Figure 2-8: Water relations of expanded leaves. A) stomatal conductance and B) leaf water potential *versus* bulk soil gravimetric water content. Filled circles denote low bulk density treatment (1.4 g cm^{-3}), hollow triangles denote high bulk density treatment (1.74 g cm^{-3}). Each symbol is an individual plant, with trendlines fitted where relationship is significant (p < .05) and p values reported for remaining model predictors (SWC, bulk density and their interaction).

Plants grown under high bulk density soil had significantly lower stomatal conductance than low bulk density plants at the same bulk soil water content (Figure 2-8A and Figure 2-9A). Moreover, stomatal conductance decreased linearly with decreasing soil water content similarly in both bulk density treatments (as indicated by no significant treatment x SWC interaction), and in both expanding and expanded

leaves. Soil water content and Ψ_{leaf} were not correlated in expanded leaves (Figure 2-8). However, plants in high bulk density soil displayed a slight but statistically significant decrease in Ψ_{leaf} in expanding leaves, averaging -0.16 MPa across a range of soil water contents (Figure 2-9B).

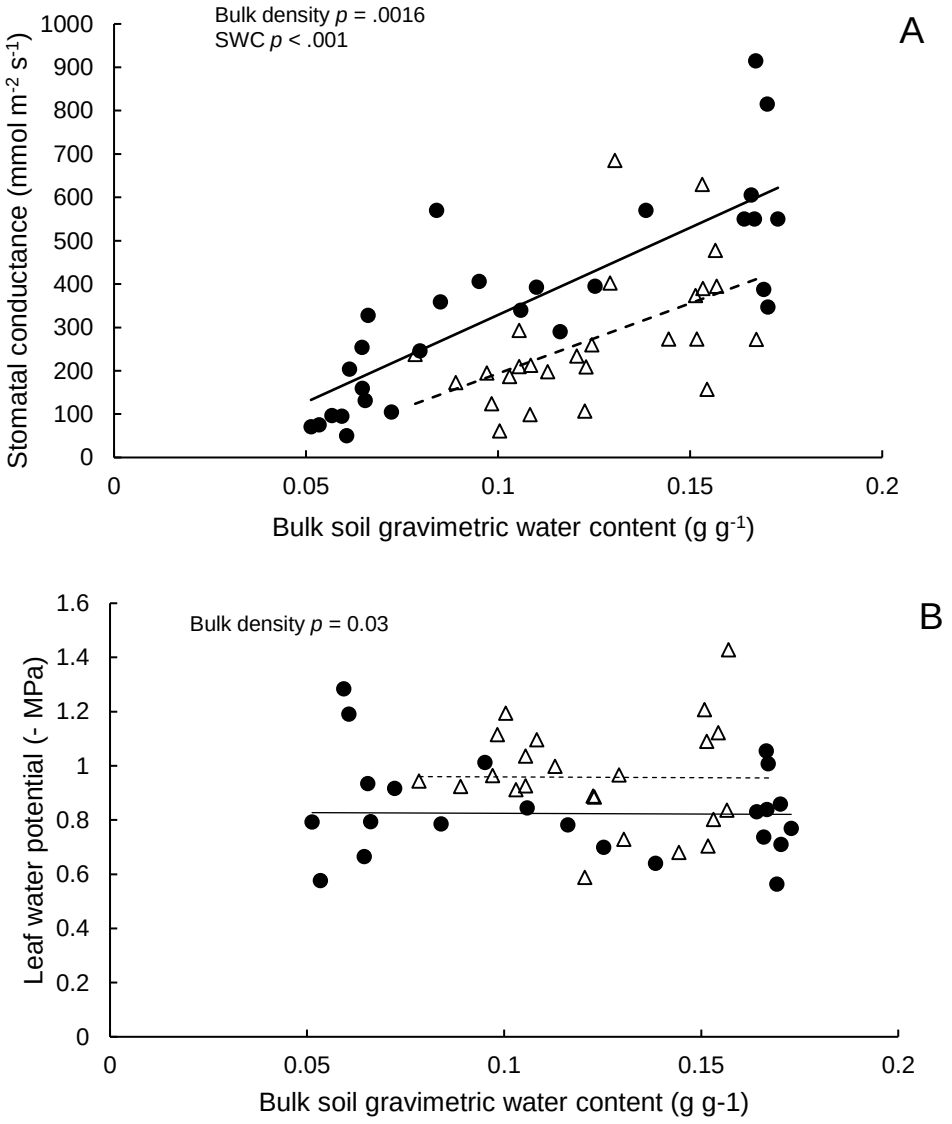


Figure 2-9: Water relations of expanding leaves. A) stomatal conductance and B) leaf water potential *versus* bulk soil gravimetric content. Filled circles denote low bulk density treatment (1.4 g cm^{-3}), hollow triangles denote high bulk density treatment (1.74 g cm^{-3}). Each symbol is an individual plant, with trendlines fitted where relationship is significant ($p < .05$) and p values reported for remaining model predictors (SWC, bulk density and their interaction).

Multi-hormone analyses

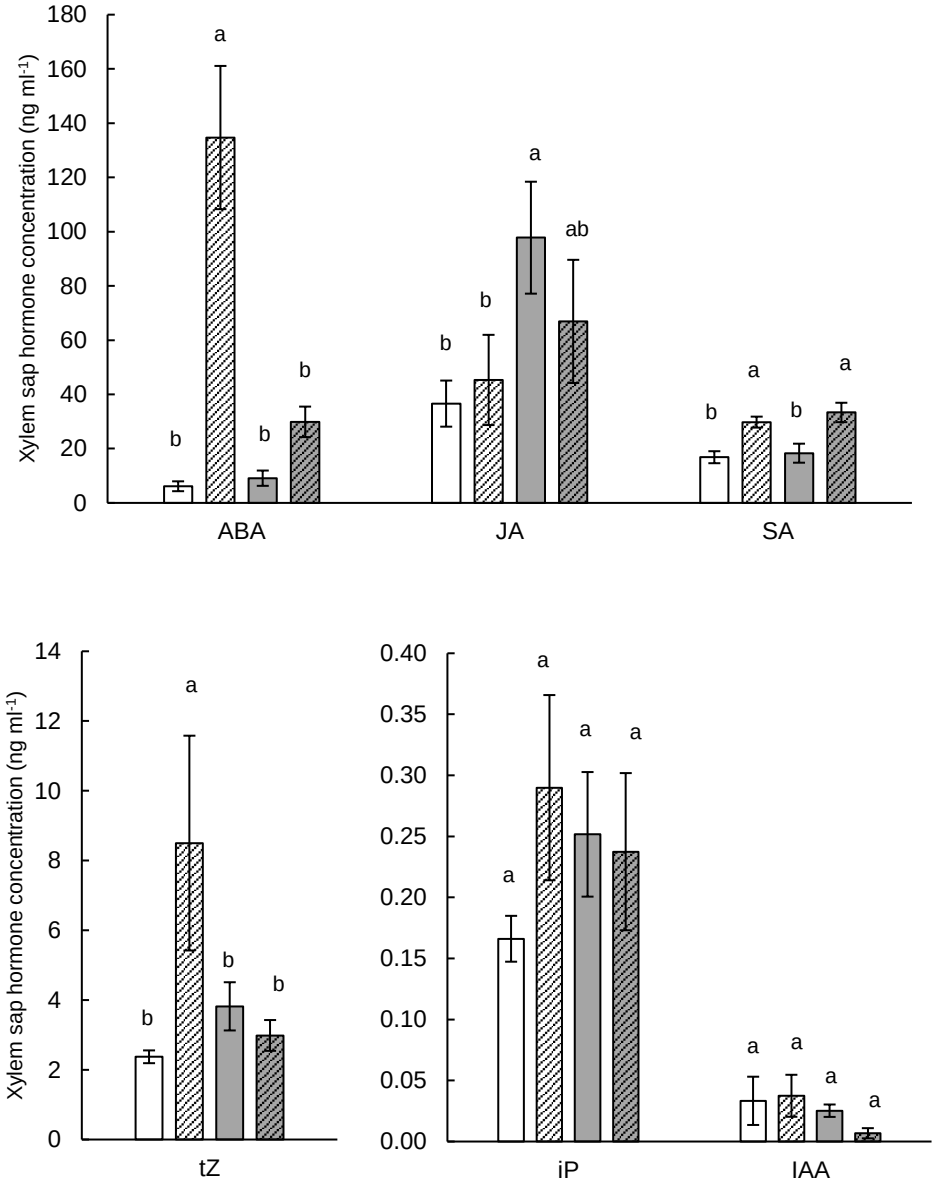


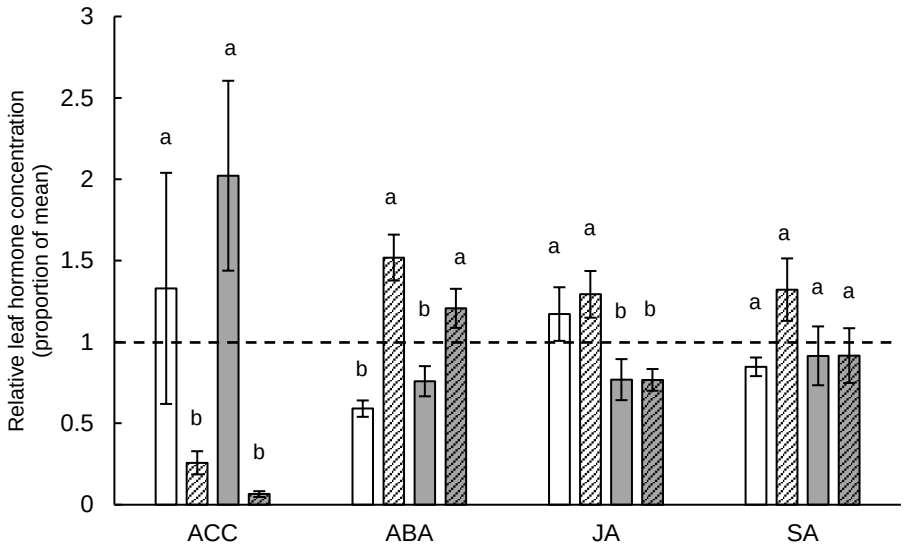
Figure 2-10: Concentrations of phytohormones detected in root xylem sap from tomatoes grown in high or low bulk density sandy loam soil, under well-watered (WW) or water-deficit (WD) conditions. White bars: Low bulk density/WW; White/striped bars: Low bulk density/WD; Grey bars: High bulk density/WW; Grey/striped bars: High bulk density/WD. Bars are means ± S.E. of 5 replicates, with different letters denoting significant differences between means within an analyte (post-hoc LSD $p < 0.05$).

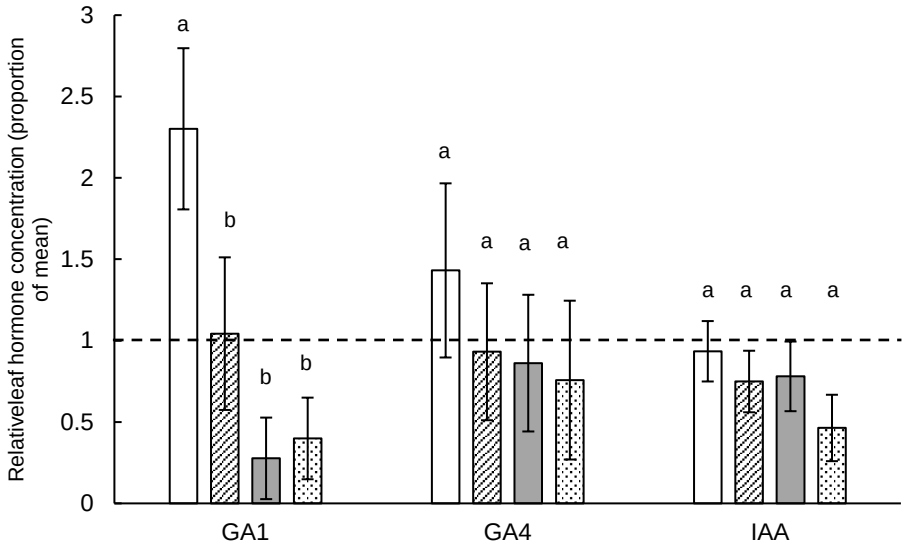
Plants were categorised as well-watered (WW) or water-deficient (WD) based on Ψ_{root} measurements, as these decreased at different rates relative to soil water content in the two bulk density treatments (Figure 2-7). When Ψ_{root} was higher than -0.2 MPa, plants were classified as WW. IAA, cytokinins (tZ, iP), ABA, JA and SA were detected in over 50% of root xylem sap samples.

In well-watered plants, soil compaction had no effect on xylem ABA and SA concentrations. However, decreased soil water availability increased xylem ABA concentration in both high and low bulk density treatments ($p < 0.001$; Figure 2-10; Table 2-5). However, this increase was far greater (29-fold) in plants grown at low bulk density than in plants grown under higher compaction (3-fold) ($p = 0.008$). Additionally, soil drying increased xylem [tZ] overall ($p = 0.035$), but there was a significant bulk density x soil drying interaction ($p = 0.002$), as this increase was only observed in low bulk density soil. Xylem SA concentration doubled under water deficit in both low and high bulk density treatments ($p < 0.001$). Water deficit did not significantly influence JA concentration but increasing bulk density significantly increased root xylem JA concentration ($p = 0.024$). There were no significant effects of soil drying and bulk density on IAA and iP concentrations.

Table 2-5: ANOVA *p* values of root xylem phytohormone concentrations. Significance of *p* values reported thus: · p is marginally non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Interaction is 3-way (Block*Watering*Bulk density = BL x W x BD) unless otherwise indicated.

Hormone	Transformation	Watering	Bulk density	Interaction
ABA	$\log_{10}$(ABA)	< 0.001 ***	0.084 ·	0.008 **
JA		0.52	0.024 *	0.26
SA		< 0.001 ***	0.41	0.73
tZ	$\log_{10}$(tZ)	0.035 *	0.28	0.002 **
iP		0.28	0.74	0.18
IAA	$\log_{10}$(IAA + 0.01)	0.55	0.45	0.081 ·


Relative leaf hormone concentration (proportion of mean)
3
2.5
2
1.5
1
0.5
0
a
b
a
b
b
a
b
a
a
b
b
a
a
b
b
a
a
a
a
ACC
ABA
JA
SA


Relativeleaf hormone concentration (proportion of mean)
3
2.5
2
1.5
1
0.5
0
a
b
b
b
a
a
a
a
a
a
a
a
GA1
GA4
IAA

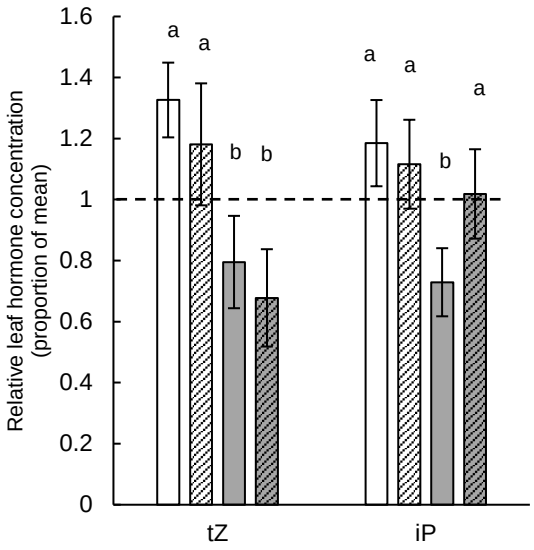


Figure 2-11: Relative phytohormonal concentrations in expanding leaf tissues. Values are expressed as proportions of the means of 2 separate analyses. Dashed line represents the average level of phytohormone across all treatments in both analyses. White bars: Low bulk density/WW; White/striped bars: Low bulk density/WD; Grey bars: High bulk density/WW; Grey/striped bars: High bulk density/WD. Bars are means ± S.E. of 5-7 replicates, with different letters denoting significant differences between means within an analyte (post-hoc LSD $p < 0.05$).

Zeatin riboside was not detected in any leaf tissue samples, and GA_3 detected in only 29% of samples. Thus, ZR and GA_3 are excluded from the analysis. All other hormones (except GA_3) were detected in foliar tissues with >50% frequency.

Increased soil bulk density significantly reduced levels of tZ ($p = 0.012$; Figure 2-11; Table 2-6), JA ($p = 0.002$) and GA_1 ($p = 0.005$) in actively expanding leaves. Increased soil density also tended to decrease IAA ($p = 0.061$) and iP ($p = 0.071$) levels. There was an interactive effect of block and bulk density on ACC concentration, as ACC was significantly higher in high bulk density/WW plants in Block 1, relative to controls. Soil compaction had no significant effect on ABA, SA or GA_4 concentrations. Increased soil bulk density decreased plant growth and the concentration of the growth promoter GA_1 in foliar tissues.

Withholding water did not affect foliar concentrations of gibberellins, tZ, iP, JA or SA. However, withholding water significantly reduced ACC ($p < 0.001$) and IAA ($p < 0.022$) concentrations and significantly ($p < 0.001$) increased ABA levels. Bulk density affected the soil drying-induced increase in foliar ABA levels (interaction $p = 0.031$), as the magnitude of ABA increase was greater in plants grown in low bulk density soil.

Table 2-6: ANOVA *p* values of foliar phytohormone concentrations. Significance of *p* values reported thus: · *p* is marginally non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.. Interaction is 3-way (Block*Watering*Bulk density = BL x W x BD) unless otherwise indicated.

Hormone	Transformation	Block	Watering	Bulk density	Interaction
ACC	$\log_{10}$(ACC + 0.01)	0.09 ·	< 0.001 ***	0.12	Bl x BD < 0.001 W x BD 0.009 **
ABA		0.78	< 0.001 ***	< 0.001 ***	W x BD 0.030 *
JA		0.97	0.91	0.002 **	0.053 ·
SA		0.99	0.30	0.71	W x BD 0.096 ·
GA_1		0.99	0.31	0.006 **	0.31
GA_4		0.99	0.58	0.085 ·	Bl x BD 0.010 *
IAA		< 0.001 ***	0.022 *	0.034 *	Bl x BD 0.079 ·
tZ		0.95	0.14	0.012 *	0.089 ·
iP		0.95	0.60	0.072 ·	W x BD 0.072 · 3-way 0.055 ·

Correlations between phytohormones and water status

Table 2-7: Pearson's r correlation coefficients between root xylem sap phytohormone concentrations and plant/soil water status parameters. Significance of *p* values reported thus: · *p* is marginally non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

	tZ	**iP**	**ABA**	**JA**	**SA**	**IAA**	**Ψ_{root}**	**GSWC**	**g_s**
tZ		0.33	**0.76 *****	-0.21	0.33	**0.36 ·**	**-0.56 ****	**-0.52 ****	**-0.44 ***
iP			0.31	-0.13	0.14	-0.06	**-0.4 ***	-0.26	-0.33
ABA				-0.29	**0.48 ***	0.19	**-0.86 *****	**-0.78 *****	**-0.63 *****
JA					-0.1	-0.12	0.16	0.02	-0.04
SA						0.12	**-0.64 *****	**-0.6 ****	**-0.69 *****
IAA							0.03	0.16	0.04
Ψ_{root}								**0.81 *****	**0.76 *****
GSWC									**0.75 *****
g_s									

Both xylem ABA concentration ([X-ABA]) (Table 2-7) and foliar ABA concentration ([L-ABA]) (Table 2-8) were highly significantly inversely correlated with plant water status and soil water content parameters ($p < 0.001$), warranting further investigation of these relationships across bulk density treatments. Furthermore, [X-tZ] and [X-SA] were also negatively correlated with soil drying and reduced Ψ_{root} ($p < 0.05$), but such associations were not observed in foliar tissues. [X-ABA] and [X-tZ] were significantly positively correlated ($p < 0.001$). Foliar GA_1 was positively correlated with foliar tZ and JA ($p < 0.01$).

Table 2-8: Pearson's r correlation coefficients between relative foliar phytohormone concentrations and plant/soil water status parameters. · Significance of *p* values reported thus: · *p* is marginally non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

	ACC	**tZ**	**iP**	**GA_1**	**IAA**	**ABA**	**JA**	**SA**	**g_s**	**Ψ_{root}**	**GSWC**
ACC		0	-0.23	-0.3	-0.1	**-0.42 ***	**-0.42 ***	-0.05	0.07	**0.5 ***	**0.48 ***
tZ			**0.42 ***	**0.63 *****	0.22	-0.14	**0.54 ****	0.18	**0.4 ·**	0.31	-0.08
iP				0.39 ·	0.02	0.02	**0.52 ****	0.03	0.28	0.06	0
GA_1					0.36 ·	-0.1	**0.74 *****	-0.04	**0.37 ·**	0.24	-0.12
IAA						-0.36 ·	0.16	-0.18	**0.46 ***	0.28	0.12
ABA							0.27	**0.54 ****	**-0.67 *****	**-0.76 *****	**-0.79 *****
JA								**0.48 ***	0.26	0.07	-0.28
SA									-0.19	-0.11	-0.34
g_s										**0.72 *****	**0.54 ****
Ψ_{root}											**0.77 *****
GSWC											

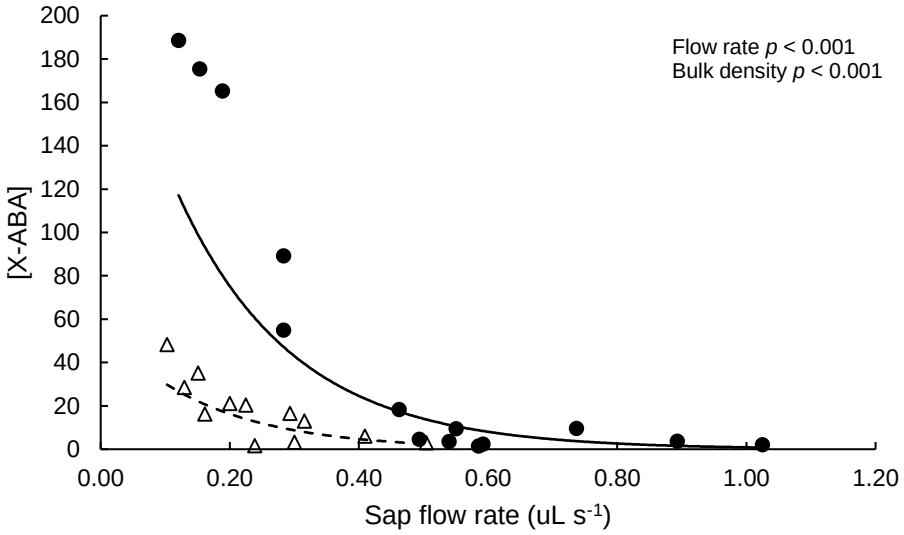


Figure 2-12: [X-ABA] increases as transpirational flow rate decreases in tomato grown in low (circles) and high (triangles) bulk density soil. Each symbol is an individual plant, with trendlines fitted where relationship is significant ($p < .05$) and p values reported for remaining model predictors (flow rate, bulk density and their interaction).

As transpirational sap flow rate decreased, [X-ABA] increased exponentially ($p < 0.001$; Figure 2-12A). [X-ABA] was increased at the same flow rate under low bulk density conditions ($p < 0.001$).

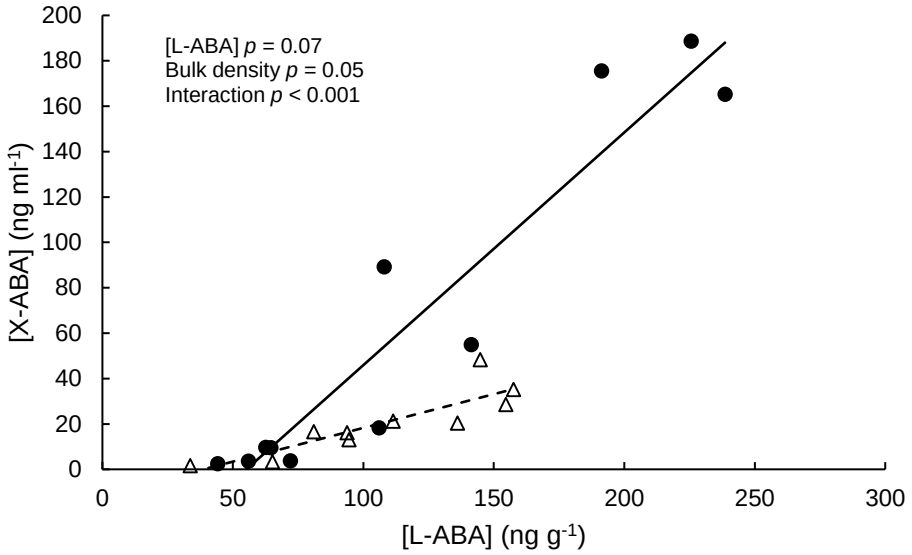


Figure 2-13: Relationship between [X-ABA] and [L-ABA] in tomatoes grown in low (circles) and high (triangles) bulk density soil. Each symbol is an individual plant, with trendlines fitted where relationship is significant (p < .05) and p values reported for remaining model predictors ([L-ABA], bulk density and their interaction).

[X-ABA] tends to increase as [L-ABA] increases (p = 0.07; Figure 2-13). However, the sensitivity of this relationship increases in plants grown in low bulk density soil (p < 0.001) such that these plants had a higher [X-ABA] at a specific [L-ABA].

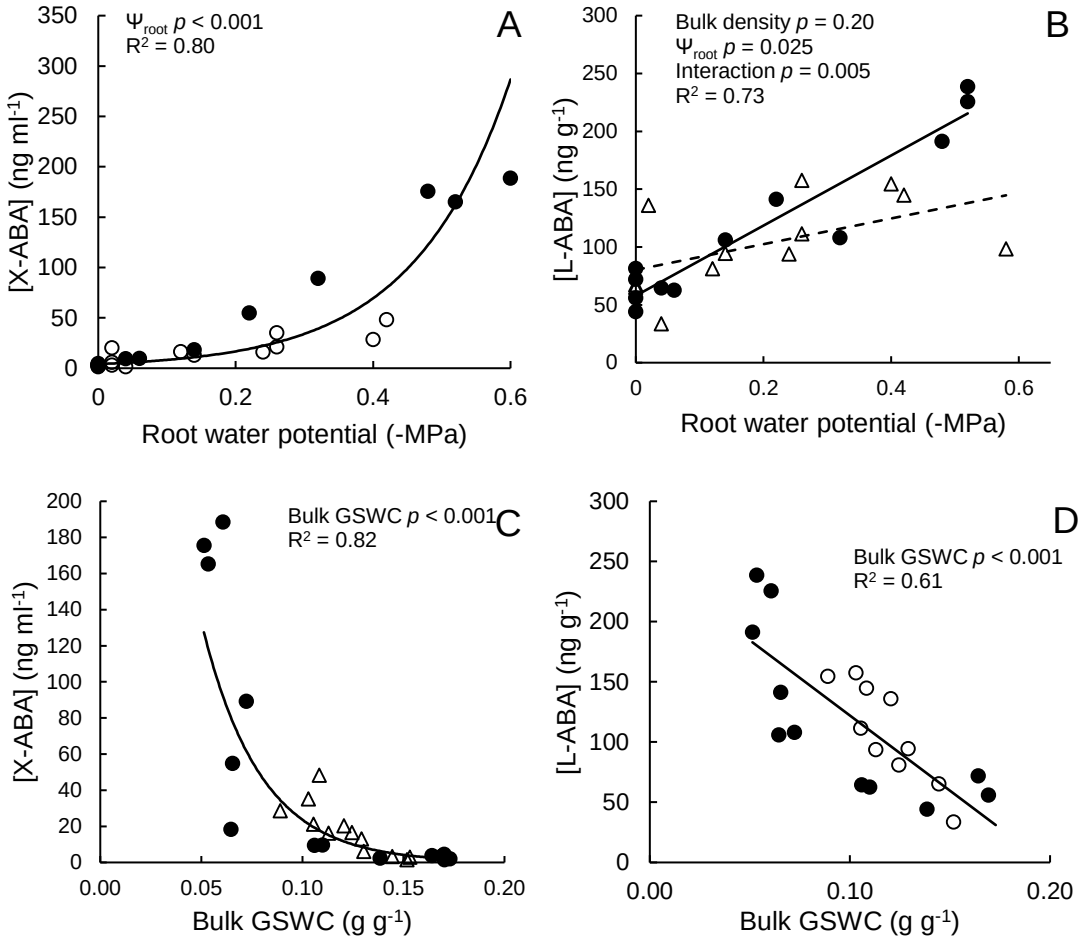


Figure 2-14: Relationships between xylem sap [ABA] (left column) or foliar [ABA] (right column) and plant and soil water status parameters. Black circles represent low bulk density treatment, white triangles correspond to high bulk density. Each symbol is an individual plant and *p* values reported for remaining model predictors (x-variable, bulk density and their interaction). Trendlines fitted where relationship is significant ($p < .05$), with solid lines corresponding to low bulk density or a single trend, and dashed lines corresponding to high bulk density .

[X-ABA] increased exponentially as Ψ_{root} decreased ($p < 0.001$; Figure 2-14A). A single relationship across both bulk density treatments explained the 80% of the variation in [X-ABA]. [L-ABA] linearly increased as Ψ_{root} decreased ($p = 0.025$), but there was a significant interaction between bulk density and Ψ_{root} ($p = 0.005$). Thus [L-ABA] increased more sensitively as Ψ_{root} decreased in low bulk density soil (Figure 2-14B). Relationships between xylem or leaf ABA and soil water content were unified across bulk density treatments (Figure 2-14C & D), with soil water content explaining more (82%) of the variation in [X-ABA] than [L-ABA] (61%).

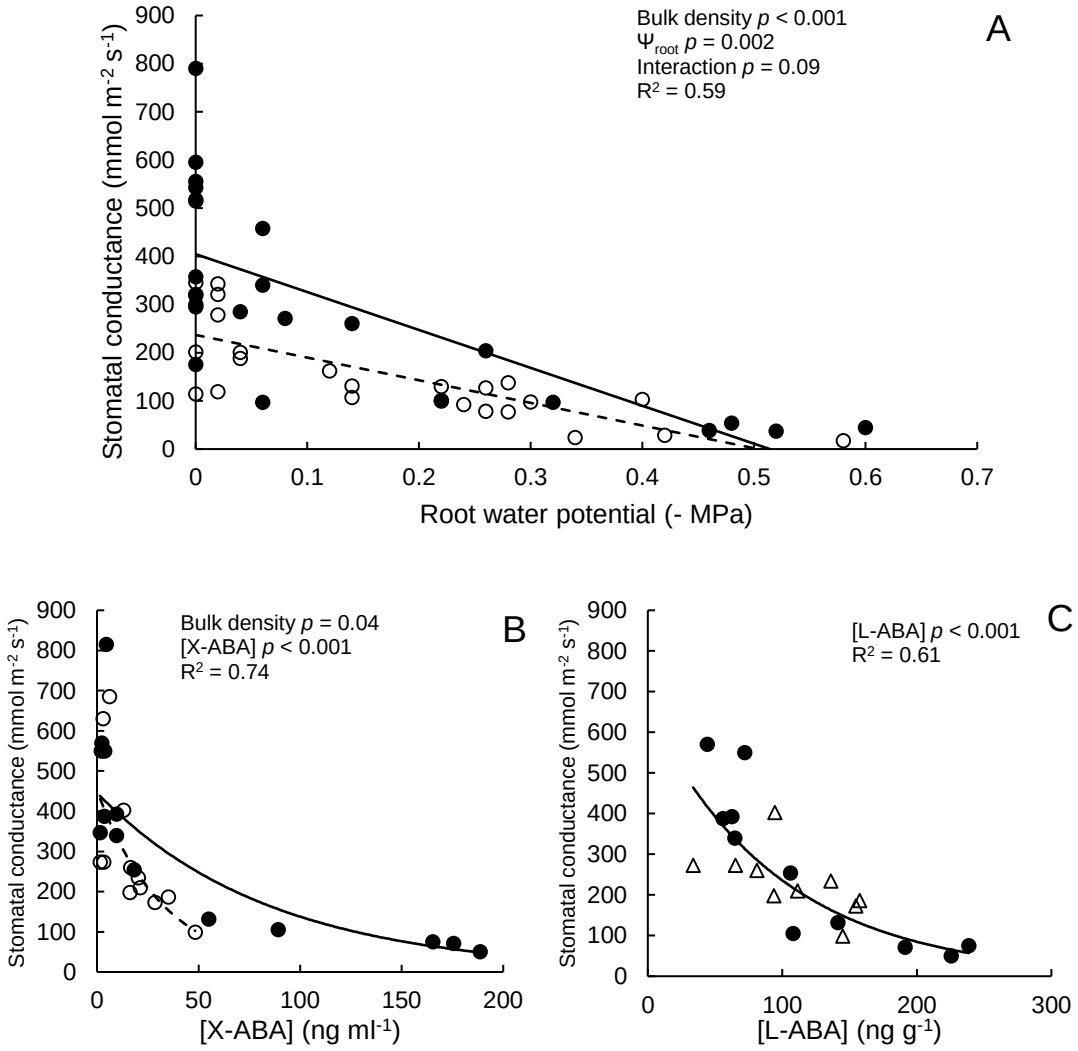


Figure 2-15: A) Relationship between g_s of expanding tomato leaves and Ψ_{root} in plants used to measure phytohormone concentrations. When sap spontaneously exuded from the de-topped root system, a Ψ_{root} value of 0 was recorded. B & C) Relationships between g_s and [X-ABA] and g_s and [L-ABA]. Black circles denote low bulk density treatment, white triangles represent high bulk density treatment. Each symbol is an individual plant and *p* values reported for remaining model predictors (x-variable, bulk density and their interaction). Trendlines fitted where relationship is significant (p < .05), with solid lines corresponding to low bulk density or a single trend, and dashed lines corresponding to high bulk density .

Stomatal conductance declined linearly with root water potential across both soil bulk densities (Figure 2-15A), although this response tended to be accentuated in plants grown at low bulk density (bulk density x Ψ_{root} interaction (p = 0.09). [L-ABA] explained 61% of the variation in g_s (p < 0.001; Figure 2-15C) with a single relationship between these variables. Although [X-ABA] explained more (74%) of

variation in g_s ($p < 0.001$; Figure 2-15B), increased bulk density significantly decreased g_s at the same [X-ABA] ($p = 0.04$).

To summarise, unifying relationships across bulk densities are observed between [X-ABA] and Ψ_{root}, and [L-ABA] and g_s. Both [X-ABA] and [L-ABA] may be predicted by soil water content, but soil water content explains more of the variance in [X-ABA].

2.4 Discussion

Tomato growth in compact soil

Total leaf area of *S. lycopersicum* decreased with increasing bulk density (Table 2-3). Although soil compaction can decrease leaf water potential (Hartung et al. 1994), other studies show that compacted soil decreases leaf expansion even though leaf water status remained unchanged (Andrade et al., 1993). Here, measurements of Ψ_{leaf} by thermocouple psychrometry showed that leaf water status was unaffected (Figure 2-8) or reduced by a small but statistically significant degree by increasing soil bulk density in Experiment 2.2 (Figure 2-9). However, in Experiment 2.2 there was also no effect of decreasing soil water content on Ψ_{leaf}, despite both g_s and Ψ_{root} decreasing with soil water content. It seems likely that soil-drying induced stomatal closure maintains Ψ_{leaf} as the soil dries (Zhang and Davies, 1989). Thus, it is difficult to attribute decreased leaf expansion to perturbed leaf water status.

Soil bulk density did not change root:shoot ratio of tomato at ~28 DAT, indicating similar growth responses of both roots and shoots. Although root:shoot ratios in grassland species also remained constant as soil impedance increased, root:shoot was measured only once (Cook et al., 1996). In contrast, root and shoot growth of wheat were both reduced during the first week after emergence on high bulk density soil, but biomass accumulation rates were greater in roots compared to shoots in compact soil after the first week (Masle et al., 1990). It is not clear how this biphasic root response relates to root-to-shoot signalling, and deserves further investigation.

However, significant reductions in specific leaf area and shoot nitrogen were observed in the high bulk density treatment (Table 2-4), suggesting nutrient

limitation in compact soil. As expected, nitrogen content of shoots was reduced. Reduced access to nutrients is a common result of soil compaction (Lipiec and Stępniewski, 1995), particularly as roots become restricted. Increased soil bulk density correlates with increased soil strength (Colombi et al., 2018), which was true of this sandy loam soil (Appendix 2).

Plant water status and ABA content in drying soils of contrasting bulk density

Increased soil bulk density caused greater decline of Ψ_{root} with decreasing bulk soil water content, likely due to reduction in local soil water content closer to the roots (Tardieu, Bruckler, et al., 1992). Differential physiological responses of plants to drying soil when grown in soils of differing compaction will affect the modelling of soil water uptake. Consequently, a better understanding of this relatively inaccessible measure of plant water status and its relationship with soil water availability in the bulk soil is required to better understand both crop growth and physiological responses in heterogeneously-structured field soils (Whitmore and Whalley, 2009).

Stomatal distribution and aperture across the leaf surface regulate leaf water balance (Dodd and Davies, 2010), and ABA's potent effect on stomatal closure has been widely studied. However, there is still debate as to the relative importance of root or leaf-synthesised ABA in the control of stomatal aperture under soil water deficit (McAdam, Manzi, et al., 2016). Here, stomatal conductance was related to both leaf tissue ABA concentration and root xylem sap ABA concentration (Figure

2-15B), with relatively little different in total variance explained by each model ([X-ABA]: R^2 = 0.54; [L-ABA]: R^2 = 0.57). Thus, it is not clear from this data whether bulk leaf or xylem ABA is a better predictor of stomatal aperture. As the classical paradigm is root-sourced (Davies et al., 2005), this work will refer primarily to xylem ABA data.

Several workers have reported increased [X-ABA] in response to soil compaction (Hartung et al., 1994; Mulholland, Black, et al., 1996). However, increased [X-ABA] was transient, returning to control levels after several days, potentially as the roots acclimatise to the mechanical resistance of the soil. High soil bulk density did not increase WW [X-ABA] (Figure 2-10). Supplying detached leaves of WT tomatoes with 264 ng ml^{-1} ABA (similar concentrations to low bulk density-WD plants here) via the transpiration stream halved transpiration rate, while 26 ng ml^{-1} decreased transpiration by 17% (de Ollas et al., 2018). Here, [X-ABA] of high bulk density plants was consistently lower than controls regardless of sap flow rate (Figure 2-12), but stomatal conductance halved even at WW conditions (Figure 2-15A). Instead, the action of other anti-transpirant signals may regulate stomatal aperture compacted tomato plants.

Since phytohormone concentrations were only measured once (at harvest), any transient fluctuations of [X-ABA] as roots adapt to growing through compact soil were not captured. Instead, the change in the slope of relationship between [X-ABA] and Ψ_{root} as soil dries in this work suggests that soil compaction may alter root sensitivity to soil drying or shoot sensitivity to [X-ABA], or the action of alternative signals transported from roots to shoots.

At similar xylem flow rates, [X-ABA] was significantly lower in plants grown under high soil bulk density conditions (Figure 2-12). While previous studies collected xylem sap at slow flow rates from de-topped roots (Mulholland, Black, et al., 1996; Hussain et al., 2000), this study obtained xylem sap at transpirational flow rates from plants grown in compact soil. Opposing responses occurred, with soil compaction increasing (Mulholland et al 1996a) and decreasing (Figure 2-12) xylem ABA concentration. Decreased sap flow rates from de-topped roots of plants grown in compact soil, perhaps caused by a hypoxia-induced decrease in root hydraulic conductance (Jackson et al., 1996), may explain the increased xylem [ABA] of Mulholland et al. (1996a), as xylem [ABA] increases exponentially with decreased sap flow rate (Else et al. 1995; Figure 2-12). When collected at transpirational flow rates, ABA delivery in flooded plants was decreased to 11% of controls (Else et al., 1995). Thus, an appropriate xylem sap sampling methodology is essential to interpret the effects of changes in soil properties on xylem sap hormone composition.

Roles of other phytohormones in compaction stress

Within the literature, different workers have measured phytohormone concentrations in different tissues (shoots, roots), and concentrations may be affected by sampling techniques or plant age. Alternative approaches may enhance our understanding of root-to-shoot signalling of compaction stress. For example, it may be difficult to compare xylem sap hormone concentrations between workers due to differences in sap flow rate caused by the application of arbitrary overpressures, or an inability to match transpirational flow rates. ABA concentrations

may be artificially inflated or diluted depending on sampling method (Dodd, 2005). Collecting root xylem sap at transpirational flow rate is not possible in the field, as it requires applying pressure to the root system. Therefore, matching pressure-induced flow rate with *in vivo* transpiration rate may improve the accuracy of measured phytohormone concentrations in xylem saps emanating from the root system (Dodd, 2005; Netting et al., 2012). This method has not yet been employed in plants growing in compact soil and may offer new insights into signalling of soil compaction stress.

Multi-hormone analyses of foliar tissues and sap samples aimed to quantify 11 phytohormones simultaneously (although not all were detected as discussed above), which has so far not been done for plants grown in compact soil. Furthermore, this methodology assessed other hormones beyond those classically-associated with compaction stress.

Ethylene has been implicated in compaction stress, since increased ethylene evolution and shoot growth restriction was observed in wild-type tomato growing in uniformly compact soil (Hussain et al., 1999; Hussain et al., 2000). However, in this work no consistent significant effect of increasing bulk density was observed on foliar ACC (Figure 2-11), the precursor of the gaseous hormone ethylene. Foliar ACC was significantly decreased by water deficit (Figure 2-11), contrary to observations that foliar ethylene evolution increases with partial soil drying (Sobeih et al., 2004), but consistent with other findings (Morgan et al., 1990). Increased ethylene biosynthesis may only occur under specific environmental conditions, such as hypoxia: xylem sap ACC delivery rates from the roots were raised 3.1-fold in flooded tomatoes (Else et al., 1995). Similarly, ACC delivery increased nearly 4-fold after 48 h of flooding, while

petiole ethylene evolution increased 7-fold (English et al., 1995) and [L-ACC] was positively correlated with increased foliar ethylene evolution (Else and Jackson, 1998). Either ethylene synthesis had no effect here, or its effect was overridden by another signal: like ABA, it is possible that foliar ethylene evolution is only able to attenuate the effect of bulk density below a critical level of stress (Hussain et al., 1999).

Foliar [tZ] decreased in response to increased soil bulk density (Figure 2-11). This agreed with the decreased shoot nitrogen status; as cytokinins are highly correlated with nitrogen availability (Kiba et al., 2010). Nutrient limitation may explain some of the growth reduction exhibited by plants growing in compact soil, but stunted growth has been reported in many systems where nutrients are not limiting (Masle and Passioura, 1987; Whalley et al., 2006; Jin et al., 2015). In contrast, [X-tZ] did not respond to changes in bulk density (Figure 2-10); yet xylem sap cytokinins are known to increase in response to increased nitrogen availability (Rahayu et al., 2005). Taken together, there is no compelling evidence that compaction-induced differences in N uptake are translated into a root-to-shoot CK signal.

Under well-watered conditions, plants grown in compact soil had significantly higher xylem JA concentrations (Figure 2-10). JA is typically associated with herbivory and wounding responses, and its biosynthesis and perception activate defence mechanisms including proteinase inhibitors and synthesis of toxic metabolites (Wasternack and Hause, 2013), and are conserved across almost all plant species including tomatoes (Sun et al., 2011). Perception of JA signals enhance plant defences at the expense of growth. Exogenous JA applications to inhibit root growth

are often used in mutant screening (Wasternack and Hause, 2013). Application of high concentrations of JA (0.1-10 μM) to isolated tomato roots stunted axile root growth and lateral root initiation, with significantly increased diameter close to root tips (Tung et al., 1996). However, JA may also promote root thickening (Tung et al., 1996), an important trait for successful root growth in strong soil (Bengough et al., 2011). Furthermore, there is some evidence of JA biosynthesis in response to root mechanical pressure: when external pressure was applied to roots using an agar block, increased JA biosynthesis was quantified through fluorescence of JA perception biosensor Jas9-VENUS (Larrieu et al., 2015). Further work is needed to determine whether enhanced root JA concentrations allow continued root elongation in compact soil.

JA also restricts shoot growth: (Moore, Taylor, et al., 2003) showed that infection of a pathogen in leaf 4 of *Rumex obtusifolis* reduced leaf expansion rates of subsequently emerging leaf 8; further experiments revealed that exogenous JA applications produced the same response by reducing cell wall extensibility and expansion (Moore, Paul, et al., 2003). JA has been implicated in mechanoresponse signalling: WT Arabidopsis subjected to touch treatments over a four-week period increased endogenous JA almost 3-fold and reduced rosette size by 28%, while JA-deficient *aos* displayed no response (Chehab et al., 2012). However, foliar [JA] decreased (Figure 2-11), calling into question the physiological significance of increased xylem JA as a root-to-shoot signal. Further work must investigate the physiological action of increased [X-JA] in plants grown in high bulk density soil, particularly its subsequent action in shoot tissues.

Increased soil bulk density significantly reduced relative levels of bioactive GA_1 in leaf tissues. Gibberellins promote germination, stem elongation, leaf expansion and flowering (Hedden and Sponsel, 2015). Gibberellins are biosynthesised from isoprenoid precursors via the MEP pathway, similar to ABA, CKs and brassinosteroids (Schwartz and Zeevaart, 2010). The bioactive gibberellins destroy DELLA proteins by forming the GA-GID1-DELLA complex, preventing sequestration of transcription factors involved in growth by DELLA and promoting growth (Harberd et al., 2009). Reductions in foliar GA levels seen here (Figure 2-11) may explain reduced growth even under WW conditions, but the significance of root-to-shoot GA signalling is unclear. Bioactive GAs have already been implicated in compaction responses. Levels of bioactive GAs were reduced in wheat growing in high soil strength conditions (Colebrook et al., 2014), and applying bioactive GA_3 to roots of plants grown under high soil strength conditions (in the absence of water and nutrient limitations) improved shoot growth (Coelho-Filho *et al.* 2013). However, the concentrations of bioactive GAs in roots are typically far lower in roots than in shoots (Tanimoto, 2005) and so the role of root-sourced GAs in compaction stress signalling remains to be established. However, bioactive gibberellins, particularly GA_3, are used to improve vegetative growth of many horticultural and agricultural crops (Stuart and Cathey, 1961; Rademacher, 2016), and there are potential uses for exogenous GAs to alleviate restricted shoot growth of plants in strong soil.

Conclusions

Taken together, increased soil bulk density alters the relationship between plant water status and bulk soil water content, potentially by limiting water extraction from the bulk soil. However, Ψ_{root} remains a good indicator of [X-ABA] regardless of soil bulk density. Furthermore, this study is the first time multi-hormone analyses have been utilised on foliar and sap samples from plants grown in compact soil. Potential roles for jasmonic acid and bioactive gibberellins have been revealed in this study. Further work will look to ameliorate the negative effects of increased bulk density on shoot growth rates by applying gibberellic acid, a bioactive gibberellin and known growth promoter.

Chapter 3 GA_3 soil drenches rescue leaf expansion in compact soil, but alter plant water and phytohormonal status

3.1 Introduction

Soil compaction reduces plant growth by increasing soil mechanical resistance and potentially restricting access to crucial resources including water and nutrients (Hamza and Anderson, 2005). In Chapter 2, slight (but statistically significant) decreases in leaf water potential were detected in expanding leaves of plants growing in high bulk density soil, suggesting water limitation. However, shoot growth rate may be inhibited even when changes in shoot water status were not detected (Masle and Passioura, 1987; Andrade et al., 1993), suggesting the action of a root-sourced signal produced in response to soil mechanical resistance. Phytohormonal analyses of foliar tissues and root-sourced xylem sap revealed possible roles for jasmonic acid, gibberellins and cytokinins in regulating plant growth responses in compact soil (Chapter 2). Since cytokinins and jasmonic acid have not previously been implicated in regulating physiological responses to soil compaction, whereas gibberellins (GAs) have (see p. 84), the current chapter focused on the role of gibberellins in plant growth regulation. Moreover, manipulating endogenous GA levels to promote or reduce growth is common in commercial agriculture (Rademacher, 2016), and growth promotion via exogenous GA_3 application may offer possibilities to overcome the impacts of strong soil.

GAs are diterpenoid acids found in many species of plant, fungi and bacteria. In plants, GAs are involved in a number of developmental processes including seed

germination, cell division and elongation, and transitions between vegetative and reproductive growth phases (Colebrook et al., 2014). High levels of GAs are present in growing tissues, and levels of bioactive GAs are maintained by feedback regulation, where bioactive GAs repress the expression of genes encoding for GA biosynthesis (Hedden and Phillips, 2000). GAs promote growth by destroying DELLA proteins, which inhibit plant growth by sequestering transcription factors and blocking their activity (Harberd et al., 2009). The GA-GID1-DELLA complex formed with bioactive GA species reduce the efficacy of DELLA proteins to interact and inhibit growth-promoting transcription factors by allowing binding of DELLA to $SCF^{SLY/GID2}$ E3 ubiquitin ligase, which is then destroyed by the proteasome (Harberd et al., 2009). Thus, bioactive GA species play an important role in developmental processes by degrading DELLA proteins, thus promoting activity of growth-related transcription factors.

The concentrations of endogenous bioactive GAs decrease in response to abiotic stresses including osmotic, salt and temperature (reviewed in Colebrook et al., 2014), thus decreasing plant growth and yield. GA_3 levels in maize leaves declined by 75% after 7 days of growth in medium supplied with 12% polyethylene glycol (osmotic potential of -0.4 MPa), with a corresponding decrease in plant height of 20% relative to controls (Wang et al., 2008). Reduced levels of bioactive GAs were reported in the leaves of wheat with mechanically-impeded roots, in the absence of water deficit (Coelho Filho et al., 2013; Colebrook et al., 2014).

Exogenous applications of GAs to crops can improve growth, even under optimal conditions, thus promoting fruit production in grapes (Hedden and Phillips, 2000)

and maintaining citrus fruit quality (Lacey and Walsh, 2017). GA_3 increased numbers of potato tubers when applied between 38-40 days after planting (Struik et al., 1989). However, the effect of GA_3 application may be dependent on whether crop growth is restricted by abiotic stress.

Exogenous GAs have been widely used to alleviate growth restrictions from abiotic stress. In maize, foliar applications of 50 mg L^{-1} GA_3 during the vegetative growth phase increased shoot dry weight by 50% under in plants receiving 75% less water than controls (Akter et al., 2014). Foliar GA_3 application (50 and 100 ppm) on salt-stressed maize (100 mM NaCl) improved root and shoot dry matter accumulation, increased nutrient status and cell membrane permeability (Tuna et al., 2008). GA may also be applied to the roots, incorporated into growing media or as a soil drench. When tomato plants exposed to different salinity levels (28-88 mM Na and 55-177 mM Cl) were irrigated with 100 mg GA_3,total water use and fruit yield was increased under low salinity conditions (Maggio et al., 2010). Notably, root-supplied GA_3 improved leaf expansion in wheat grown in a strong substrate (Coelho Filho et al., 2013). Many rootzone stresses increase root:shoot ratio (Bloom et al., 1985), and this may be reversed by GA_3 application. Exogenous GA_3 may reduce root elongation (Morris and Arthur, 1985; Coelho Filho et al., 2013; Wang et al., 2015), perhaps as plants redistribute available resources to the shoot.

Endogenous GA interacts with other phytohormones, influencing developmental and growth responses (Weiss and Ori, 2007), thus manipulating GA status with exogenous applications may alter wider phytohormonal profiles. Root drenches of 150 ppm GA_3 increased expression of biosynthesis genes of cytokinin, ABA and

brassinosteroids in carrot leaves (Wang et al., 2015). Multi-hormonal analyses of *S. lycopersicum* receiving foliar GA_3 sprays while growing in saline conditions (100 mM NaCl) revealed increased foliar tZ and iP concentrations (Khalloufi et al., 2017). Foliar GA_3 application inhibited cytokinin responses in tomato, particularly repression of primary response genes, reduced anthocyanin accumulation and simplified leaf shape (Fleishon et al., 2011), suggesting further effects of GA_3 on sensitivity to phytohormonal signals. Taken together, interactions between multiple endogenous phytohormones may affect plant responses to abiotic stress in unpredictable ways. The effects of exogenous GA_3 applications on the concentrations of various other phytohormones have been scarcely investigated.

This study aimed to investigate the growth and physiological responses of plants grown in compact soil to GA_3 soil drenches. It was hypothesised that GA_3 would be translocated to the shoots and promote plant growth in compact soil by enhancing concentrations of bioactive gibberellins in sap and foliar tissues. GA_3 application was hypothesised to increase transpiration rates, due to possible effects on ABA sensitivity, and that root-supplied GA_3 enhanced water use in tomato (Maggio et al., 2010). Furthermore, the effects of exogenous GA_3 on phytohormonal profiles of root-sourced xylem sap and leaf tissues were investigated.

3.2 Materials and methods

Growing conditions

Soil was prepared and pots were filled to high and low bulk densities, as described in Chapter 2.2.

Tomato seeds (*Solanum lycopersicum* cv. Ailsa Craig) were surface sterilised in 10% thick bleach (5% sodium hypochlorite) for 5 minutes, rinsed thoroughly with deionised water and placed on filter paper (Whatman No. 1) in 90 mm petri dishes. Dishes were dark-incubated at 21°C for 48-72 hours, until most radicles had emerged and were at least 2-3 mm long. Three seeds per pot were transplanted into holes to a depth of 2 cm below the surface of the soil, and loosely covered with small amount of soil. Plants were grown for approximately 28 days in controlled environment rooms at 24°C/19°C (day/night), with a 12-hour photoperiod (07:00 to 19:00). Pots were placed in a random arrangement which was changed every two days to minimise effects of environmental variation. Plants were watered daily between 14:00 and 16:00 with tap water to approximately 0.16 ± 0.01 g g^{-1} GSWC, and pot weights recorded daily to monitor ET.

Gibberellin treatment

Powdered GA_3 (>90% total gibberellins; Sigma-Aldrich, U.K.) was dissolved in a few drops of 1 M KOH, and made up to 1 L with deionised water (supplying 100 µM/34.6 mg L^{-1}/34.6 ppm GA_3). The pH of the solution was adjusted to approximately pH 7 using 0.1 M HCl. The Control solution contained the same volume of KOH in 1 L of

deionised water, adjusted to pH 7. GA or Control solutions were applied from 8 days after transplanting (DAT). Solutions were applied twice weekly between 2-5 hours after the start of the photoperiod, and plants received 6 root drenches in total over the experimental period.

Plant measurements at harvest

Four replicate experiments were grown between September 2017 and January 2018 (n = 5-6 per replicate). Measurements are tabulated by experiment (Table 3-1). Expanding leaf tissue was cut, frozen in liquid nitrogen and stored at -20 C for multi-hormone analyses (See Chapter 2.2). In all experiments, stomatal conductance (g_s) of a fully-expanded leaf was measured using an AP4 Porometer (Delta-T Devices, Cambridge, UK), and Ψ_{leaf} of the same leaf was measured using a tall Scholander pressure vessel (Soilmoisture Equipment Corp., Santa Barbara, CA., USA). Plants were harvested approximately 28 DAT. Furthermore, in 2 experiments, pots were weighed 1 hour prior to and immediately before harvest to determine soil water content and transpiration rate. Plants were de-topped approximately 6 cm from the soil surface and the root system was pressurised in the same pressure chamber to obtain Ψ_{root}. Subsequently, pressure was increased in 0.04 MPa increments to collect xylem sap at transpirational flow rate. Leaves longer than 1 cm were counted, and total leaf area determined with a Leaf Area Meter (Li-3100 Leaf Area Meter, Li-Cor inc., Lincoln, Nebraska, USA) and shoots were dried at 80°C for at least 48 h to obtain biomass and specific leaf area. Roots from a subset of 1 experiment (*n* = 4) were washed to remove soil, scanned (Expression 11000XL, EPSON, Seiko Epson Corp.,

Japan) and architecture analyses performed using WinRHIZO Pro 2013 (Regent Instruments Inc., Canada).

Phytohormonal analyses

Frozen foliar tissues were freeze-dried and ground, and 10 mg samples were reserved for phytohormonal analyses. At least 100 µL of root xylem sap was collected per plant at transpirational flow rates for phytohormonal analyses. Analyses were kindly carried out by Dr. Alfonso Albacete (CEBAS-CSIC, Murcia, Spain), as described in Chapter 2.2. Nine (of 11) hormones were detected with over 50% frequency in foliar tissues, and six (of 11) in xylem sap samples.

Nitrogen analyses

Shoot tissues were dried for at least 48 h at 80°C. Samples were prepared and analysed as detailed in Chapter 2.2.

Table 3-1: Measurements taken at harvest for each of four replicate experiments (September 2017-January 2018).

Experiment	Measurements
3.1	Biomass, Ψ_{leaf}, g_s, root length and diameter
3.2	Biomass, g_s
3.3	Biomass, Ψ_{leaf}, Ψ_{root}, root hydraulic conductivity, transpiration rates, shoot nitrogen, sap and foliar phytohormone
3.4	Biomass, Ψ_{leaf}, Ψ_{root}, root hydraulic conductivity, transpiration rates, shoot nitrogen, sap and foliar phytohormone

Statistical analyses

Root data was collected in one batch and analysed as a 2-way (bulk density x root drench) design. Post-hoc LSD tests ($p < 0.05$) were used to distinguish between groups. Treatment differences in shoot biomass, water status and phytohormonal profiles were analysed using 3-way ANOVA (experiment x bulk density x root drench design). Statistical analyses were conducted on phytohormones with a frequency detection rate of at least 50% of analysed samples. Appropriate transformations were applied to improve normality of residuals and are indicated where used. Data are presented as back-transformed means across blocks. One-way ANOVA and post-hoc LSD were used to differentiate between group means of phytohormonal data.

Pearson's correlations were conducted to explore relationships between foliar and sap phytohormone concentrations and measurements of plant water status. Pearson's r coefficients are reported, and *p* values included when statistically significant.

Multiple linear regressions were used to explore relationships between continuous and categorical predictors, non-linear data were subject to appropriate transformation (e.g. $\log_{10}$). Full interaction models were built with categorical and one continuous predictor, and terms sequentially dropped when their significance was below the 5% level (drop1() function, F-test; R Core Team, 2018). *p*-values for final model predictors are presented.

3.3 Results

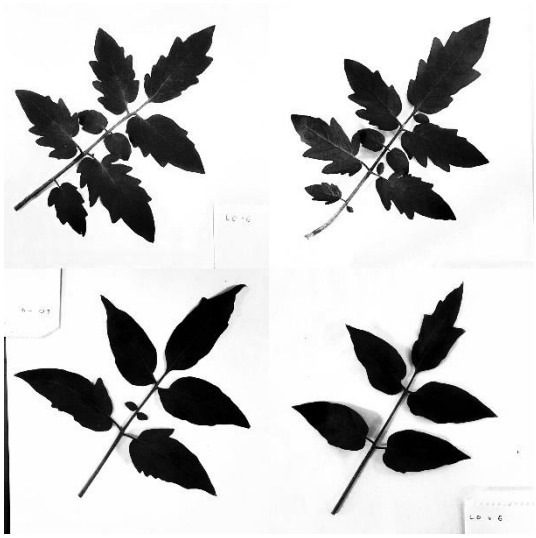


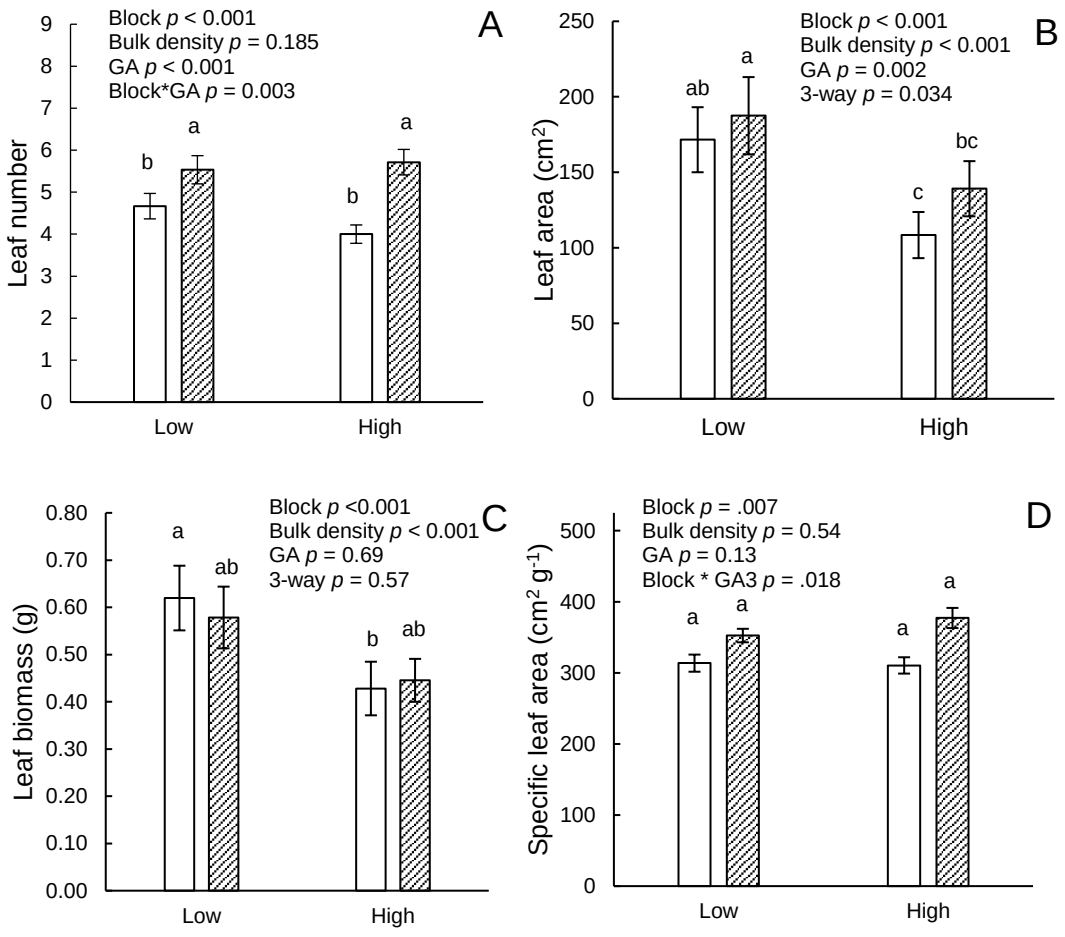
Figure 3-1: GA_3 soil drenches changed leaf morphology, particularly by smoothing the leaf edges. Top row: Control. Bottom row: GA_3 drench.

Plant growth and water relations

Exogenous application of GA_3 to the rootzone resulted in plants with a simplified leaf phenotype, displaying smoother leaf edges than control plants (Figure 3-1). Plants receiving the bi-weekly GA_3 root drench also increased leaf number by up to 50% ($p < 0.001$) at harvest (Figure 3-2A).

Across all blocks, soil compaction decreased leaf area by 37% ($p < 0.001$; Figure 3-2B). GA_3 application significantly rescued leaf area ($p = 0.002$) by 9% in the low bulk density treatment and 28% in the high bulk density treatment. There was a significant effect of block on leaf area as plants in Block 4 exhibited 3-fold greater leaf area overall. A significant 3-way interaction between block, soil compaction treatment and GA_3 application ($p = 0.034$), suggested variation in the response of leaf area to compaction and GA_3 application across blocks. Overall, leaf area increased in response to GA_3 application, and decreased in response to increasing bulk density.

Although increased bulk density significantly reduced the total leaf biomass obtained ($p < 0.001$; Figure 3-2C) at harvest, this did not translate into significant changes in specific leaf area, even with the rescue of leaf expansion (Figure 3-2D). Leaf biomass varied across blocks ($p < 0.001$), but the effect of bulk density was conserved. However, there was a significant block effect and block*GA interaction, as GA_3 application increased specific leaf area in Block 4. However, percentage shoot nitrogen content (Figure 3-2E) was significantly reduced by both increased bulk density ($p = 0.002$) and GA_3 application ($p = 0.007$). There was a significant block effect, as contents were higher in block 2 ($p = 0.003$).



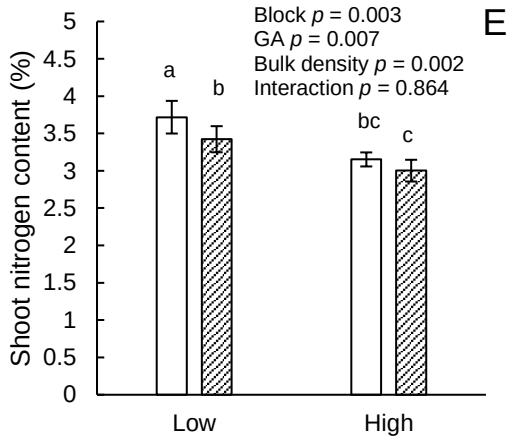


Figure 3-2: Mean leaf number (A), leaf area (B), leaf biomass (C), specific leaf area (D) and shoot nitrogen content (E) of plants growing in low or high bulk density soil. White bars represent control soil drenches, shaded bars represent plants receiving GA_3 soil drenches. Bars are means ± S.E. of 10-20 replicates, with different letters denoting significant differences between means (post-hoc LSD $p < 0.05$). p values reported for block, GA_3 treatment, bulk density and their interactions.

Mean Ψ_{leaf} was significantly reduced by both increasing soil bulk density and GA_3 application (Figure 3-3B). Although bulk GSWC was maintained at approximately 0.16 ± 0.01 g g^{-1} by daily watering near the end of the photoperiod, bulk GSWC was significantly reduced in the low bulk density treatment ($p < 0.001$) at harvest (midway through the photoperiod), but there was no effect of GA_3 application. There was no significant relationship between bulk GSWC and Ψ_{leaf}, despite the range of water contents (0.10-0.15 g g^{-1}) within treatments at harvest (Figure 3-3A). Instead, GA_3 application significantly reduced Ψ_{leaf} at the same GSWC ($p = 0.026$), and high bulk density tended to decrease Ψ_{leaf} ($p = 0.075$).

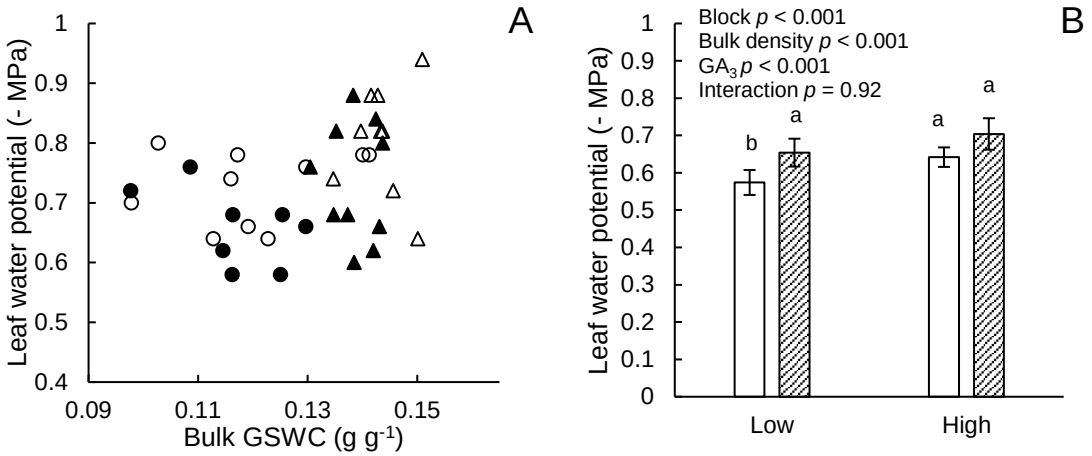


Figure 3-3: A) Bulk GSWC and Ψ_{leaf} were not correlated, despite the range of soil water contents at harvest (and significantly lower SWC in low bulk density treatment). Circles: low bulk density soil. Triangles: high bulk density soil. Each symbol is an individual plant from the control (filled) or GA_3 (hollow) treatments. . B) Mean leaf water potential (- MPa) of plants grown in low or high bulk density soil. White bars represent control soil drenches, shaded bars represent GA_3 treatment. Leaf water potential was significantly reduced by both increased soil bulk density and GA_3 application. Bars are means ± S.E. of 15 replicates, with different letters denoting significant differences between means (post-hoc LSD $p < 0.05$). *p* values reported for block, GA_3 treatment, bulk density and their interactions.

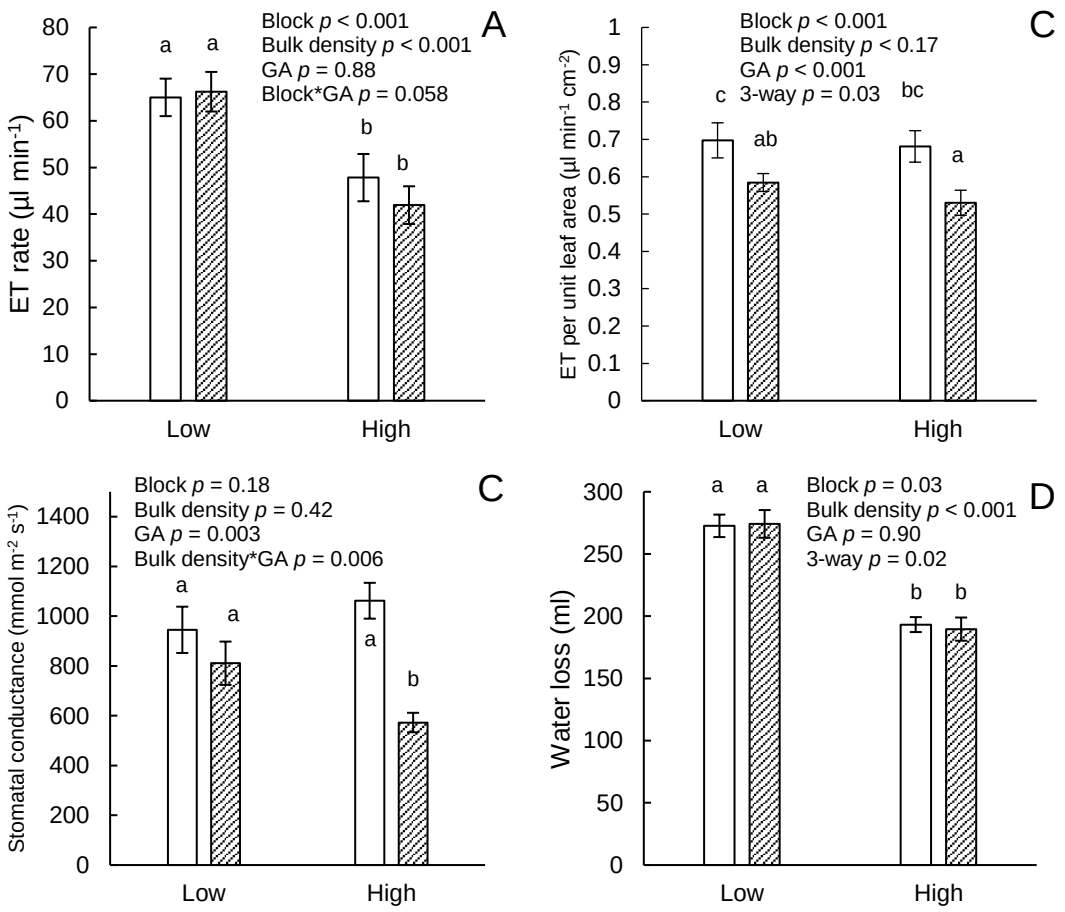


Figure 3-4: Mean A) ET rate, B) ET rate per unit leaf area, C) stomatal conductance at harvest and D) total water lost in the week prior to harvest, under low and high bulk density soil. White bars represent the control root drench and shaded bars represent the GA_3 treatment. ET rate over 80 minutes prior to harvest was reduced by increasing soil bulk density (A), but when normalised to total leaf area this was significantly influenced by GA_3 application. GA_3 application significantly reduced mean stomatal conductance (C), as measured directly using the AP4 porometer, at harvest. D) Total water lost in the week prior to harvest was significantly reduced in high bulk density soil, but not affected by GA_3 treatment, consistent with ET rate. Bars are means ± S.E. of 10 replicates, with different letters denoting significant differences between means (post-hoc LSD p < 0.05). p values reported for block, GA_3 treatment, bulk density and their interactions.

Soil compaction significantly (p < 0.001) decreased absolute ET by 33% (averaged across GA_3 application treatments) in the hour prior to harvest (Figure 3-4A), but there was no effect on relative ET which was normalised by leaf area (Figure 3-4B). GA_3 application decreased relative ET by 20% (averaged across soil bulk density treatments), which was consistent with GA_3-induced stomatal closure (Figure 3-4C).

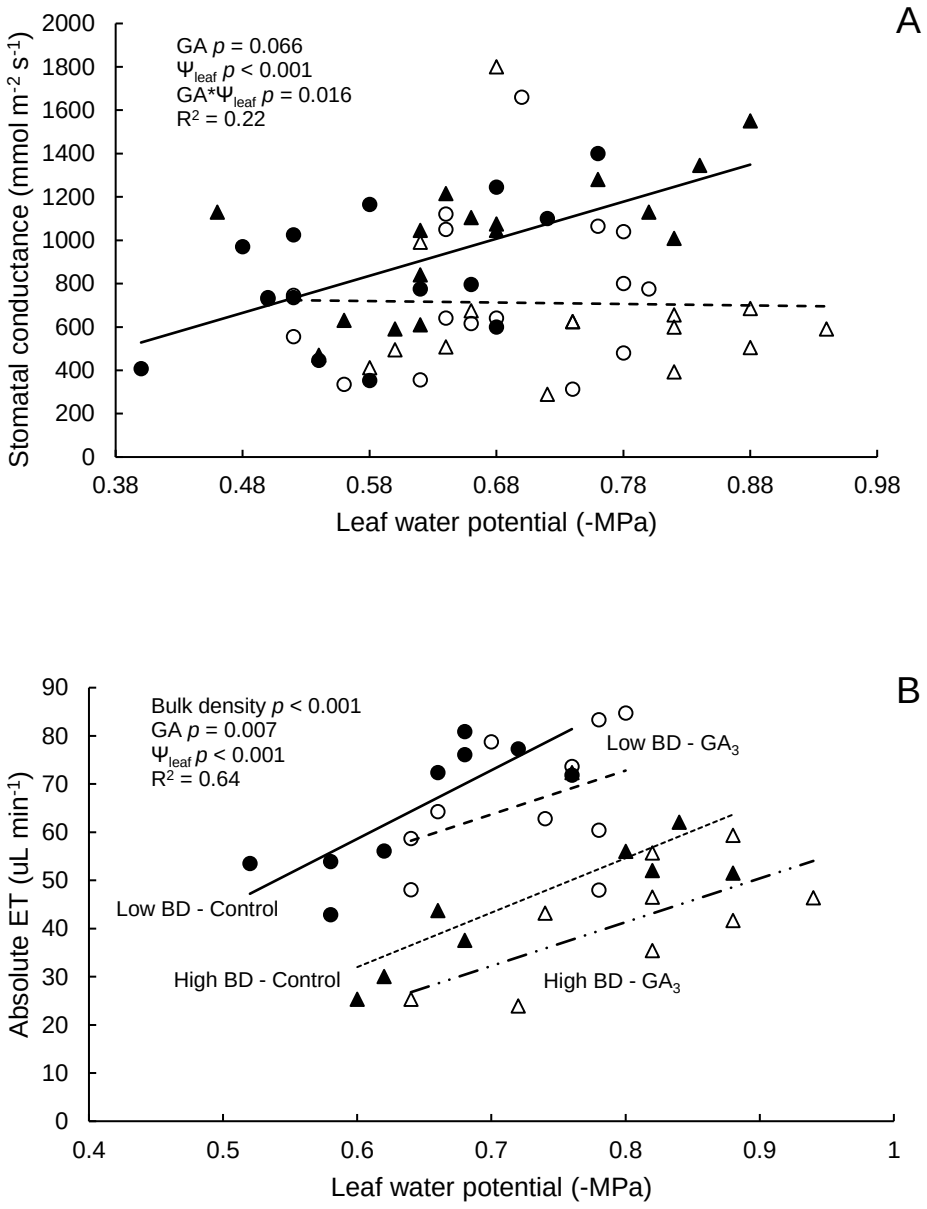


Figure 3-5: The relationship between A) stomatal conductance and Ψ_{leaf} across 4 replicate experiments, B) ET rate and Ψ_{leaf} in 2 experiments, under 4 combinations of soil bulk density and root drench treatments. Circles represent individual plants grown at low soil bulk density, triangles represent high bulk density treatments. Filled markers correspond to control root drenches and hollow markers represent GA_3 treatments. Trendlines denote linear relationships (BD = Bulk density). *p* values reported for remaining model predictors (leaf water potential, bulk density and their interaction).

Co-variation of stomatal conductance with leaf water potential changed with GA_3 treatment, as indicated by a significant interaction ($p = 0.016$) between Ψ_{leaf} and root drench (Figure 3-5A). In Control plants, Ψ_{leaf} decreased with increasing stomatal

conductance but in plants receiving the GA_3 root drench, g_s and Ψ_{leaf} were not correlated (as indicated by the flat trendline). Nevertheless, both increased bulk density and GA_3 application reduced absolute ET at the same Ψ_{leaf} ($p < 0.001$ and $p = 0.007$ respectively), with Ψ_{leaf} decreasing as absolute ET increased (Figure 3-5B). Taken together, GA_3 root drenches decouple g_s and Ψ_{leaf} in tomato, and reduce total transpirational losses.

Increased soil bulk density significantly decreased total root length ($p < 0.001$) by 49% but significantly increased root diameter by 42% ($p < 0.001$; Figure 3-6A & B). Neither root trait was affected by GA_3 drench. Ψ_{root} at harvest was maintained at positive pressure (recorded as 0 MPa) in plants grown at low soil bulk density but was reduced by 0.1-0.15 MPa in high bulk density treatments.

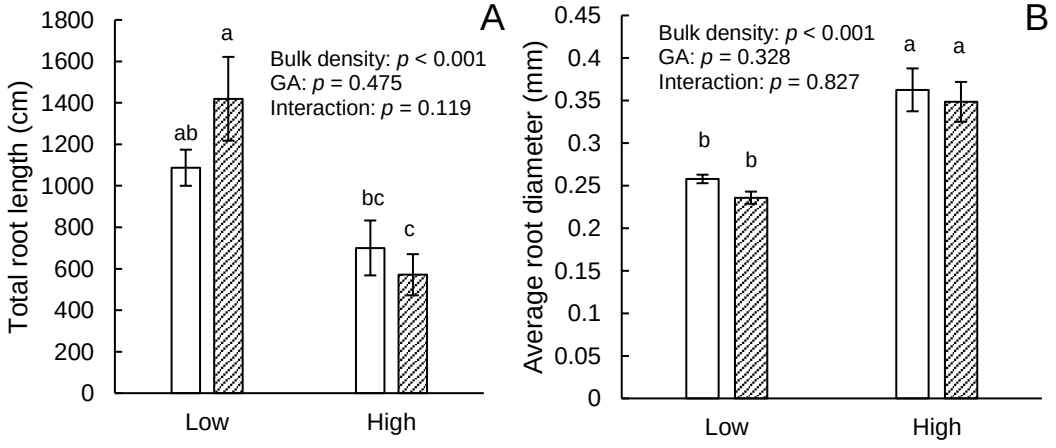


Figure 3-6: A) Total root length was significantly reduced by increased soil bulk density. B) Root thickness was increased under high soil bulk density. GA_3 application exerted no significant influence on either parameter. Bars are means ± S.E. of 4 replicates, with different letters denoting significant differences between means (post-hoc LSD $p < 0.05$). p values reported for bulk density, GA_3 treatment, and their interaction.

The slope of the relationship between applied pressure and xylem sap flow rate constitutes hydraulic conductivity of the root system (Figure 3-7A). Increased soil bulk density significantly decreased the mean regression slope by 24% (p = 0.025; Figure 3-7B). There was a significant effect of block (p = 0.016), as the conductivity of Low-GA_3 was higher in Block 1. However, exogenous GA_3 application significantly reduced root hydraulic conductivity by 30% (p = 0.008) overall.

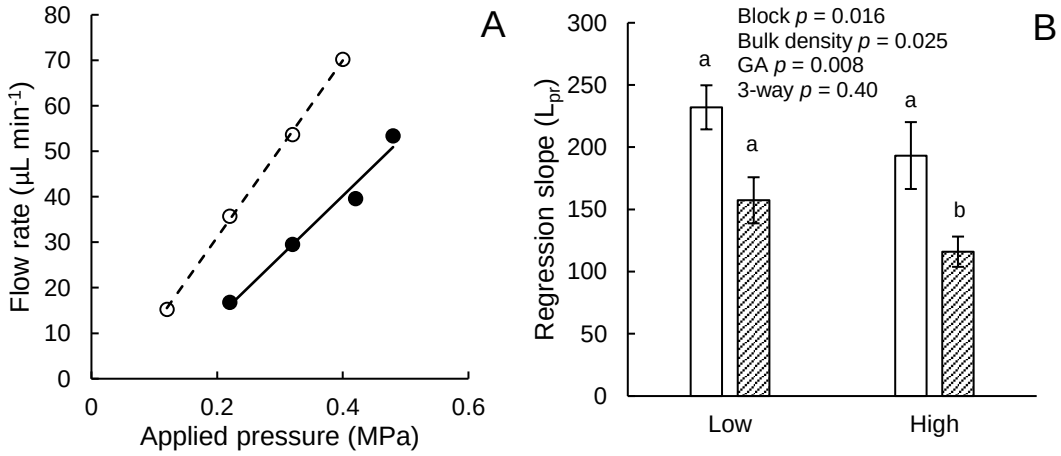


Figure 3-7: A) A linear relationship exists between pressure applied to the rootzone and xylem sap exuded from the cut surface of the detopped stem. Hollow symbols are sequential flows from a plant grown in loose soil, while filled symbols are from a plant grown in compact soil. The slope of the trendline constitutes the hydraulic conductivity of the root system. B) Mean values of the regression slopes for flow rate vs. pressure relationship in tomatoes grown in loose or compact soil. White bars represent plants receiving control root drench, shaded bars received GA_3 drenches. Bars are means ± S.E. of 8-10 replicates, with different letters denoting significant differences between means (post-hoc LSD $p < 0.05$). p values reported for block, bulk density, GA_3 treatment, and their interaction.

Phytohormonal profiles

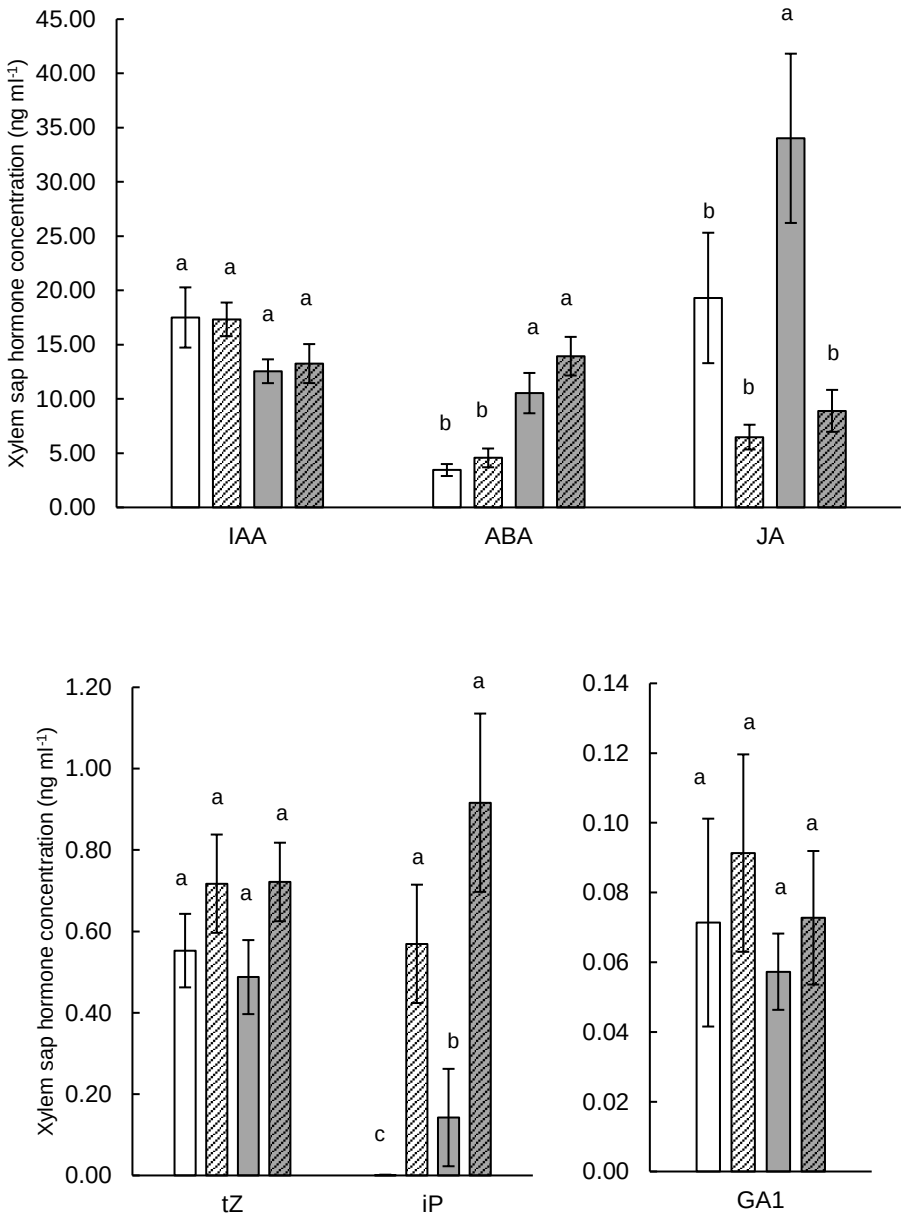


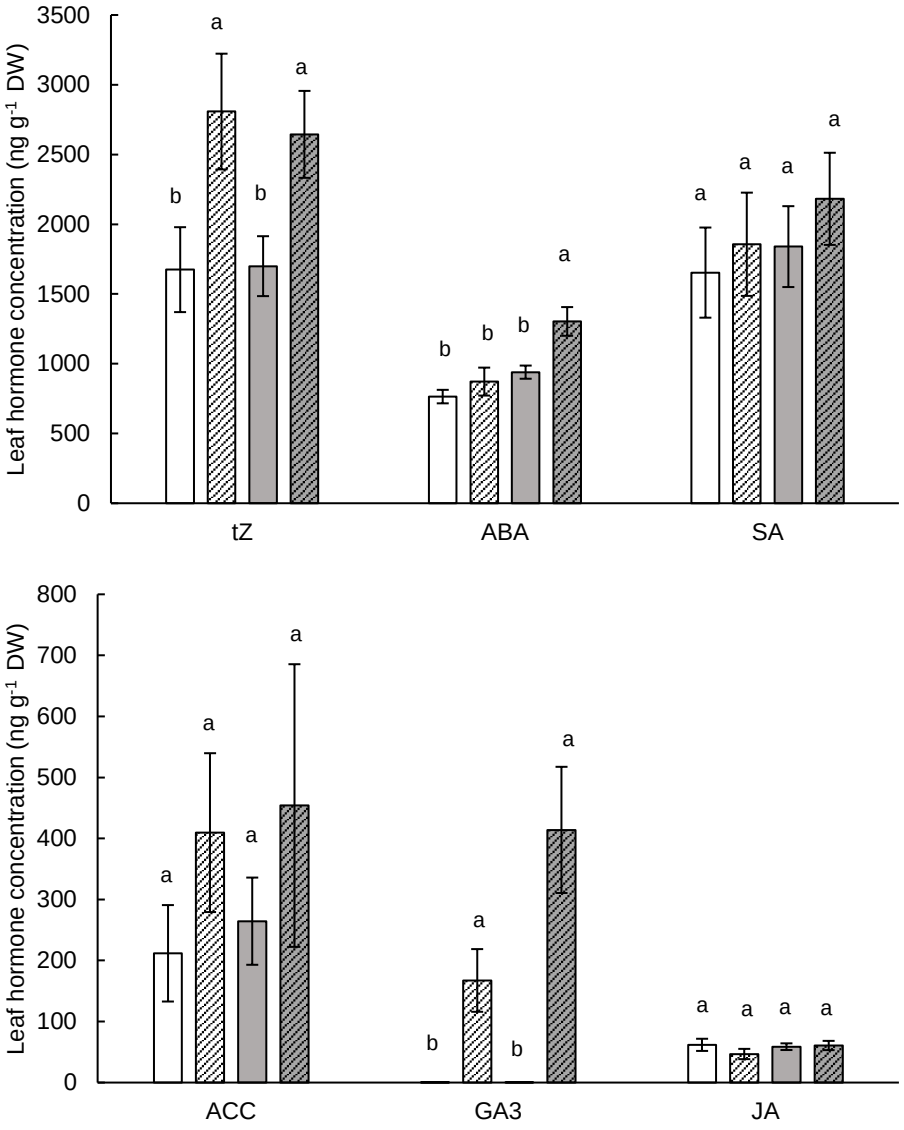
Figure 3-8: Phytohormonal concentrations of root xylem sap collected from plants grown in low bulk density soil/control root drench (white bars), low bulk density soil/GA_3 root drench (striped bars), high bulk density soil/control root drench (grey bars), and high bulk density soil/GA_3 root drench (grey striped bars). Bars are means ± S.E. of 8-10 replicates, with different letters denoting significant differences between means within an analyte (post-hoc LSD $p < 0.05$).

Xylem [JA] was significantly increased by 69% in plants grown in high bulk density soil ($p = 0.034$; Figure 3-8; p values in Table 3-2), but GA_3 application decreased JA

concentrations by 71% in plants grown in high bulk density soil ($p < 0.001$). Increasing soil bulk density significantly increased ABA concentrations in root xylem sap ($p < 0.001$) by 2-fold and reduced levels of IAA by 26% ($p = 0.019$), but no effects of GA_3 root drench on either ABA or IAA concentrations were observed. Both increased bulk density and GA_3 applications significantly raised concentrations of the cytokinin iP ($p = 0.018$ and < 0.001 respectively). Neither GA_3 or bulk density significantly affected tZ or GA_1 concentrations in xylem sap. Thus, high bulk density increased [ABA], [iP] and [JA], while reducing xylem [IAA]. GA_3 drenches significantly enhanced xylem [iP] and interacted with bulk density treatment to significantly reduce [JA] in plants growing in high bulk density conditions.

Table 3-2: *p*-values from 3-way ANOVA analyses of root xylem sap phtyohormone concentrations. Significance of *p* values reported thus: · p is marginally non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Interaction is 3-way (Block*GA_3*Bulk density = BL x GA x BD) unless otherwise indicated.

Hormone	Transformation	Block	Bulk density	GA	Interaction
IAA		0.98	0.019 *	0.99	0.90
ABA		0.40	< 0.001 ***	0.15	0.91
JA	$\log_{10}$(JA + 0.01)	0.63	0.034 *	< 0.001 ***	0.08 ·
tZ		0.90	0.73	0.089 ·	0.99
iP	$\log_{10}$(iP + 0.01)	0.058 ·	0.018 *	< 0.001 ***	0.46
GA_1		0.20	0.31	0.69	0.87


Leaf hormone concentration (ng g-1 DW)
3500
3000
2500
2000
1500
1000
500
0
b
a
b
a
b
b
b
b
a
a
a
a
a
a
a
a
tZ
ABA
SA
Leaf hormone concentration (ng g-1 DW)
800
700
600
500
400
300
200
100
0
a
a
a
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b
a
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ACC
GA3
JA

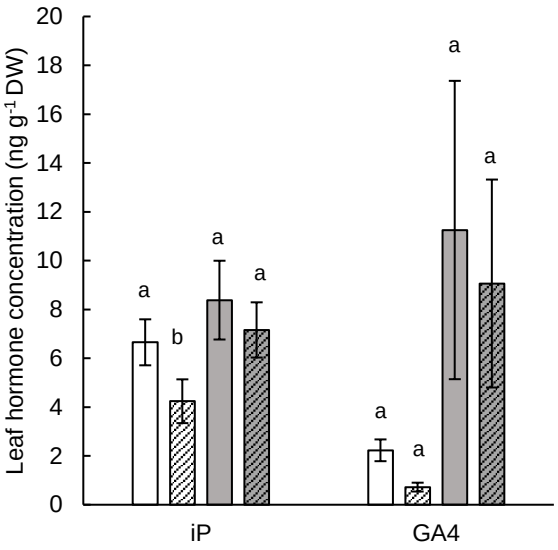

Figure 3-9: Phytohormonal concentrations of leaf tissues from plants grown in low bulk density soil/control root drench (white bars), low bulk density soil/GA_3 root drench (striped bars), high bulk density soil/control root drench (grey bars), and high bulk density soil/GA_3 root drench (grey striped bars). Bars are means ± S.E. of 8-10 replicates, with different letters denoting significant differences between means within an analyte (post-hoc LSD $p < 0.05$).

GA_3 root drenches significantly increased concentrations of GA_3 (over 1000-fold) in actively growing leaf tissues ($p < 0.001$; Figure 3-9; p values in Table 3-3). Concentrations of the cytokinin tZ were also significantly elevated in plants receiving GA_3 drenches ($p = 0.002$), and there was a significant block effect on this response, as values were higher overall in Block 2 ($p = 0.041$). The cytokinin iP was significantly increased in foliar tissues at high bulk density ($p = 0.045$). ABA was significantly increased by both GA_3 application ($p = 0.003$) and increasing soil bulk density ($p < 0.001$). There was a significant interactive effect of block and bulk density ($p = 0.007$), and a marginally non-significant interaction between bulk density and GA_3 application ($p = 0.05$). A significant effect of block was also observed on JA concentrations, as concentrations were increased in Block 2, but with no effects of GA_3 drench or bulk density ($p< 0.001$). No effects of block, GA_3 or bulk density were

observed on SA or ACC. Thus, increased bulk density enhanced foliar [iP] and [ABA], while GA_3 application increased [tZ] and [ABA].

Table 3-3: *p*-values from 3-way ANOVA analyses of foliar phtyohormone concentrations. Significance of *p* values reported thus: · *p* is marginally non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. is 3-way (Block*GA_3*Bulk density = BL x GA x BD) unless otherwise indicated.

Hormone	Transformation	Block	Bulk density	GA	Interaction
tZ		0.041 *	0.88	0.002 **	0.39
ABA		0.47	< 0.001 ***	0.003 **	0.007 ** BL x BD
SA		0.52	0.51	0.48	0.40
ACC		0.066 ·	0.83	0.19	0.090 · BL x BD
GA_3	$\log_{10}(GA_3 + 0.01)$	0.46	0.88	< 0.001 ***	0.090 · GA x BD
JA		< 0.001 ***	0.38	0.44	0.58
iP	$\log_{10}(iP + 0.01)$	0.43	0.045 *	0.12	0.59
GA_4	$\log_{10}(GA_4 + 0.01)$	0.56	0.12	0.14	0.18

Correlations between phytohormones and plant/soil water status

Table 3-4: Pearson correlation coefficients of root xylem sap phytohormone concentrations and corresponding measures of leaf water status. Significance of *p* values reported thus: · *p* is marginally non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

	tZ	**iP**	**GA_1**	**IAA**	**ABA**	**JA**	**Ψ_{leaf}**	**Rel. ET**	**g_s**	**Abs. ET**
tZ		0.18	0.25	**0.51 ****	0.12	-0.09	-0.02	-0.16	-0.08	0.02
iP			0.02	-0.09	**0.3 ·**	-0.24	**-0.54 ****	**-0.51 ****	**-0.36 ***	-0.09
GA_1				0.21	0.01	0.07	-0.04	0	0.03	-0.02
IAA					-0.02	0.13	0.26	-0.1	0.07	0.3
ABA						0.11	-0.27	**-0.41 ***	-0.24	**-0.59 *****
JA							-0.06	**0.38 ***	**0.36 ***	-0.11
Ψ_{leaf}								**0.5 ****	0.05	-0.13
Rel. ET									0.19	-0.15
g_s										**0.34 ***
Abs. ET										

Xylem [ABA] and [iP] were significantly negatively correlated with water status parameters (at the $p < 0.05$ level or lower; Table 3-4), while xylem [JA] was positively associated with relative ET and gs. Furthermore, iP was negatively correlated with leaf, relative ET and g_s at the $p < 0.05$ or lower. iP and ABA displayed a marginally non-significant positive correlation, and these sap phyothormones were best associated with plant water status, under relatively WW conditions (compared to Chapter 1).

[L-ABA] showed most significant associations with plant water status, relative to other foliar phytohormones (Table 3-5). [L-ABA] tended to increase with decreasing

Ψ_{leaf} (r = 0.38, p = 0.019), and was negatively related to g_s, absolute and relative ET (r = -0.43, -0.40 and -0.38 respectively, $p < 0.05$). [L-GA_3] was also negatively correlated with relative and absolute ET and g_s ($p < 0.05$) and increased [L-GA_3] was significantly associated with [L-ABA] (r = 0.79, $p < 0.001$) and [L-ZR] (r = 0.42, p = 0.007). [L-JA] correlated positively with Ψ_{leaf} (r = 0.38, p = 0.027), and cytokinins tZ (r = 0.51, $p < 0.001$) and iP (r = 0.42, p = 0.009). Under low water stress conditions, [L-ABA] exhibits enhanced associations with leaf water status than [X-ABA], suggesting increased sensitivity to small changes in plant water status. GA_3 applications were significantly associated with increased [L-ABA] and [L-tZ].

Table 3-5: Pearson correlation coefficients of foliar tissue phytohormone concentrations and corresponding measures of leaf water status. Significance of *p* values reported thus: · *p* is marginally non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

	ACC	**tZ**	**ZR**	**iP**	**GA_3**	**GA_4**	**ABA**	**JA**	**SA**	**Ψ_{leaf}**	**Rel. ET**	**g_s**	**Abs. ET**
ACC		0.01	**0.38 ***	-0.18	0.04	0.17	-0.03	-0.17	**0.27 ·**	0.04	0.07	-0.01	-0.03
tZ			0.03	-0.06	**0.31 ·**	-0.04	0.27 ·	**0.51 *****	0.26	**-0.37 ***	**-0.51 ****	**-0.29 ·**	0.17
ZR				0.11	**0.42 ****	**0.36 ***	**0.4 ***	-0.07	**0.42 ****	-0.06	0.17	-0.21	-0.25
iP					0.03	**0.31 ·**	0.2	**0.42 ****	**0.27 ·**	-0.13	0	-0.01	-0.12
GA_3						0.15	**0.79 *****	-0.1	**0.28 ·**	-0.2	**-0.36 ***	**-0.6 *****	**-0.43 ****
GA_4							0.24	-0.01	**0.34 ***	0.09	-0.14	-0.26	**-0.42 ****
ABA								0.15	**0.3 ·**	**-0.38 ***	**-0.36 ***	**-0.43 ****	**-0.4 ***
JA									0.13	**-0.36 ***	-0.26	-0.15	0.26
SA										-0.05	-0.11	0.06	-0.07
Ψ_{leaf}											**0.49 ****	0.04	-0.13
Rel. ET												0.21	-0.14
g_s													**0.37 ***
Abs. ET													

3.4 Discussion

Gibberellic acid treatment

The concentration of 100 µM GA_3 (equivalent to 34.6 mg L^{-1} or ppm) was chosen as it is commonly used to produce a saturating growth response (Rieu et al., 2008; Coelho Filho et al., 2013). Exogenous GA_3 application enhanced leaf expansion of plants grown in both low and high bulk density soils (Figure 3-2). Its action in non-stressed plants suggests that GA_3 acts as a general growth promoter. Endogenous bioactive GA levels are regulated by feedback mechanisms: increased bioactive GA promotes up-regulation of genes encoding for GA oxidases, e.g. *GA2ox*, thereby preventing excessively high concentrations of bioactive GAs from accumulating in tissues under control conditions. GA_1 and GA_4 are the primary bioactive GAs in higher plants (Hedden and Sponsel, 2015), but GA_3 is widely commercially available, produced from the fungi *Gibberella fujikuroi* (Rademacher, 2016). Bioactive GA action in the tomato shoots is clear even without directly measuring endogenous GA concentrations, as GA accumulation alters leaf phenotype (Figure 3-1). Leaf dissection and leaflet formation in tomato is regulated by KNOX proteins. The sensitivity of growing leaf tissue to KNOX is regulated by endogenous GA levels: reduction of GA leads to increased leaf complexity (greater number of leaflets, serrated shape), while increased GA levels produces a smoother phenotype (Jasinski et al., 2008).

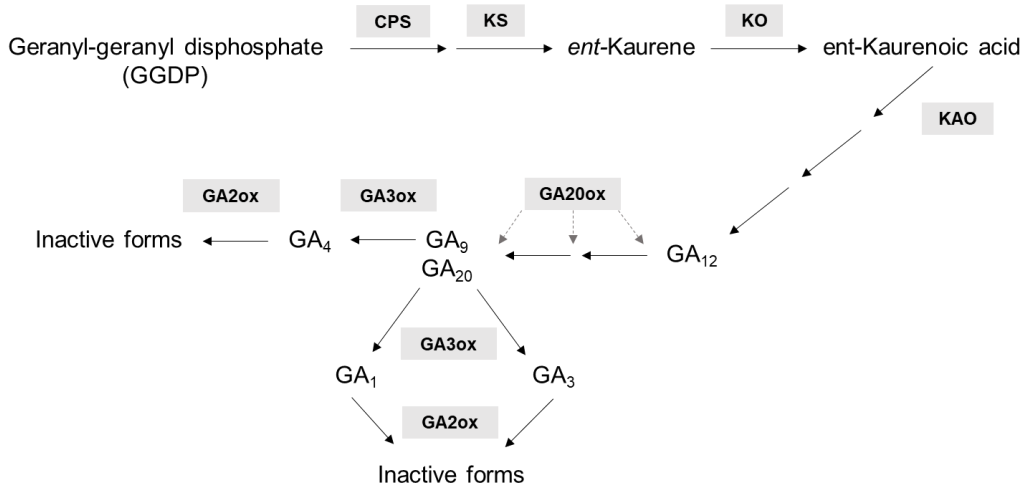

Figure 3-10: Biosynthesis pathway of bioactive GAs from geranyl-geranyl diphosphate precursor, to inactivation by GA2ox. CPS: ent-copalyl diphoshate synthase; KS: ent-kaurene synthase; KO: ent-kaurene oxidase; KAO: ent-kaurenoic acid oxidase. GA_{12} is a potential long-distance signalling candidate (Regnault et al., 2015).

Although GA_1 was identified as the primary bioactive GA that responded to increased soil bulk density (Figure 2-11), exogenous GA_3 application can increase concentrations of other bioactive GA species (Hamayun et al., 2010; Khalloufi et al., 2017). GA_3 application is unlikely to directly stimulate the biosynthesis of other bioactive GA species (GA_1, GA_4, GA_7), as the next step in GA pathways are inactive species (Figure 3-10). Thus, the increase in foliar GA_1 observed in treated plants (Figure 3-11), while GA_4 did not change, may be due to low purity of GA_3. GA_3 offered by many suppliers (including Sigma-Aldrich/Merck, Duchefa Biochemie, Fisher Scientific) is 90% of total gibberellin content.

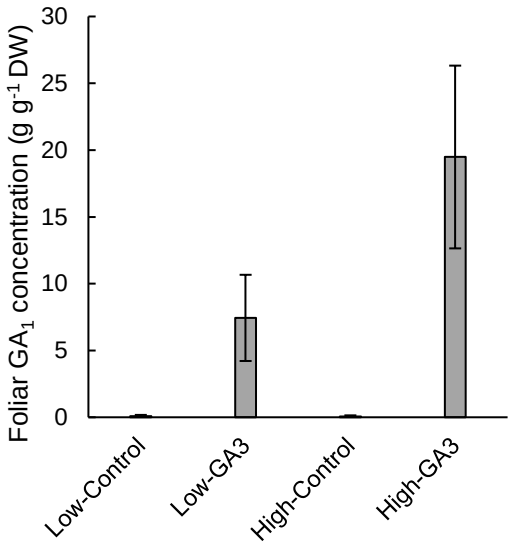

Figure 3-11: GA_3 root drenches tended to increase foliar GA_1 concentrations, despite being detected in < 50% of leaf samples.

GA_3 drenches and plant water status

GA_3 root drenches enhanced shoot growth at the expense of plant water status (Figure 3-3B). Stomatal conductance and relative evapotranspiration rate were also reduced in response to GA_3 application, under both high and low bulk density. Root-supplied GA_3 increased total water use of tomato by 12% by reducing stomatal resistance (Maggio et al., 2010). No significant effects were observed on Ψ_{leaf}. However, foliar sprays decreased transpiration rates and increased water use efficiency in spring wheat, but the ameliorative effect was greater in the salt-sensitive Barain-83 cultivar (Ashraf et al., 2002). Foliar GA_3 applications to grape cultivars also decreased g_s, but there were cultivar-specific effects on water use efficiency (Teszlák et al., 2013). Here, total transpirational losses in the week prior to harvest were lower in GA_3-treated plants (Figure 3-4D). Furthermore, root hydraulic conductivity was reduced in GA_3-treated plants (Figure 3-7B), suggesting increased

resistance to water transport through the plant. While GA_3 promoted leaf expansion, root length did not increase (Figure 3-6), suggesting that a similar size rootzone is supporting a larger plant. However, reduction in Ψ_{leaf} in plants receiving control root drenches in high bulk density soil suggests that water is limiting, even with daily irrigation. Therefore it is unclear whether decreased plant water status is due to water limitations imposed by the bulk density treatment (clustering of the rootzone limits water uptake from bulk soil – Chapter 2), exacerbated by increased plant size under GA_3 treatment, or a direct response to GA_3 application. Furthermore, xylem and foliar [ABA] increased with bulk density and GA_3 application (Figure 3-8, Figure 3-9), but was well-correlated with decreasing plant and soil water status. While increased endogenous GAs have been associated with decreased stomatal sensitivity to ABA (Nir et al., 2017) perhaps indicating feedback regulation of ABA levels, it is not immediately possible to disentangle the cause of increased [X-] and [L-ABA] in GA_3-treated plants here due to possible confounding soil water deficits.

Nevertheless, exogenous applications of GA_3 enhanced shoot growth in response to a range of abiotic stresses, including increased soil strength. While 100 µM GA_3 improved shoot growth of wheat grown in this sand culture system (Coelho Filho et al., 2013), a role of GAs as a long-distance signal of soil strength has been dismissed (Colebrook et al., 2014). Primarily, shoots were regarded as independent of root GA supply, and instead shoot GA levels may be regulated by another root-sourced signal. However, reciprocal grafting of WT tomato and constitutive-GA response mutant *procera* demonstrated that *pro* rootstocks enhanced WT leaf area and shoot biomass under both control and water-stressed conditions (Gaion et al., 2018). Foliar bioactive gibberellins were highest in *pro*/WT plants (rootstock/scion), while lowest

GA levels were present in WT/*pro*, suggesting an important role of the rootstock in regulating GA status of the plant. Furthermore, intricate work with Arabidopsis micro-grafts demonstrated the ability of WT rootstocks to rescue shoot growth of scions harbouring mutations at the early stages of bioactive GA synthesis (e.g. CPS, KAO: see Figure 3-10), suggesting long-distance signalling activity. Crucially, these mutants were not altered at the later steps of the bioactive pathway (GA20oc, GA3ox: Figure 3-10), allowing production of bioactive GAs. Growth was not restored when WT rootstocks were grafted to scions with mutations at the final stages of bioactive GA synthesis (Regnault et al., 2015). The intermediate GA_{12} was proposed as the mobile GA form, as mutations in the later stages prevented progression from GA_{12} onwards, and endogenous bioactive GAs were not detected. Thus, there is some evidence for the role of long-distance GA-signalling in abiotic stress responses. Furthermore, soil applications of bioactive GA species allows shoot GA accumulation (Figure 3-9) and growth response (Figure 3-2), demonstrating acropetal movement. Lack of GA_3 in xylem sap is likely since bioactive GAs are thought to move via the phloem (Lacombe and Achard, 2016).

Effects of exogenous GA_3 on phytohormonal profiles and interactions with bulk density

However, acropetal bioactive GA transport influences concentrations of other hormones in xylem sap and leaves. Concentrations of endogenous CKs ([X-iP] and [L-tZ]; Figure 3-8 & Figure 3-9) were increased by GA_3 root drenches. GAs and CKs exert reciprocal interactions upon each other, where CKs inhibit GA biosynthesis and

promote deactivation of bioactive species, while GAs nullify plant responses to CKs (Weiss and Ori, 2007). Regulating GA-CK levels is required to maintain appropriate shoot apical meristem function, ideally "high CK-low GA" ratio for optimal shoot growth (Jasinski et al., 2005), and *GA2ox* may be promoted by CK to reduce levels of bioactive GAs. Additionally, KNOX proteins that promote expression of CK-biosynthesis genes, e.g. *ISOPENTYL TRANSFERASE7* (Jasinski et al., 2005), also repress expression of *GA20ox* and *GA3ox*, enzymes which catalyse conversion of intermediate GAs to bioactive forms (see Figure 3-10; Weiss and Ori, 2007). There is little evidence that KNOX transcription is regulated by GA, as GA-deficient tomato mutants (*gib1*) exhibited similar transcript levels of KNOX genes compared to the constitutive GA-response mutant *procera*, suggesting that GA instead modulates sensitivity to KNOX (Jasinski et al., 2008). However, no comparison was made with WT plants, and the lack of functioning *SlDELLA* in the *procera* mutant does not necessarily result in higher endogenous GA levels, and may also possess reduced GA20ox activity (George Jones, 1987). Exogenous GA applications inhibited CK-related responses (by repressing primary CK response genes – Fleishon et al., 2011), but little information is available regarding the effects of GA_3 applications on endogenous CK levels. Enhancement of CK concentrations by GA_3 application may therefore result from reduced sensitivity to CK, and simultaneous transcriptomic analyses would allow for further exploration of this relationship.

Xylem CK concentrations have been implicated as a root-to-shoot signal of nitrogen availability, and CK levels are thought to control biomass partitioning between roots and shoots, where low CK levels promote root growth and high CK promotes shoot growth (van der Werf and Nagel, 1996). Nitrogen supplementation to previously N-

deprived maize rapidly (< 1 h) induced root CK accumulation and increased xylem sap CK concentrations (Takei et al., 2001), with tZ-CKs being the primary CK species in xylem sap (Kiba et al., 2011). However, [X-iP] was generally elevated in response to high soil bulk density (Figure 3-8), despite restrictions in root and shoot growth and reduced shoot nitrogen status (Figure 3-2E). Thus, in this work, it seems unlikely that the changes in endogenous CKs are regulating growth *per se*, since exogenous CKs usually promote leaf growth (Ulvskov et al., 1992).

CKs have also been implicated in enhancing transpiration by promoting stomatal opening, but as CK-overproducing genotypes often have small rootzones, premature wilting often masks the stomatal effects of CK (Dodd, 2003). Foliar [tZ] was inversely correlated to Ψ_{leaf} (Table 3-5). In tomato, Ψ_{leaf} declined with increased transpiration (Dodd et al., 2009) thus Ψ_{leaf} was inversely correlated to relative ET (Table 3-5) as expected. However, despite the Ψ_{leaf}/ET relationship, [L-tZ] was also negatively correlated to relative ET, resulting the conclusion that [L-tZ] reduces transpiration. However, detached leaf transpiration assays are required to determine whether xylem-supplied CKs can affect stomatal conductance of tomato.

Xylem [JA] tended to increase under high soil bulk density conditions (Figure 3-8). This is consistent with findings in Chapter 2 (Figure 2-10) and suggests a role of JA in responses to root mechanical stress. Jasmonates are typically considered to be signals of herbivory and mechanical wounding and may be produced in both shoot and root tissues and transported long distances through the plant vascular system as part of plant defence responses (Fragoso et al., 2014; Lu et al., 2015). JA biosynthesis in response to wounding is hypothesised to be triggered by sudden increases in

xylem turgor (Farmer et al., 2014). The LOX6 promoter of JA biosynthesis was expressed in cells adjacent to the xylem vessels, suggesting sensitivity to the changes in xylem tension on wounding. How this hypothesis may relate to hydraulic signalling of water deficit (decreased xylem turgor) remains to be explored (Farmer et al., 2014). However, increasing endogenous JA concentrations have been observed in response to water deficit in several species, and JA is known to possess anti-transpirant properties (de Ollas and Dodd, 2016). As Ψ_{leaf} decreases in response to both increased soil bulk density and GA_3 root drenching (Figure 3-3B), it is not immediately possible to dissect the cause of increased [X-JA] in this system.

Cross-talk between JA and GA_3 occurred, as GA_3 root drenches significantly reduced xylem JA concentration (independently of bulk density; Figure 3-8). At the molecular level, antagonistic interactions occur between DELLA and JAZ (JASMONATE ZIM-domain) proteins which can modulate shoot growth (Wasternack and Hause, 2013), typically allowing plants to prioritise plant defences over shoot growth when JA biosynthesis is triggered (Yang et al., 2012). JAZ proteins repress JA-associated developmental and defence response in the absence of JA. JAZ inhibits DELLA action, freeing transcription factors for the promotion of plant growth. However, degradation of JAZ by JA (produced in response to external stress) stabilises DELLA proteins and therefore restricts GA-induced plant growth, while JA-related plant defences are activated (Wasternack and Hause, 2013). Conversely, during GA-induced DELLA degradation, JAZs inhibit MYC2 which can decrease the sensitivity of JA-induced growth restriction (Song et al., 2014). At the whole plant level, silencing of calcium-dependent protein kinases in tobacco resulted in stems containing 140-240-fold greater JA than WT, with a stunted growth phenotype and dark green leaves

(Heinrich et al., 2013). Furthermore, JA-accumulating genotypes of tobacco were deficient in GAs, and bioactive GA_1 was five-fold lower than in WT. While foliar GA_3 application (3 μM) restored growth to 80% of WT, no data on JA levels in GA_3-treated plants was provided. Thus, it is possible that the GA-mediated reduction in xylem sap [JA] seen here is due to the antagonistic action of bioactive GA_3 on DELLAs and JAZ cross-talk, but further gene expression analyses would be required to confirm this hypothesis.

Conclusions

Taken together, GA_3 root drenches improve shoot growth in compact soil, despite decreasing plant water and nitrogen status. GA_3 applications interact with other phyothormones present in root xylem sap and foliar tissues, perhaps allowing growth to be decoupled from hydraulic and nutrient limitations. These responses cannot solely be attributed to increased mechanical impedance to root growth, as soil water content and plant water status also changed and difficult to control for. Phytohormone concentrations have been correlated with particular physiological responses even when plants are grown at different bulk densities (Chapters 2, 3). Additionally, it is unclear whether these responses would be conserved across different soil types, as similar bulk density changes may result in different stress combinations depending on soil conditions. Further work is required to elucidate the effects of these treatments (root impedance and GA_3) from co-occurring stresses (particularly soil water deficit and nutrient availability) with an experimental system that isolates mechanical resistance from resource limitation.

Chapter 4 GA_3 root drenches enhance shoot growth when roots are mechanically impeded

4.1 Introduction

The growth and physiological responses of plants to many isolated abiotic stresses have been well-studied, including drought, salinity, heat and anoxia. Many of these stresses occur in the rootzone, and plants utilise a range of hydraulic and chemical signals to communicate adverse conditions to their growing shoots. Increasing soil strength is an important component of not only compaction stress, but also soil water deficit. Plants likely encounter increased mechanical resistance before soil water becomes limiting, as soil mechanical strength increases rapidly with limited soil drying (Whalley et al., 2005; Bengough et al., 2011; Valentine et al., 2012). However, increasing soil strength by compacting soil may inadvertently impose other simultaneous abiotic stresses on plants. Compaction alters soil physical, chemical and biological properties, particularly retention and infiltration of water, nutrients and air (Hamza and Anderson, 2005). Thus, in compact soil, it can be difficult to separate the effects of increased soil strength from other abiotic stresses

Consequently, studies of plant responses to substrate strength have utilised a range of experimental systems to vary mechanical strength independently of resource (water, nutrients) availability. Many studies isolating mechanical stress use glass ballotini: selection of ballotini sizes controls pore spaces by ensuring consistent particle sizes, and manipulation of impedance experienced by different root size classes (Goss, 1977). Experiments by Goss (1977) used specially designed perspex cells filled with glass ballotini and a constant flow of aerated nutrient solution,

thereby maintaining plant water and nutrient status. External pressure applied to the outside of the cell was linearly related to increasing root elongation resistance in the form of pressure required to inflate a neoprene probe inserted into the medium. Root elongation rate of barley seminal roots decreased by 50% when 20 kPa of external pressure was applied, and 80% at 50 kPa, however these external pressures are up to 100-fold lower than penetrometer pressures required to reduce root growth in the field (Bengough et al., 2011).

Analyses by Bengough and Mullins (1990b) showed Goss (1977) underestimated root elongation resistance by at least 5-fold, as the penetrometer resistance within the cell was 60 times higher than the externally-applied pressure. Penetrometer resistance also varied throughout the cell, increasing with depth, and it was not possible to accurately determine mechanical strength for any given external pressure. Furthermore, while the ballotini pressure cell system varies strength independently of resource availability, plants growing in non-pressurised controls may still encounter considerable resistance to root elongation as they push aside ballotini to grow (Bengough and Mullins, 1990b).

Sand cultures have been employed by several workers to assess growth responses to increased substrate strength. Several designs have been employed, with many consisting of tubes of incompressible sand with weights placed on the upper surface, standing in nutrient solution allowing watering by capillary action (Materechera et al., 1991; Whalley et al., 1999; Clark et al., 2002). Many workers determined the resulting penetrometer resistance of the sand for a particular weight. Materechera et al. (1991) placed 5 kg weights upon tubes of diameter 7 cm to achieve a

penetrometer resistance of 4.0 MPa, while 17 kg weights placed on tubes with 15 cm diameter produced a penetrometer resistance of 0.75 MPa (Whalley et al., 2006; Coelho Filho et al., 2013; Jin et al., 2015). The combination of weight and surface area varies the pressure exerted on the surface of the sand (*Pressure = Force/Area*) and resulting force required for roots to move through sand depends on the size of substrate particles and remaining pore spaces.

Previous work has shown an unidentified role of GA signalling in shoot responses to increasing soil strength. GA_3 application to the nutrient solution of wheat grown under both low and high soil strength conditions improved leaf elongation but at the expense of tiller production (Coelho Filho et al., 2013). Furthermore, semi-dwarf wheat genotypes carrying different *Rht* genes for partial GA-insensitivity were less sensitive to the stunting effects of increased root strength, with leaf length decreased by 35% compared to 55% in the tall Cadenza genotype (Jin et al., 2015). Taken together, GAs seem to regulate shoot growth of plants grown in strong soils, but how this is communicated from root to shoot, or the effects on other plant hormones, including ABA, remains to be explored.

Soil compaction decreased the concentrations of bioactive GAs in growing leaves of tomato (Figure 2-11). GA3 root drenches rescued leaf expansion (Figure 3-2B) but altered phytohormonal profiles (Figure 3-8; Figure 3-9). However, as previously discussed, it is difficult to distinguish the physiological response of plants to soil strength from other possible stresses related to soil compaction, such as lower plant water status (Figure 2-9; Figure 3-3B), which have already been well-documented in literature (e.g water deficit). Consequently, the sand culture system (Whalley et al.,

2006) was used to independently determine the effects of increased root strength on growth and physiological responses of tomato. To determine whether the physiological responses to GA_3 application were consistent in plants grown in compact soil (Chapter 3) and the sand culture system (Chapter 4), a GA_3 root drench was applied to investigate its effects on shoot growth, water relations and phytohormone profiles in the absence of water or nutrient limitations.

4.2 Materials and Methods

2 replicate experiments were carried out in February-March 2018 (Experiment 4.1) and August-September 2018 (Experiment 4.2).

Sand culture preparation

Nutrient solution (Table 4-1A) was adapted from (Clark et al., 2002) to contain similar concentrations of macronutrients as half-strength Hoagland's Solution (Table 4-1B) to improve suitability for growing tomatoes hydroponically for the first 4 weeks (Hochmuth and Hochmuth, 2015). Stock solutions were adjusted to pH 6 using 1 M potassium hydroxide and 0.5 M hydrochloric acid. Plastic tanks were initially filled with 20 L of nutrient solution and covered with a lid. The lid allowed 6 PVC tubes (550 mm height, inner diameter 152 mm) to be placed into the tanks. The tubes were raised from the bottom of the tank using metal supports, allowing solution uptake by capillary action (Figure 4-1). Silica sand (Chelford T-grade, Sibelco, UK) and nutrient solution were poured into the tubes such that sand was always falling into solution (Figure 4-2), facilitating settling and preventing air bubbles. A plastic mould placed around the top of the tube allowed sand to be packed approximately 8 mm above the top of the tube (Figure 4-2). Nutrient solution was poured into the tank to a depth of 15 cm from the bottom of the tubes and topped up with DI water daily. Nutrient solution was replaced with fresh stock after 3 weeks of growth.

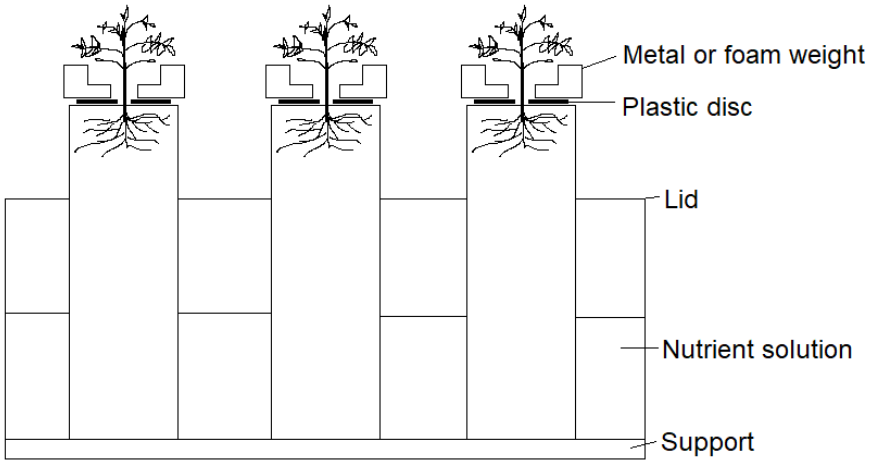

Figure 4-1: Sand culture system based on (Materechera et al., 1991) and adapted by (Clark et al., 2002). Six tubes were arranged in each tank. A 17 kg weight was placed upon the surface of the sand, corresponding to a penetrometer pressure of 0.75 MPa (Whalley et al., 2006). GA_3 solution (20 ml of 100 µM) was applied to the rootzone at the opening at the stem base, twice weekly using a dropper.

Figure 4-2: Top: Sand poured into nutrient solution to facilitate settling without air bubble formation. Bottom: Sand packed up to 8 mm above the top of the tube. Plastic discs placed on sand surface to distribute weight evenly.

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Table 4-1: A) Nutrient solution recipe adapted from (Clark et al., 2002) to suit tomato growth. B) Macronutrient composition of adapted nutrient solution compared to half-strength Hoagland's solution

A

Stock solution	Compound	Working stock concentration
1	$Ca(NO_3)_2.4H_2O$	2.5 mM
	FeEDTA.2Na	5 mM
2	KH_2PO_4	1 mM
	KCl	2 mM
	$MgSO_4.7H_2O$	0.5mM
	H_3BO_3	50 µM
	$MnCl_2.4H_2O$	10 µM
	$ZnSO_4.7H_2O$	1 µM
	$CuSO_4.5H_2O$	1 µM
	$H_2MoO_4.H_2O$	0.5 µM

B

Macronutrient	Working stock concentration (ppm)	50% Hoagland's Solution concentration (ppm)
N	70.03	88.22
P	30.97	31.18
K	119.84	120.62
Mg	12.15	12.13
S	16.03	16.05
Ca	100.20	70.09

Plant material and growing conditions

Tomato (*Solanum lycopersicum* cv. Ailsa Craig) seeds were surface-sterilised in 10% v/v thick bleach (of approximately 5% sodium hypochlorite) for 5 minutes, then rinsed with DI water. The seeds were placed in petri dishes on filter paper moistened with DI water, sealed and kept in the dark at 21°C until radicles emerged (72 – 96 h). A single germinated seed was carefully transplanted into the sand core to a depth of 15 mm through a hole at the centre of the plastic disc (Figure 4-1). A metal weight (17 kg) or foam control of the same shape was then placed on top of the plastic disc to exert pressure onto the sand core. The penetrometer resistance produced by the metal weight is approximately 0.75 MPa, and 0.19 MPa by the foam weight (Jin et al., 2015).

Plants were grown in a fluorescent-tube lit controlled environment growth room (12 hr photoperiod, day/night temperature 24/19°C, RH 50%).

20 mL of 100 µM GA_3 (prepared as in Chapter 3.2) or a control solution containing the same volume of 1 M KOH was applied to the sand at the opening of the plastic disc using a 1 mL plastic dropper, twice weekly.

Measurements

Leaf emergence, number and stem height were recorded every other day in Experiment 4.2 only.

Chlorophyll concentration of leaf 3 (counting from the base of the plant, excluding the cotyledons) was measured 22 DAT (SPAD-502 Meter, Konica Minolta, Japan).

Leaf water potential of leaf 3 was measured using a Scholander pressure vessel (Soilmoisture Equipment Corp., Santa Barbara, CA, USA). The leaf was excised from the plant in the controlled environment room, sealed in a plastic bag for transfer to the laboratory for measurement. In Experiment 4.2, this was conducted in the 3rd week of growth, and in Experiment 4.1 this was measured at harvest, approximately 4 weeks after transplanting.

Stomatal conductance was measured on an expanded leaf using an AP4 Porometer (Delta-T Devices, Cambridge, UK). The distal leaflet from an actively growing leaf was excised and transferred to a 1.5 mL Eppendorf before flash-freezing in liquid nitrogen. Samples were stored at -80°C prior to phytohormonal analyses.

Shoot water potential (Ψ_{shoot}) was measured in Experiment 4.2 using a tall Scholander pressure vessel. The detopped shoot was transferred to the laboratory similarly to leaves. An overpressure of -0.2 MPa was applied to obtain 100 µL of xylem sap, pipetted into a 1.5 mL Eppendorf tube, flash-frozen and stored at -80°C. A core of sand was extracted from the upper layer of the column immediately after the plant was detopped and dried at 105°C for 48 hours to obtain moisture content.

Roots were extracted, washed, flash-frozen and stored at -80°C before further phytohormonal analyses

For each plant, a photograph of all leaves spread on a white background was taken using an iPhone 6 (Apple, Cupertino, CA, USA). A 4 cm^2 reference area of red electrical tape was positioned in each image. Leaf area was determined using Easy Leaf Area (Easlon and Bloom, 2014; Figure 4-3), which corresponded well to leaf area measurements made with the Li-1000 Leaf area meter used in previous work (R^2 = 0.97; Figure 4-4).

Leaf and stem fresh weights were recorded: tissues were then bagged and dried at 80°C for 48 h to obtain dry weights. The sand columns were extracted from tubes and sand was gradually washed away. The maximum depth of roots were recorded.

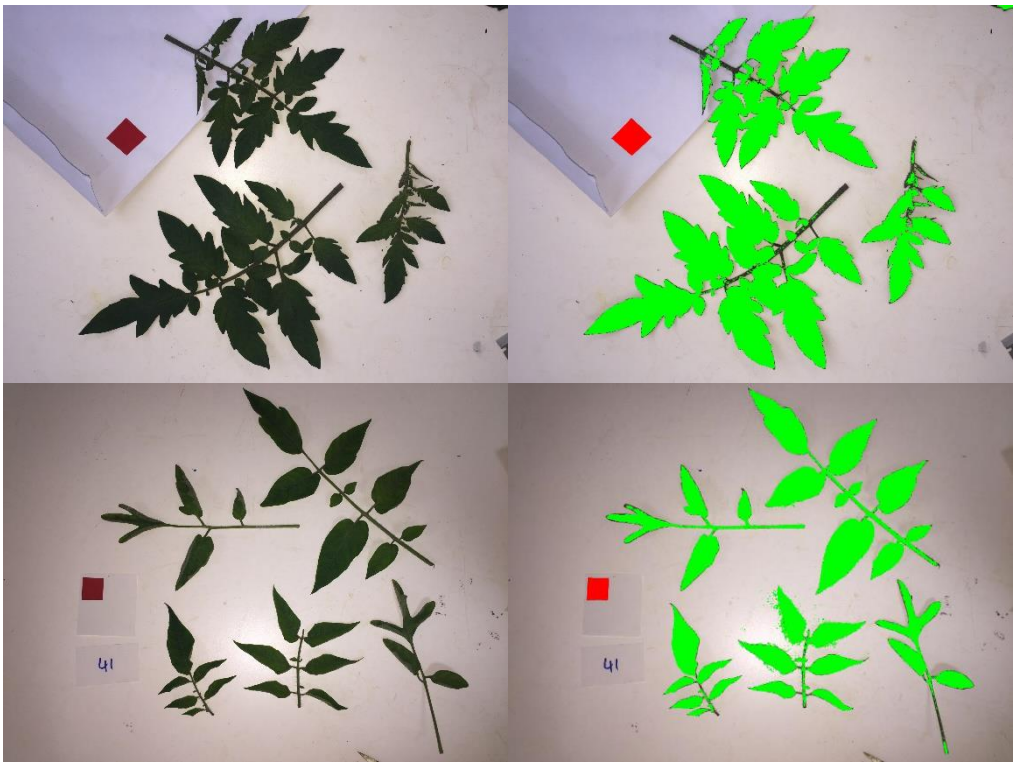


Figure 4-3: Determination of total leaf area using Easy Leaf Area (Easlon and Bloom, 2014). A red calibration square of known area (4 cm^2) allows the program to calculate area of green leaf against a plain background (no green or red). Images on the left show the raw images (taken on iPhone 6; Apple Inc. California), and images on the left show post-processing with the software to distinguish and determine the pixel area of the red calibration square and the contrasting green leaf area.

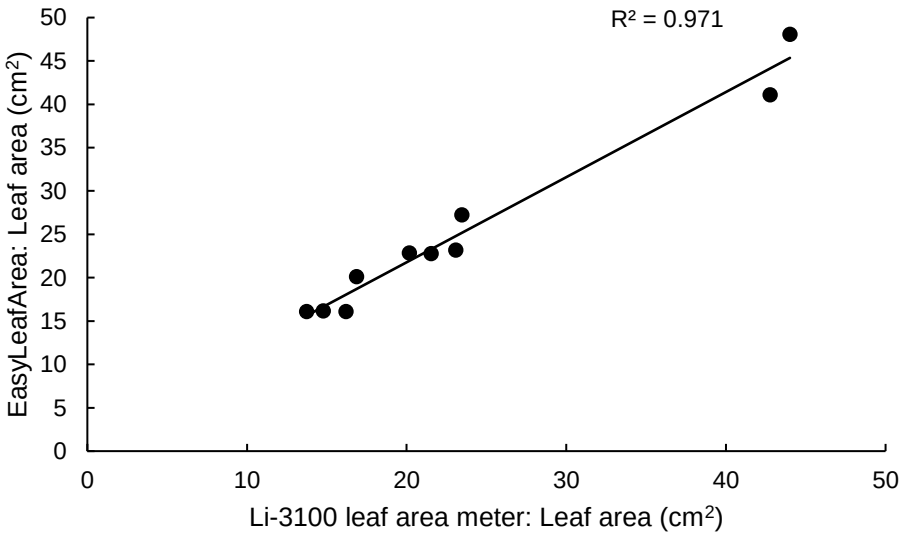


Figure 4-4: Comparison of leaf area measurements made using Li-3100 leaf area meter (Li-Cor inc., Lincoln, Nebraska, USA) and images processed using Easy Leaf Area. Each symbol represents an individual leaf measured using both methods.

Nitrogen analyses

Shoot tissues were dried for at least 48 h at 80°C. Samples were prepared and analysed as detailed in Chapter 2.2.

Multi-hormone analyses

Leaf and root tissues were freeze-dried and ground with scissors. Ground tissues and shoot xylem sap were prepared and analysed as described in Chapter 2.2 by Dr. Alfonso Albacete (CEBAS-CSIC, Murcia, Spain). Of the 11 hormones for which standards were added in the analyses, 7, 8 and 10 were detected with over 50% frequency in shoot xylem sap, foliar and root tissues.

Statistical analyses

Growth data is presented the means of two combined experiments (unless otherwise indicated), however, phytohormone and nutrient analyses were conducted in Experiment 4.2 only. Data were analysed using restricted maximum likelihood (REML) linear mixed-effects models (nlme package, (R Core Team, 2018)). Root drench and soil strength were assigned as fixed factors. The error term was Block/Tank/Plant. For data sensitive to time of day (plant water status, phytohormone content), “Time” period of measurement (morning or afternoon) was also included as a fixed factor.

Pearson's correlations were conducted to explore associations between root and sap phytohormone concentrations. Pearson's r coefficients are displayed, and *p* values included when statistically significant.

One-way ANOVA was used to determine daily differences between mean stem height and leaf number, group-level differences were determined using post-hoc LSD.

4.3 Results

Growth

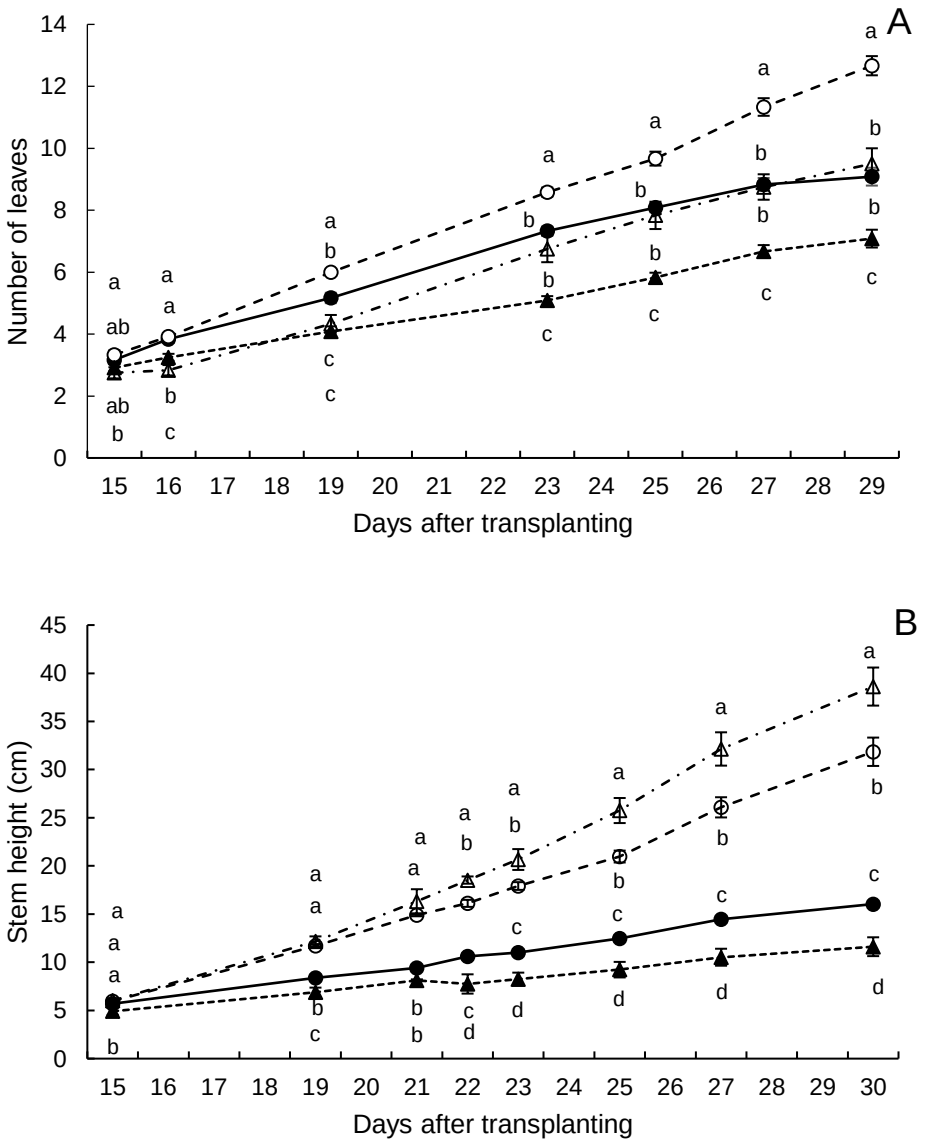


Figure 4-5: A) Leaf emergence (number of leaves of length > 1 cm) and B) stem height of tomato grown in low and high strength substrate (Experiment 4.2). Circles represent low and triangles represent high substrate strength. Filled symbols correspond to control root drenches and hollow symbols denote GA_3 treated plants. Symbols are means ± S.E. of 12 replicates. Different letters denote significant differences between means (post-hoc LSD $p < 0.05$) on each day.

Increased substrate strength reduced leaf emergence, as high strength-controls consistently exhibited lower leaf numbers than other treatments (Figure 4-5A).

Exogenous GA_3 application increased rate of leaf emergence in tomato plants regardless of substrate strength, and GA_3 root drench increased leaf emergence in plants growing in strong sand such that the number of emerged leaves were not different between low strength-control and high strength-GA_3 on the final day of measuring.

High strength stunted stem elongation, which became apparent by 19 DAT in Experiment 4.2 (Figure 4-5B). GA_3 drench improved stem elongation, and high strength plants were more sensitive to GA_3 treatment, exhibiting significantly greater stem length during the majority of the measuring period.

Increased substrate strength significantly decreased shoot biomass accumulation, total leaf area and leaf expansion of individual leaves ($p < 0.001$) (Figure 4-6A, C & E). GA_3 application increased shoot biomass ($p = 0.011$) and leaf area ($p = 0.006$) in plants grown under both low and high soil strength conditions. There was a significant interactive effect of GA_3 and strength, as plants in low strength treatments exhibited greater increase in leaf area in response to GA_3 ($p = 0.045$). GA_3 application increased leaf biomass by 14 and 18% in the low and high strength treatments, but greater improvements were seen in stem dry weight which increased by 2- and 3-fold respectively (Figure 4-6B). Thus, specific leaf area was significantly increased in GA_3 treated plants ($p = 0.023$), despite similar changes in both total shoot dry mass and total leaf area (Figure 4-6D). Final stem height was not reduced by strength at harvest (Figure 4-6F) but was significantly increased by GA_3 ($p < 0.001$). This contradicts the results of Figure 4-5B, which indicated a significant effect of increased strength on stem height, but this may be a function of an increased

number of replicates in Figure 4-6F, and the use of a different statistical test. There was a significant interaction between strength and GA, as high strength plants were more responsive to GA_3 applications (p = 0.001).

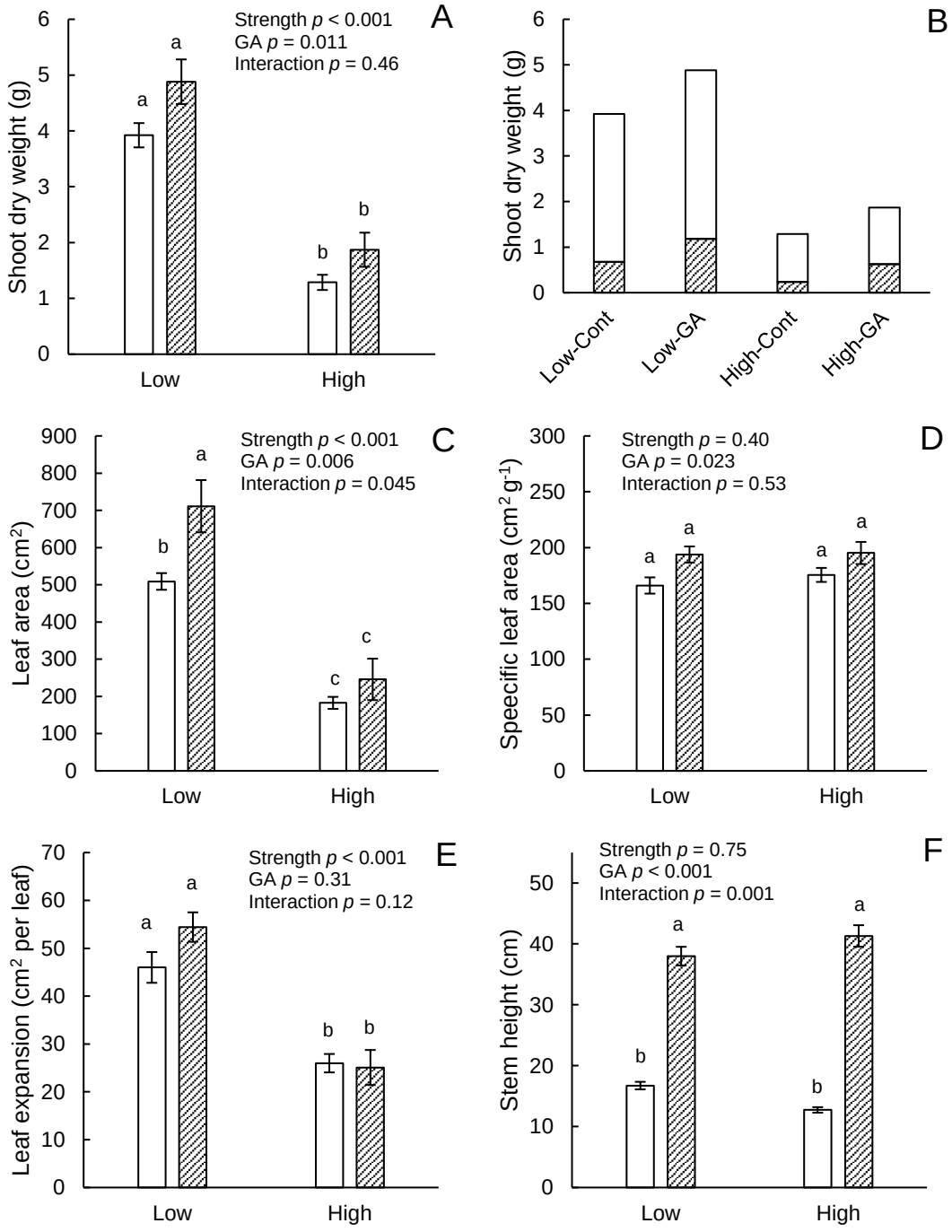


Figure 4-6: Shoot growth parameters at harvest of tomato grown in low or high strength substrate. A) Total shoot dry weight; B) Proportion of shoot biomass allocated to stem (shaded) or leaves (white); C) total leaf area; D) specific leaf area; E) leaf expansion, expressed as cm^2 per leaf; F) final stem height. In A, C, D & E: white bars represent control root drench, shaded bars represent GA_3 root drench. Bars are means ± S.E. of 24 replicates, with different letters denoting significant differences between means (post-hoc LSD $p < 0.05$). *p* values reported for strength, GA_3 treatment, and their interaction.

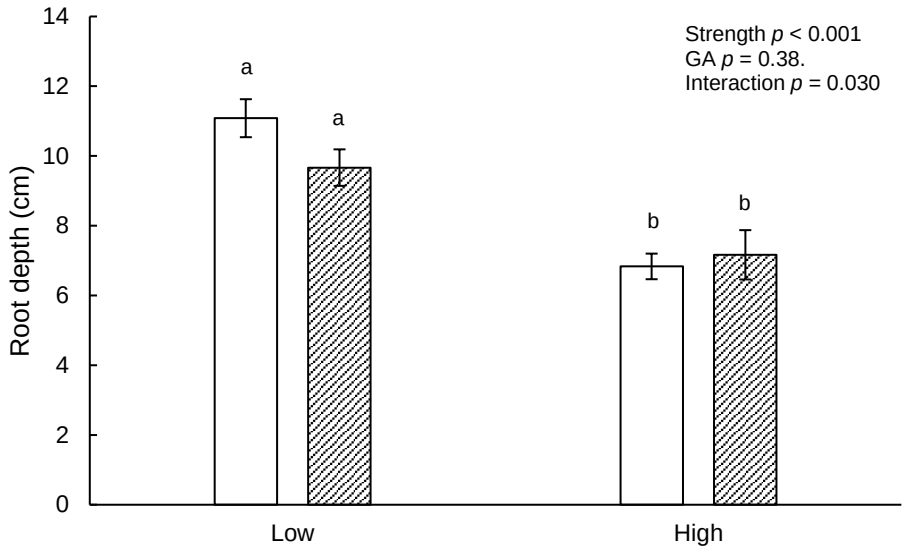


Figure 4-7: Maximum rooting depth of tomato grown under low or high rootzone strength. White bars represent control root drench treatment, shaded bars correspond to GA_3 root drench. Bars are means ± S.E. of 24 replicates, with different letters denoting significant differences between means (post-hoc LSD $p < 0.05$). p values reported for strength, GA_3 treatment, and their interaction.

However, while increased strength reduced rooting depth ($p < 0.001$), this was not improved by GA_3 application (Figure 4-7). GA_3 application decreased root depth in the low strength treatment (by 12.7%) but had no effect in the high strength treatment (Interaction $p = 0.03$).

Nutrient status

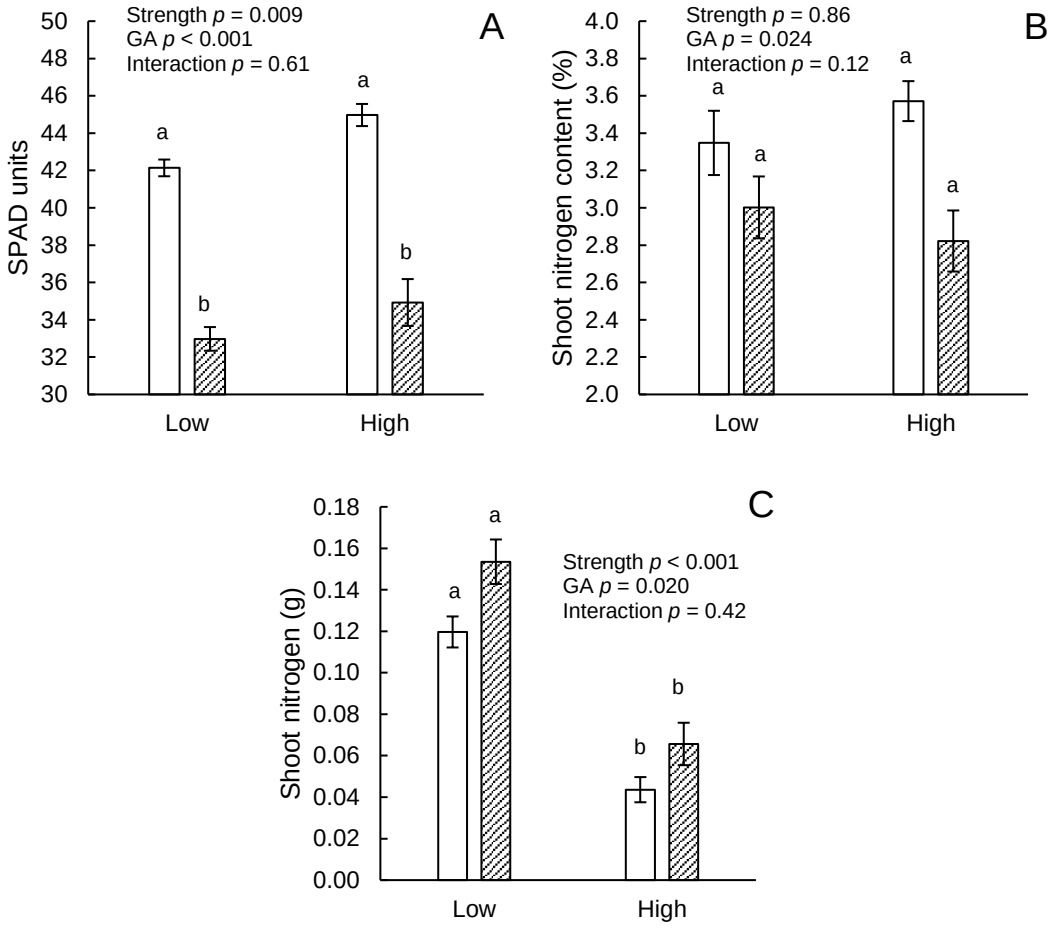


Figure 4-8: Nitrogen status of tomato grown in low or high strength substrate. A) SPAD measured as an indicator of chlorophyll content; B) Shoot nitrogen content; C) Total shoot nitrogen. White bars represent plants receiving a control root drench, shaded bars represent GA_3-treated plants. Bars are means ± S.E. of 12 replicates, with different letters denoting significant differences between means (post-hoc LSD $p < 0.05$). p values reported for strength, GA_3 treatment, and their interaction.

SPAD units were measured to estimate chlorophyll content. GA_3 application significantly reduced SPAD ($p = 0.001$), while increased substrate strength significantly increased SPAD ($p = 0.009$; Figure 4-8A). Shoot nitrogen concentration was also significantly reduced by GA_3 root drench ($p = 0.024$; Figure 4-8B), but as GA_3 treated plants were generally larger than controls, total shoot nitrogen was significantly higher in these treatments ($p = 0.02$; Figure 4-8C). Increased substrate

strength significantly ($p < 0.001$) decreased total shoot nitrogen, as plants were smaller than low strength controls.

Water status

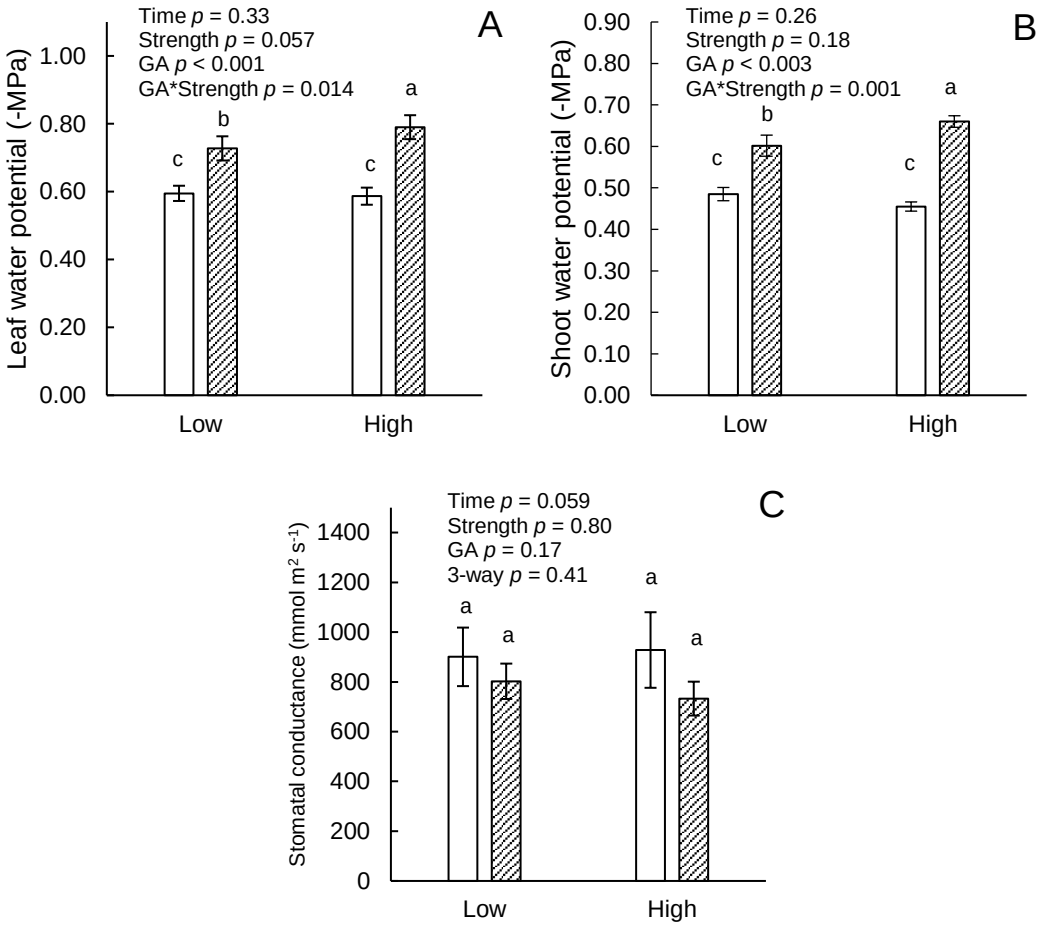


Figure 4-9: Plant water status of tomato grown in low or high strength substrates. A) Leaf water potential (n = 24); B) Shoot water potential (n = 12). C) Stomatal conductance (n = 24). White bars represent control root drench, shaded bars represent GA_3 root drench treatment. Bars are means ± S.E., with different letters denoting significant differences between means (post-hoc LSD $p < 0.05$). p values reported for time, strength, GA_3 treatment, and their interactions.

Increased strength had no statistically significant effect on plant water status (Figure 4-9). GA_3 application significantly decreased both Ψ_{leaf} and Ψ_{shoot} ($p < 0.001$), and this

effect was greater for plants grown under high strength treatments. GA_3 decreased Ψ_{shoot} and Ψ_{leaf} by 0.2 MPa under high strength, compared to 0.13 and 0.11 MPa respectively under low strength conditions. Furthermore, there was no effect of time, GA_3 or strength on g_s when foliar tissues were sampled for phytohormone analyses. In Experiment 4.1, soil water content was significantly lower in GA_3-treated plants (Figure 4-10A), but this was not the case in Experiment 4.2 (Figure 4-10B).

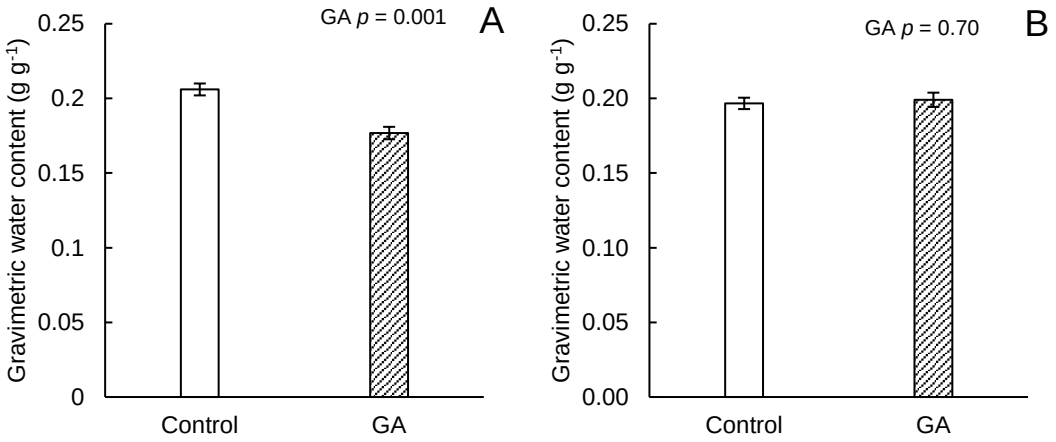


Figure 4-10: Gravimetric water content of sand in upper layer of columns in Experiment 4.1 (A) and 4.2 (B). White bars represent control treatments, shaded bars represent GA_3 root drench. A) Bars are means ± S.E. of 4-6 replicates; B) Bars are means ± S.E. of 24 replicates

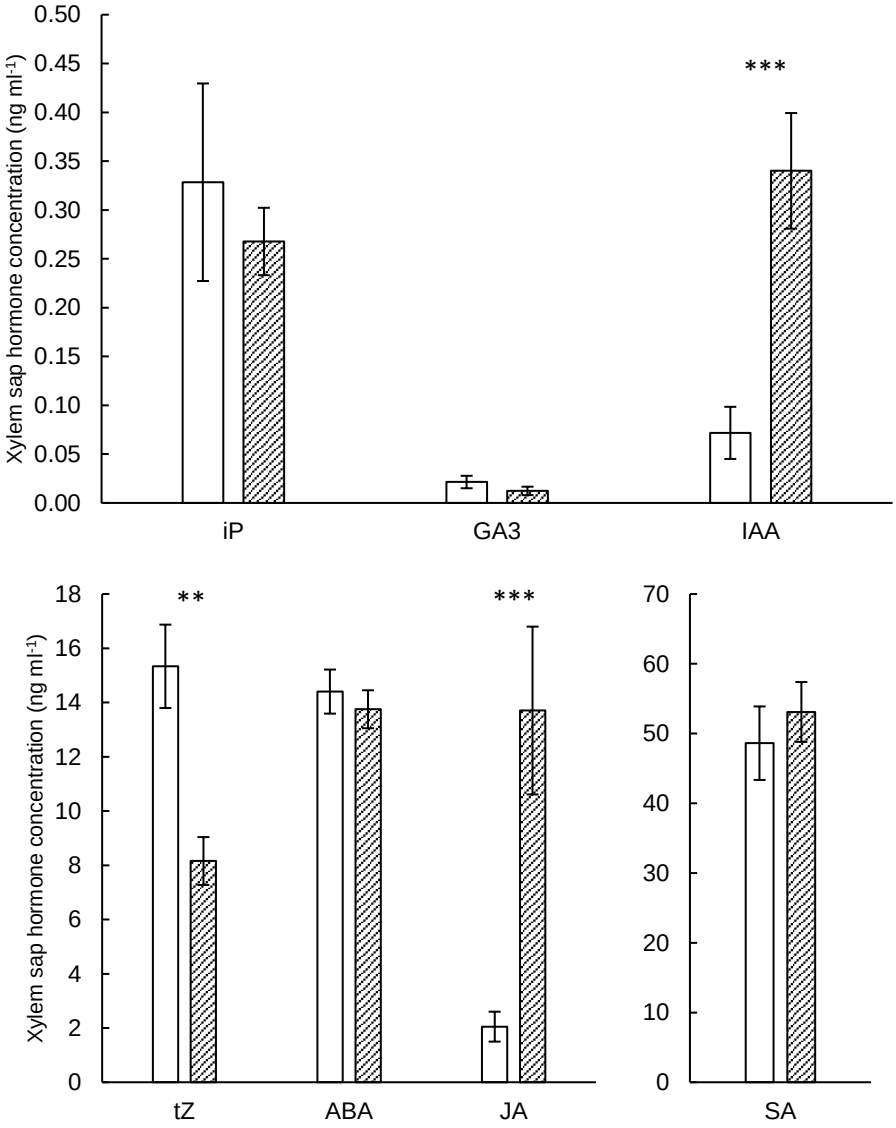


Figure 4-11: Phytohormonal profile of shoot xylem sap collected from tomato plants grown in low (white bars) or high (shaded bars) strength sand. Error bars ± S.E. n = 12. Asterisks denote significant effect of substrate strength (linear mixed effect model): * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 4-2: *p*-values from ANOVA summary tables from linear mixed models of shoot xylem sap phytohormones. Significance of *p* values reported thus: · *p* is marginally non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. JA data was $\log_{10}$ transformed.

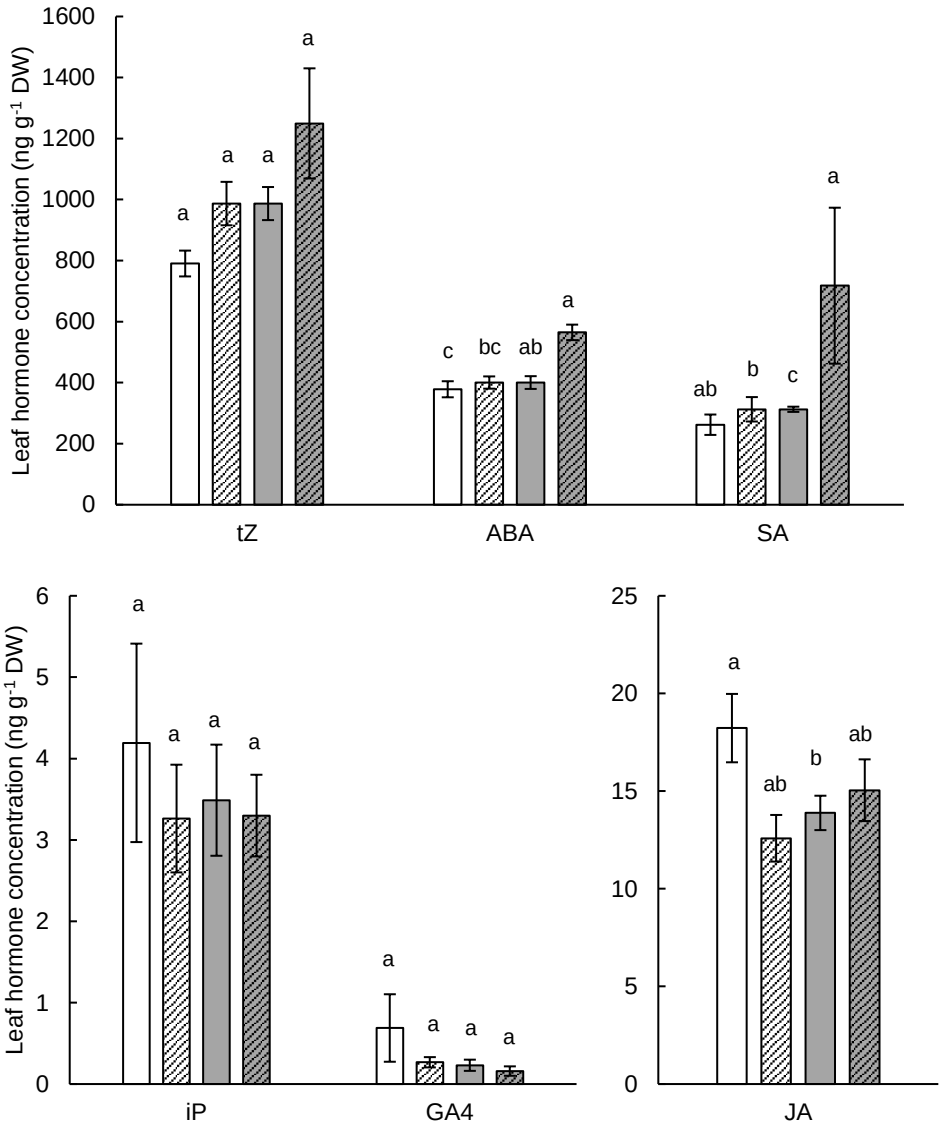
		Factor	
Hormone	**Time**	**Strength**	**Time*Strength**
tZ	0.369	0.004 **	0.071 ·
iP	0.377	0.607	0.244
ABA	0.727	0.546	0.340
JA	0.330	< 0.001 ***	0.218
SA	0.288	0.424	0.346
IAA	0.238	< 0.001 ***	0.279
GA3	0.508	0.262	0.873

Increased substrate strength significantly increased xylem sap IAA concentration 5-fold ($p < 0.001$; Figure 4-11; Table 4-2) and JA by 7-fold ($p < 0.001$), but decreased tZ by 50% ($p = 0.004$). Substrate strength did not affect xylem sap ABA, iP, GA_3 or SA concentrations. Sampling time of day had no significant effect on phytohormone concentrations, nor any significant interactive effect with strength.

Greater soil strength significantly increased foliar ABA ($p < 0.001$ Figure 4-12). There was a marginally non-significant effect of strength on GA concentrations (increase in GA_1 and GA_3: $p = 0.095$ and 0.071 respectively; reduction in GA_4: $p = 0.061$). However, foliar concentrations of cytokinins (tZ and iP), JA, GA_4 or SA did not change in response to greater soil strength.

GA_3 applications to soil significantly ($p = 0.048$) increased foliar [GA_3] concentrations several thousand-fold and foliar GA_1 concentrations by several hundred-fold ($p = 0.011$). There was a marginally non-significant increase in foliar [tZ] in response to GA_3 drenches ($p = 0.063$), and foliar [SA] increased significantly ($p = 0.036$).

There were significant interactions between GA_3 application and substrate strength on foliar concentrations of ABA, SA, JA and GA_1. Plants growing in high strength conditions accumulated higher concentrations of GA_1 in foliar tissues when GA_3 root drenches were applied. GA_3 drenches resulted in increases in foliar [ABA] and [SA] under high substrate strength. Different responses were observed in foliar [JA] accumulation when low and high strength plants were treated with GA_3 – low strength plants were reduced in foliar [JA], while high strength plants slightly increased [JA].


Leaf hormone concentration (ng g-1 DW)
1600
1400
1200
1000
800
600
400
200
0
a
a
a
a
c
bc
ab
a
ab
b
c
a
tZ
ABA
SA
Leaf hormone concentration (ng g-1 DW)
6
5
4
3
2
1
0
a
a
a
a
a
a
a
a
iP
GA4
25
20
15
10
5
0
a
ab
b
ab
JA

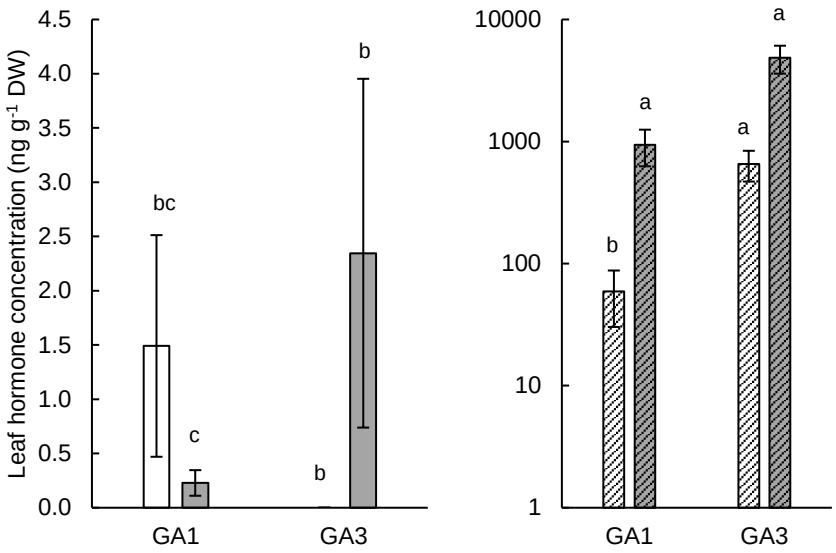
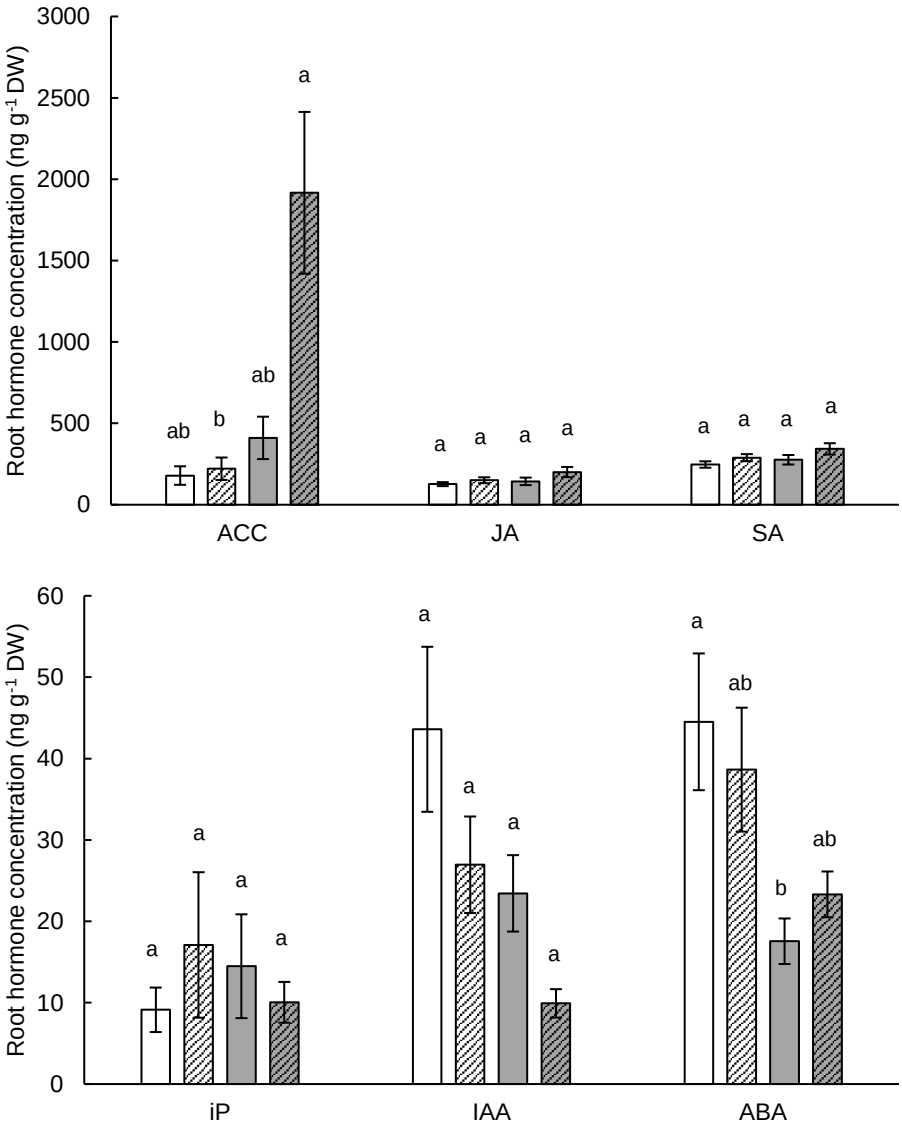


Figure 4-12: Foliar phytohormone concentrations of tomato grown in under low and high soil strength conditions, receiving control or GA_3 root drenches.White bars: Low strength-Control; White/striped bars: Low strength-GA_3; grey bars: High strength-Control; Grey/striped bars: High strength-GA_3. Bars are means ± S.E. of 12 replicates, with different letters denoting significant differences between means within an analyte (post-hoc LSD $p < 0.05$).

Table 4-3: *p*-values from ANOVA summary tables from linear mixed models of foliar phytohormones. Significance of *p* values reported thus: · p is marginally non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. For succinctness, three-way interaction between Time, Root drench (GA) and strength is presented in the Interaction column unless otherwise stated (T = Time, GA = Root drench, S = Strength).

		Factor			
Hormone	**Transformation**	**Time**	**GA**	**Strength**	**Interaction**
tZ		0.75	0.063 ·	0.13	0.083 · (G*S)
iP		0.58	0.71	0.85	0.54
ABA		0.37	0.11	< 0.001 ***	0.018 * (G*S)
JA		0.57	0.19	0.24	0.009 ** (G*S)
SA	$Log_{10}(SA)$	0.49	0.036 *	0.80	< 0.001 (G*S)
GA_1	$Log_{10}(GA_1 + 0.01)$	0.97	0.005**	0.095 ·	< 0.001 *** (G*S)
GA_3	$Log_{10}(GA_{3+} + 0.01)$	0.93	0.001**	0.071 ·	0.38
GA_4	$Log_{10}(GA_4 + 0.01)$	0.47	0.69	0.061 ·	0.008 ** (T*G*S)


Root hormone concentration (ng g-1 DW)
3000
2500
2000
1500
1000
500
0
ab
b
ab
a
a
a
a
a
a
a
a
a
ACC
JA
SA
Root hormone concentration (ng g-1 DW)
60
50
40
30
20
10
0
a
a
a
a
a
a
a
a
a
ab
b
ab
iP
IAA
ABA

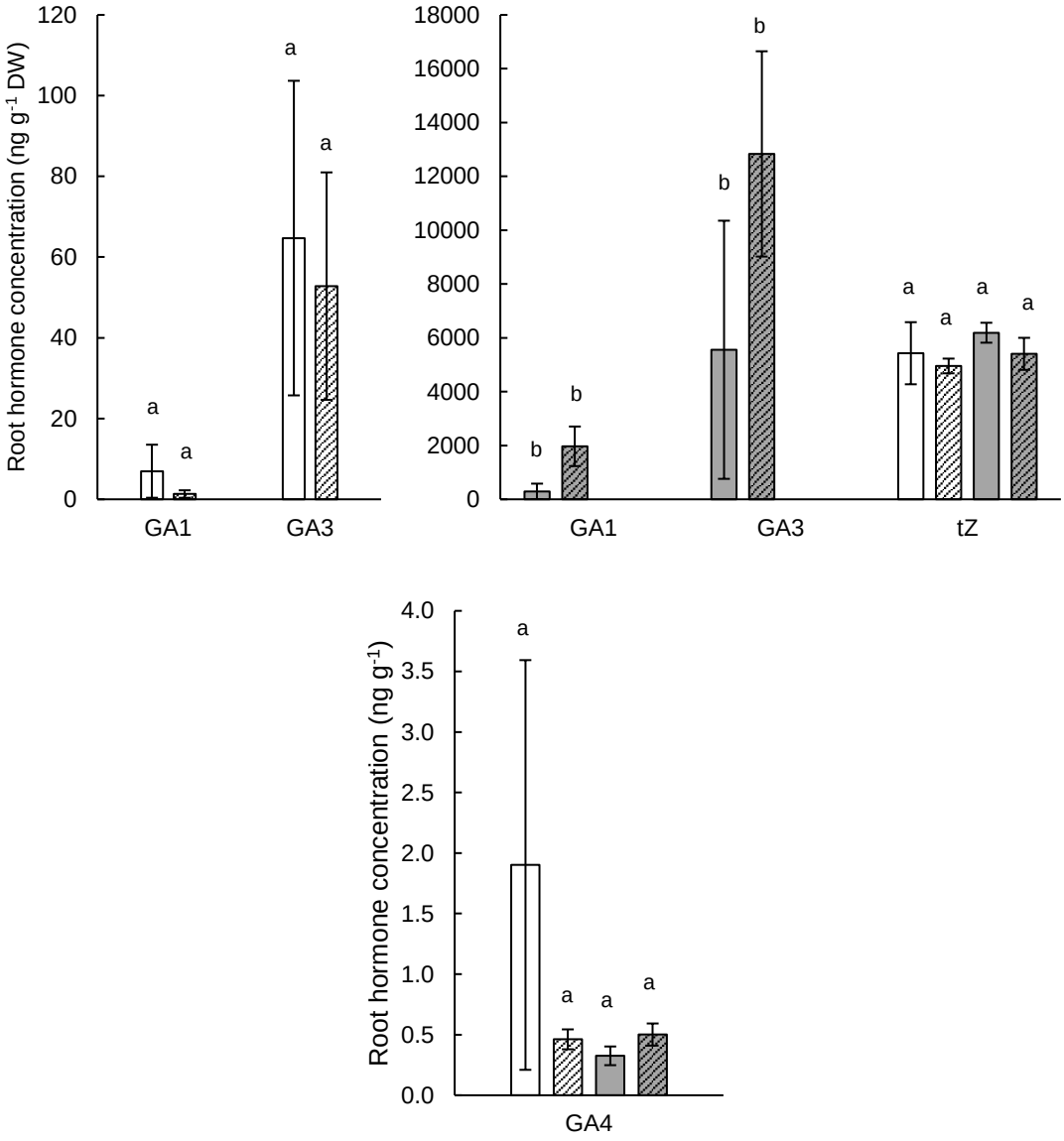


Figure 4-13: Root phytohormone concentrations of tomato grown in under low and high soil strength conditions, receiving control or GA_3 root drenches.White bars: Low strength-Control; White/striped bars: Low strength-GA_3; grey bars: High strength-Control; Grey/striped bars: High strength-GA_3. Bars are means ± S.E. of 12 replicates, with different letters denoting significant differences between means within an analyte (post-hoc LSD $p < 0.05$).

Table 4-4: *p*-values from ANOVA summary tables from linear mixed models of root tissue phytohormones. Significance of *p* values reported thus: · p is marginally non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. For succinctness, three-way interaction between Time, Root drench (GA) and strength is presented in the Interaction column unless otherwise stated (T = Time, GA = Root drench, S = Strength).

		Factor			
Hormone	**Transformation**	**Time**	**GA**	**Strength**	**Interaction**
tZ		0.28	0.27	0.067 ·	0.23
iP		0.29	0.66	0.94	0.47
ACC	Log_{10}(ACC)	0.43	0.64	0.016 *	0.011 * (GA*S)
ABA		0.95	0.99	0.002 **	0.77
JA		0.38	0.13	0.097 ·	0.052 · (T*S)
SA		0.11	0.10	0.054 ·	0.80
IAA		0.89	0.27	0.007 **	0.69
GA_1	$Log_{10}(GA_1 + 0.01)$	0.66	< 0.001 ***	0.21	0.31
GA_3	$Log_{10}(GA_{3+} + 0.01)$	0.99	0.003 **	0.10	0.83
GA_4	$Log_{10}(GA_4 + 0.01)$	0.66	0.23	0.86	0.059 · (T*S)

Roots were collected at harvest. Strong sand significantly increased ($p = 0.012$; Figure 4-13) root ACC concentrations, but this effect was affected by GA treatment (significant GA treatment x strength interaction - $p = 0.03$). Thus, root [ACC] increased nearly 8-fold in GA_3-treated plants compared to the 2-fold increase in control plants. Root [ABA] was also significantly reduced in plants grown in strong sand ($p = 0.043$), and increasing mechanical strength tended to reduce root [IAA] ($p = 0.08$). Substrate strength did not significantly affect root cytokinin (iP and tZ), JA, GA_4 or SA concentrations.

Table 4-5: Pearson's correlations between xylem sap and root tissues phyothormone concentrations in plants receiving control root drenches only. · Significance of *p* values reported thus: · *p* is marginally non-significant; * *p* < 0.05; ** *p* < 0.01; *** *p* < 0.001.

		Xylem Sap								
		ACC	**tZ**	**iP**	**GA3**	**GA4**	**IAA**	**ABA**	**JA**	**SA**
Root tissue	**ACC**	-0.16	-0.3	-0.06	0.11	0.04	0.03	-0.29	0.04	-0.13
	tZ	-0.11	**-0.35 .**	0.04	0	0.14	0.3	-0.09	0.32	0.34
	iP	-0.11	-0.32	0.05	0.09	-0.21	0.16	0.11	0.09	-0.29
	GA3	0.03	**0.39 .**	-0.16	-0.31	**0.57 ****	0.25	**0.44 ***	**0.45 ***	-0.08
	GA4	-0.14	0.12	-0.08	0.22	**0.48 ***	-0.19	-0.09	-0.09	-0.04
	IAA	-0.19	0.26	0.3	0.11	-0.28	-0.08	**0.4 .**	-0.19	-0.08
	ABA	-0.23	**0.45 ***	0.23	0.1	-0.05	-0.33	**0.36 .**	-0.27	-0.29
	JA	-0.14	-0.28	0.1	0	0.26	0.28	-0.08	0.04	-0.33
	SA	-0.08	-0.07	0.04	-0.12	0.06	0.08	-0.01	0.11	-0.21

Correlations between root and sap phytohormones in control drench plants revealed significant, positive associations between sap [GA_4] and root [GA_3] and [GA_4] concentrations (Table 4-5). Root [GA_3] was also significantly positively correlated with sap [ABA] and [JA]. Root ABA was positively correlated with sap [tZ]. However, in general, root tissue hormone concerntrations were poorly correlated with concentrations of the same hormone in xylem sap.

4.4 Discussion

Plant growth and biomass accumulation

Plants growing in strong sand are smaller, with lower leaf area and shoot biomass, even though water and nutrients were supplied in abundance and not limiting (Jin et al., 2015). Greater mechanical strength also slowed both leaf expansion and leaf emergence rates. GA_3 treatment significantly increased total leaf area at harvest in both low and high strength treatments, although these differences were quite small (40% leaf expansion promotion averaged across both soil strengths). Contrary to Figure 3-2B, here GA_3 treatment had little effect on leaf area of plants grown in compact sand, and control plants were still almost 50% larger than high strength plants receiving GA_3 treatment (Figure 4-6C). Likewise, GA_3 treatment increased wheat leaf expansion, with a significantly greater effect of GA_3 in plants growing under low strength conditions (Coelho Filho et al., 2013). However, GA_3 application restored leaf emergence rates of plants growing in high strength soil such that they were not different from low strength controls (Figure 4-5A). Thus, GA_3 treatment had a greater effect on leaf initiation than leaf expansion in tomato growing in strong sand.

Combined with increased stem elongation and biomass accumulation, GA_3 may promote growth in tomato growing in strong soil by enhancing plant development rates and reducing time to reproductive maturity (Mutasa-Gottgens and Hedden, 2009). Here, across both experiments, increased soil strength reduced the number of plants reaching inflorescence at harvest by 80%. GA application had no effect on flowering time under low strength conditions, but increased number of flowering

plants in high strength treatments almost 3-fold (Figure 4-14). In contrast, transgenic tomatoes over-expressing *SlGA20ox* (enzyme promoting bioactive GA formation) increased the number of leaves emerging by 1 before first inflorescence and delayed flowering (~5 days longer than WT; García-Hurtado et al., 2012), but this study did not record age of plant at first flowering. Thus, GA_3 applications reduced time to inflorescence, with a more sensitive response in high strength plants, suggesting that substrate strength not only reduces growth but delays flowering and reproductive development in tomato.

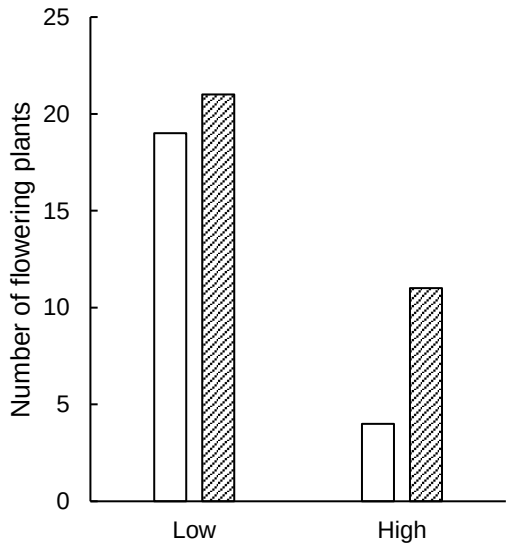


Figure 4-14: Number of tomato plants (out of 24 per strength/root drench combination) flowering at harvest (approx 4 weeks after transplanting), growing in low or high root strength conditions. White bars: control root drench; shaded bars: GA3 root drench.

Nitrogen status

While plants grown at high strength had increased chlorophyll concentrations, GA_3 treatment decreased SPAD values (Figure 4-8), corresponding to visually paler green leaves. Other workers have noted decreased leaf greenness in response to

exogenous gibberellic acid or overproduction of bioactive GAs (Wheeler and Humphries, 1963; Carrera et al., 2000; Biemelt et al., 2004). While chlorophyll content may remain unchanged or enhanced, chlorophyll per unit leaf area often decreases as leaf expansion is enhanced at the same leaf biomass, resulting in paler green leaves (Wheeler and Humphries, 1963). Although exogenous GA_3 application decreased shoot %N, GA_3-treated plants took up more nitrogen (total shoot nitrogen) over the growing period. Applying 5 µM GA_3 to the roots of cucumber seedlings increased nitrate fluxes by 25% under optimal root temperature of 22 °C (Bai et al., 2016). Therefore, GA_3 treatment enhanced N uptake, but diminished leaf chlorophyll concentrations by diluting this chlorophyll across a larger leaf area.

Decreased tZ concentration may indicate decreased N uptake, as nitrate deprivation and re-supply experiments show a tight temporal correlation between N re-supply, increased root expression of the cytokinin biosynthesis-related gene IPT (encoding isopentenyl tranferase), and increased root cytokinin export to the shoot via the xylem (Takei et al., 2001; Sakakibara et al., 2006). However, substrate strength did not change shoot %N even though xylem sap concentration of bioactive cytokinin tZ decreased under high substrate strength (Figure 4-11). Therefore, it is unlikely that this change in root cytokinin export was a response to insufficient N uptake (as expected in the sand culture system supplying supra-optimal nutrient concentrations) but rather a direct response to root mechanical impedance.

Plant water status

Increased substrate strength did not significantly reduce plant water status (Figure 4-9), indicating adequate water supply (Figure 4-10), contrary to work with compact soil (Figure 2-9; Figure 3-3B). GA_3 treatment decreased Ψ_{leaf} and Ψ_{shoot} (Figure 4-9), as in compact soil (Figure 3-3B), but without changing soil water content (Figure 4-10). Despite this decrease in plant water status, GA_3 treatment enhanced shoot growth, contrary to observations that leaf growth can be inhibited by small decreases in leaf water potential (Boyer, 1970). However, future work should incorporate direct measurements of cellular turgor to elucidate the physiological significance of changes in leaf water potential, as it is not the only water status component influencing growth rates (Boyer, 1970). Furthermore, these measurements should be made in expanding leaves, as measurements here (Figure 4-9A) were in fully expanded leaves, as these were sufficiently rigid to permit insertion in the pressure chamber.

Although GA_3 treatment significantly enhanced stem elongation, changes in gravitational potential are unlikely to significantly affect plant water status. GA_3-treated tomatoes were between 2 to 3.5-fold taller than control plants, reaching around 40 cm tall on average. The gravitational force opposing sap movement through the stem is 0.01 MPa m^{-1} (Neufeld, 2000), thus a height difference of 25 cm would only contribute 0.0025 MPa, far less than the 0.15 MPa difference between control and GA_3-treated plants. While decreased water status in response to increased transpiration rates seems a common response of tomato (Dodd et al., 2009), reductions in Ψ_{leaf} are consistent with the findings of Chapter 3 (Figure 3-3B), where GA_3 root drenches decreased g_s and ET rates (Figure 3-4). As previously

discussed, there appears to be both inter and intra-species variation in water use responses to GA_3 applications (see Chapter 3.4). While GA_3 may improve water uptake of cherry tomatoes (Maggio et al., 2010), it is clear that GA_3 reduces water status of cv. Ailsa Craig.

An alternative explanation for decreased leaf water status of GA-treated plants may relate to their root phenotype. Although GA_3 treatment increased shoot biomass, maximum rooting depth (Figure 4-6) was unaffected (at high strength) or tended to decrease (at low strength). Increased hydraulic demand of a larger shoot on a relatively smaller rootzone may lower Ψ_{leaf} and Ψ_{shoot}. However, this root-centric explanation is less plausible as GA effects on water status were conserved across soil strength treatments while GA effects on rooting depth depended on substrate strength (Figure 4-7, Interaction $p = 0.03$).

However, there is evidence that exogenous GA_3 applications upregulate aquaporin-related genes in a variety of plant species. Aquaporins are a class of membrane protein that facilitate transport of molecules of water and small neutral solutes across cell membranes (Maurel et al., 2008). They may be divided into two main classes (plasma membrane intrinsic and tonoplast intrinsic proteins –PIPs and TIPs) according to the membrane layer in which they reside. Foliar applications of GA_3 significantly upregulated the Arabidopsis tonoplast intrinsic protein (γ-TIP) associated with cell expansion (Phillips and Huttly, 1994). Exogenous GA_3 upregulates tobacco aquaporin NtAQP1 promoters by 4-fold (Siefritz et al., 2001). However, roles of aquaporins in cell elongation, particularly of TIP and PIP-type aquaporins, may allow cells to continue elongating and tissues to expand even against water potential gradients (Maurel et al., 2008) thus explaining GA-mediated growth enhancement

even at low tissue water potentials. Further work seems necessary to dissect the effects of exogenous GA_3 application on plant hydraulic conductance, particularly with respect to possibly mediating aquaporin activity.

When leaves were sampled, stomatal conductance did not vary between treatments despite an increase in foliar [ABA], particularly in GA_3-treated plants. DELLA proteins promote guard cell closure by increasing sensitivity to ABA (Nir et al., 2017), and DELLA are degraded by bioactive GAs. The increased foliar [ABA] of GA-treated plants may be counterbalanced by increased bioactive GA leading to DELLA degradation, which may explain limited g_s response to increased foliar [ABA] (Figure 4-9C).

Signalling candidates of root mechanical impedance

Increased soil strength tended to decrease bioactive GA_4, while a significant interaction between strength and GA_3 application showed that plants growing in high strength conditions had higher GA_1 contents than low strength plants. This was consistent with the observation that high strength-GA_3 plants exhibited greater stem elongation relative to high strength-control (Figure 4-6E), and this difference in sensitivity is consistent with findings of Coelho Filho et al. (2013), where stem elongation was greater in plants growing in high strength soil when receiving GA_3 drenches. Detection of GAs using UHPLC can be sporadic, and the responses of different GA species seems to vary between experiments (Figure 2-11; Figure 3-9; Figure 4-12), but in general, decreases in growth are accompanied by a decrease in

bioactive GAs, and *vice versa*. However, using multihormonal analyses, it is possible to investigate possible cross-talk between signals.

Importantly, using sand as a growing medium increased the ease and speed of root tissue sampling, thus discerning hitherto undetected effects of substrate strength on root hormone concentrations. For the first time, increased root [ACC] was detected under high mechanical strength, even if its possible transport to the shoot could not be confirmed as measurable xylem ACC concentrations were sporadic (< 50% of the total samples collected). Tissue [ACC] increased in response to increased external pressure in maize (Sarquis et al., 1991), but external pressure systems may exert unrealistic impedance upon plants (Bengough and Mullins, 1990b). Furthermore, the whole plant was pressurised within a cell for up to 10 h, and may have induced an ethylene-wounding response (Moss et al., 1988; Bengough and Mullins, 1990a; O'Donnell et al., 1996).

Increased foliar ethylene evolution in response to specific soil compaction treatments (Hussain et al., 2000) likely occurred in response to low soil oxygen concentrations at low soil porosity (0.02 m^3 m^{-3}), as 5% (v/v) air-filled porosity often described as characteristic of a soil deficient in aeration (Stępniewski et al., 2013). Increased xylem sap and foliar [ACC] in response to soil hypoxia via flooding (Else et al., 1995; Else and Jackson, 1998) are consistent with this interpretation. However, it has been argued that the sand culture system used here provides a normoxic rootzone (Whalley et al., 2006), thus enhanced root ACC concentrations likely represents a direct response to increased substrate strength.

Whether ACC in the roots is converted to ethylene remains to be investigated in this system. Although petiole ethylene evolution increased linearly with xylem [ACC] levels (Else and Jackson, 1998), ACC may also be conjugated into inactive 1-(malonylamino)-cyclopropan-1-carboxylic acid (MACC) thus regulating ethylene production. Although increasing external pressure between 25 and 100 kPa did not change free ACC concentrations in maize (increased 3-fold from 0-25 kPa), MACC levels increased along with the activity of ethylene-forming-enzyme suggesting that ACC metabolism regulates ethylene evolution (Sarquis et al., 1992). Contrastingly, root mechanical stimuli triggered upregulation of genes involved in ethylene signalling but not biosynthesis, and whole plant ethylene evolution did not change in Arabidopsis (Okamoto et al., 2008). However, it was unclear from their work whether localised (root) ethylene evolution occurred. Exogenous applications of ethylene to unimpeded maize roots increased root diameter (Moss et al., 1988), which is a crucial trait for root elongation in strong soils (Bengough et al., 2011). Thus increased root [ACC] concentrations are likely to maintain or alter root architecture, perhaps via localised ethylene evolution. Further work with this sand culture system should quantify rootzone ethylene levels, although technical challenges associated with such measurements suggests that root transcriptomic analyses (of regulatory genes in the ethylene biosynthesis pathways) may also be informative.

Although root and foliar JA concentrations did not change in response to substrate strength, xylem [JA] increased at high strength (Figure 4-11), as in compact soil (Chapters 2, 3). While the role of JA in plant responses to mechanical stimuli are well-known, soil water deficit can also increase JA concentrations throughout the plant, and JA and related precursors possess anti-transpirant properties (de Ollas and Dodd,

2016). In compact soil, distinguishing the relative importance of water deficit or mechanical strength may be difficult (Chapters 2, 3). Thus, increased shoot xylem JA concentration in plants exposed to high soil strength that were not water-limited (Figure 4-9; Figure 4-10B) suggests that xylem JA acts as a direct signal of root mechanical strength.

Classically synthesised as a response to herbivory or pathogen infection, endogenous JA levels are associated with reduced plant growth and enhanced defences, particularly through altered metabolite composition (Wasternack and Hause, 2013). However, recent literature suggests that JA forms a crucial part of plant responses to mechanostimulation. Arabidopsis JA-biosynthesis mutant *aos* did not exhibit touch-induced growth inhibition: WT *gl-1* displayed reduced inflorescence elongation, 28% smaller rosette radius and 1-2 day flowering delay (Chehab et al., 2012). Increased fluorescence of biosensor Jas9-VENUS was observed on application of an agar block to Arabidopsis roots, indicating increased concentrations of bioactive JA species in the pressurised root tips (Larrieu et al., 2015). The squeeze-cell hypothesis (Farmer et al., 2014) theorises that JA biosynthesis is induced in response to changes in turgor of plant vasculature. Disturbance of turgor may be caused by wounding (JA as a defence mechanism) but also abiotic stresses including water deficit (JA species as anti-transpirants – de Ollas and Dodd 2016). It is possible that as roots push through strong substrates, they become wounded or pressure changes induce JA biosynthesis which is immediately transported. A consistent [X-JA] response across Chapters 2, 3 and 4 strongly indicates a role as a messenger of rootzone conditions.

A significant interaction between GA_3 application and soil strength was also apparent. Increased strength reduced foliar [JA] (Figure 4-12), consistent with Chapter 2 (Figure 2-11), but GA_3 drenches removed this effect, perhaps as a result of GA_3-JA crosstalk (discussed in Chapter 3.4). Thus, lack of foliar or root increases in [JA] suggest it may not directly regulate growth responses, but that it may act through a subsequent metabolic product, or through crosstalk with other phytohormones (e.g. with bioactive GAs).

4.5 Conclusions

Increased soil strength is a potent inhibitor of plant growth, even in the absence of water or nutrient limitation. Applications of GA_3 partially rescue shoot growth, but at the expense of shoot water potential. The relationship between plant water status, cell turgor and growth rates warrants further investigation, particularly since exogenous GA_3 promoted aquaporin activity - in Arabidopsis (Phillips and Huttly, 1994). Xylem concentrations of jasmonic acid and the cytokinin tZ increased in response to increased soil resistance, and further work should investigate the physiological significance of these signals and/or their related compounds.

Chapter 5 General discussion

5.1 Soil vs sand culture system

Studies of plant responses to mechanical impedance generally fall into two categories: where impedance is varied *with* or *without* other soil physical properties such as aeration or water availability (Clark et al., 2003). Generally, compacting soil alters multiple physico-chemical properties, and so plant responses cannot be solely attributed to mechanical impedance (Hamza and Anderson, 2005; Bengough et al., 2011). However, these studies are generally more representative of field conditions (Clark *et al*., 2003), so plants were grown in compact soil in Chapters 2 and 3. The sand culture system employed in Chapter 4 aimed to isolate the effects of mechanical impedance on plant growth and physiology. Despite much work focussing on potential roles for ABA and ethylene (Moss et al., 1988; Hussain et al., 2000; Mulholland et al., 1996), no consistent evidence of a root-to-shoot phytohormonal response to increasing soil compaction or strength had been established. Furthermore, for the first time, multi-hormone analyses were conducted on tissue and xylem sap collected from tomato growing under isolated mechanical impedance. Comparison of both systems (with and without a GA_3 root drench as a possible mitigating treatment) evaluated whether particular physiological responses are due to soil compaction or increased soil mechanical impedance in isolation.

5.2 Soil compaction alters relationships between plant water status and soil water content as soil dries

Increased soil bulk density increased sensitivity of Ψ_{root} to bulk soil water content as the soil dried (Figure 2-7), in agreement with observations made by Tardieu et al. (1992a) that root clustering decouples Ψ_{root} from bulk Ψ_{soil} (Figure 5-1, from Donaldson et al., 2018).

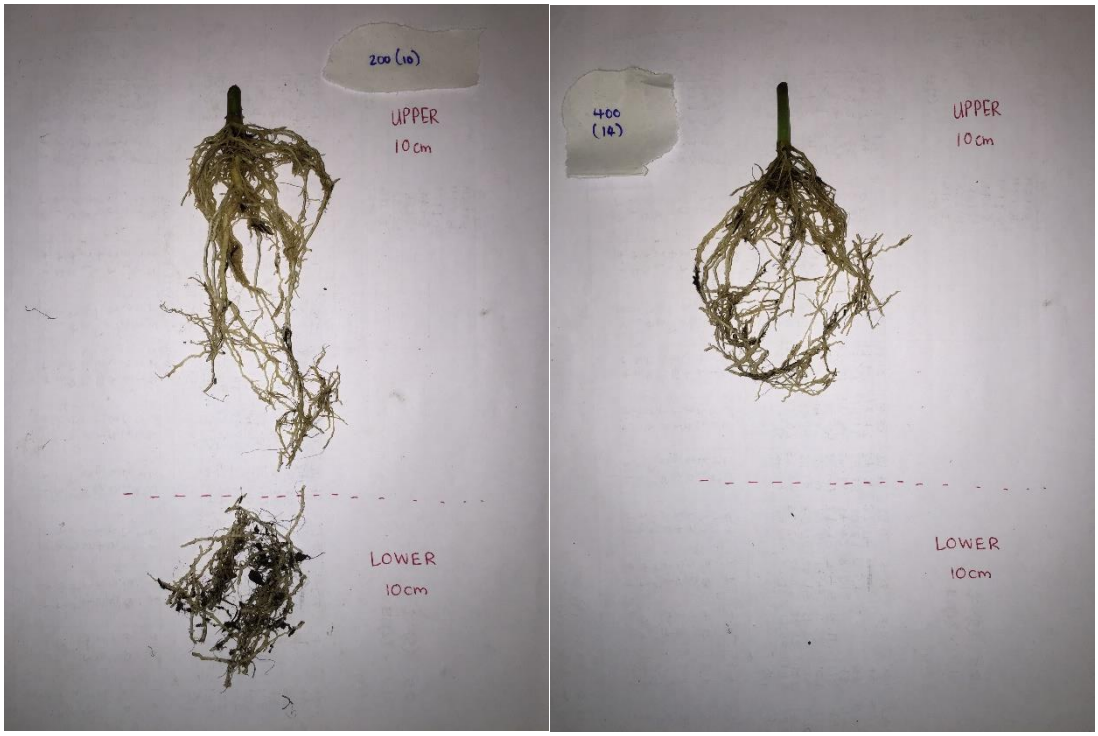


Figure 5-1: Sunflower rootzones in upper and lower 10 cm of pots when grown under low (left) and high (right) bulk density conditions (early experiments from this thesis; Donaldson et al., 2018). Roots were divided into the upper and lower 10 cm of a 20 cm soil column. Roots in high bulk density soils are clustered in the upper portion of the column.

Ψ_{root} is a measure of bulk rootzone water status, accounting for heterogeneity of the soil physical conditions. Regression models suggested that Ψ_{root} remained a good predictor of log[X-ABA] independently of bulk density (R^2 = 0.80; Figure 2-14A), as concluded by Dodd et al. (2010) when soil texture was varied. Improved understanding of GSWC-Ψ_{root}-phytohormone relationships would improve

interpretation of plant growth responses to drying soil (particularly with regards to phytohormone export). Furthermore, Ψ_{root} is scarcely considered in modelling of crop yields as its value is inaccessible in field-grown plants , and measurements of this parameter contributes to an underrepresented field of knowledge (Whitmore and Whalley, 2009).

However, despite soil compaction changing the Ψ_{root}-GSWC relationship, GSWC was still a good predictor of [X-ABA] (R^2 = 0.82; Figure 2-14C), perhaps due to a short soil profile (~22 cm), or only small changes in Ψ_{soil} in response to soil compaction in this system. Further work would be required to ascertain whether changes in Ψ_{root} could account for physiological responses when plants are grown in a deeper soil profile.

5.3 GA_3 promotes growth and rescues shoot growth in compact or strong soils

ABA likely acts as a signal of water deficit in compact soils, rather than as a signal of mechanical impedance. Variability in foliar and xylem ABA was explained by measuring plant and soil water status (Figure 2-9; Figure 3-3). Although a unifying relationship between g_s and [L-ABA] was observed across bulk density treatments (R^2 = 0.61; Figure 2-15C), the significance of [X-ABA] vs. [L-ABA] in the regulation of stomatal aperture is still debated (Wilkinson and Davies, 2002; Dodd, 2005; McAdam, Manzi, et al., 2016), and seems to depend on a range of environmental factors. For example, slow soil drying increased [X-ABA] earlier and faster than bulk [L-ABA], with [L-ABA] not differing between well-watered and unwatered maize plants, despite reduced g_s and increased [X-ABA] (Zhang and Davies, 1990). However,

the unique relationships between [X-ABA] and g_s across bulk densities (Figure 2-15B) may result from Ψ_{root} declining more sensitively as high bulk density soil dries (Figure 2-7). As previously discussed, further knowledge of the relationships between GSWC-Ψ_{root}-phytohormones could improve our understanding of growth restriction in heterogeneously-structured soil. Nevertheless, in the sand culture system, foliar [ABA] also increased with strength, and interacted with GA_3 such that high strength-GA_3 treated plants exhibited highest [L-ABA] (Figure 4-12). However, in this system, [L-ABA] did not explain g_s (which was unchanged by increasing strength or GA_3 application; Figure 4-9C), and [X-ABA] did not increase in response to mechanical impedance alone (Figure 4-11).

Soil compaction reduced concentrations of bioactive GA_1 in expanding tomato leaf tissues (Figure 2-11). GA_3, a commercially-available growth promoter (Rademacher, 2016), was applied as a soil drench to assess growth and physiological responses when plants were grown in low or high bulk density soils. GA_3 application partially rescued leaf expansion and shoot biomass accumulation of plants grown in high bulk density/strength soils, and further enhanced growth in control conditions (Figure 3-2; Figure 4-5; Figure 4-6). Since endogenous concentrations of bioactive GAs are feedback-regulated in low-stress conditions (Hedden and Sponsel, 2015), exogenous applications override this regulatory mechanism, therefore promoting further growth in plants under low bulk density/strength treatments.

However, regardless of experimental system, GA_3 root drenches always reduced plant water potentials (leaf and stem; Figure 3-3, Figure 4-9), beyond what could be attributed to increased plant height. There are varied reports regarding water uptake

and use in GA_3-treated plants, likely representing species or genotypic variation in GA_3 responses. Despite reduced transpiration rates and water potentials, GA_3 promoted tomato shoot growth, but future work should also consider leaf turgor and aspects of leaf structure and morphology, particularly as GA_3 applications caused consistent (but statistically non-significant) increases in specific leaf area (Figure 3-2D; Figure 4-6D), as well as changes in leaf shape (Figure 3-1). There is some evidence that GA_3 application enhances aquaporin activity in Arabidopsis (Phillips and Huttley, 1994), and further work should ascertain the role of aquaporins in GA_3-mediated growth promotion.

5.4 Xylem jasmonic acid concentration increases in response to high soil strength

Although JA (and associated precursors) may be synthesised in response to decreasing plant water status (de Ollas and Dodd, 2016; de Ollas et al., 2018), increased [X-JA] due to more compact soil could not be ascribed to decreased leaf water potential under WW conditions (Figure 5-2C). Furthermore, increased [X-JA] was conserved even when mechanical impedance was imposed via the sand culture system (and with a change in sap sampling methodology) (Figure 5-2D). To determine the physiological significance of this concentration, it is necessary to consider the relationship between [X-JA] and transpirational sap flow rate. Sap flow rate determines the flux of a compound through the vascular system; as transpiration decreases and sap flow is reduced, concentrations of compounds in xylem sap may increase passively, without any increase in biosynthesis (Dodd, 2005).

While [X-ABA] appeared to increase at higher soil bulk density (Figure 5-2A), [X-ABA] is highly sensitive to sap flow, since [X-ABA] increases with decreasing transpiration (Figure 5-3A and B). Assuming similar behaviour, it would be expected that [X-JA] would increase with decreasing sap flow. However, [X-JA] is stable across a range of flow rates in both Chapters 2 and 3 (Figure 5-3C and D). Furthermore, increased [X-JA] was observed in plants grown in sand culture, where water was non-limiting and thus samples were collected across a restricted range of shoot water potentials (Figure 5-2D), while [X-ABA] remained stable (Figure 5-2B). Therefore, it is concluded that [X-JA] acts as a signal of mechanical stress in the rootzone that is independent of changes in soil water availability.

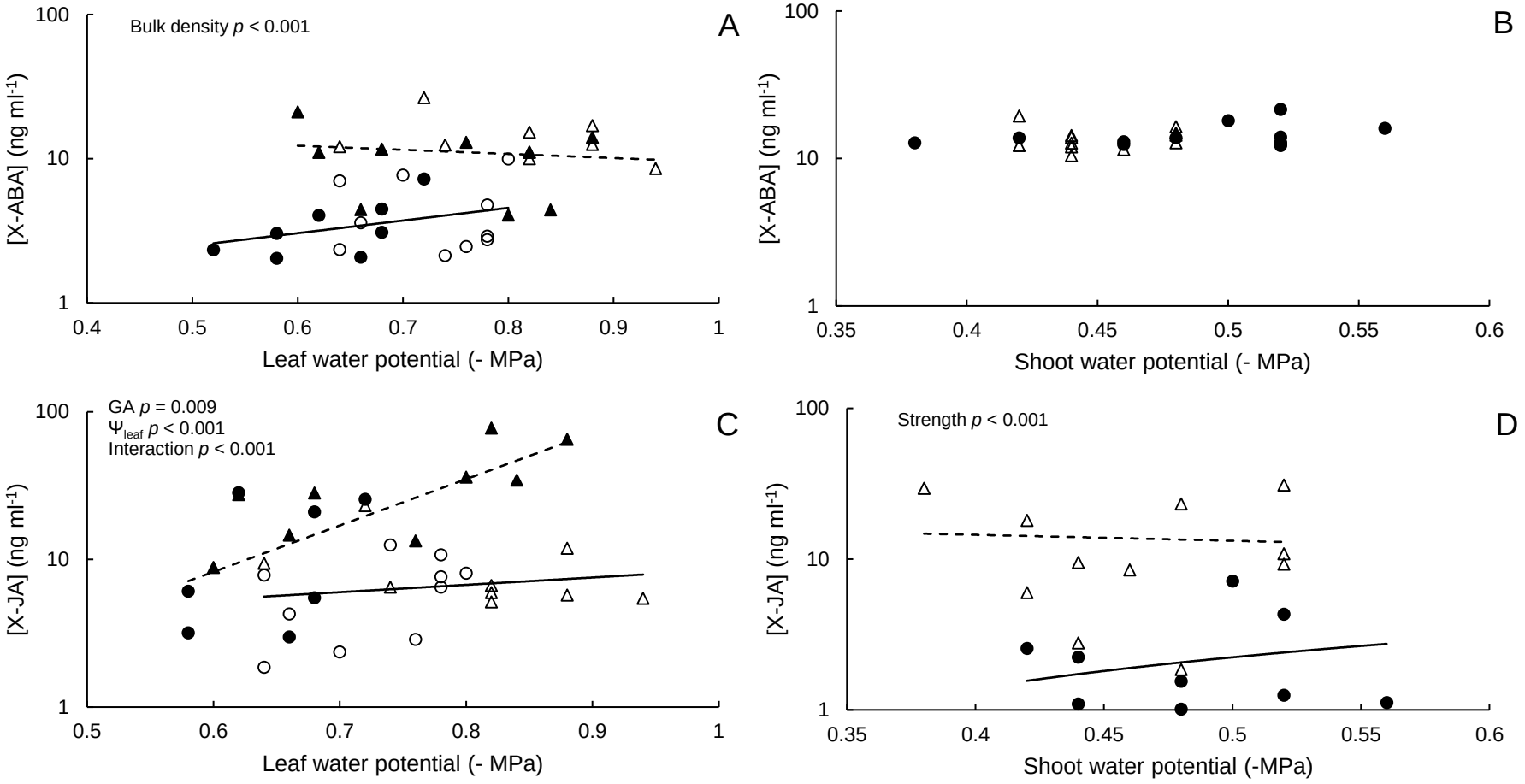


Figure 5-2: Relationships between plant water status and xylem sap concentrations of ABA and JA. A & C: (Chapter 3) Filled circles = Low bulk density-control root drench; hollow circles: low bulk density-GA_3 root drench; filled triangles = high bulk density-control root drench, hollow triangles = high bulk density-GA_3 root drench. B & D: (Chapter 4) circles = low soil strength-control root drench; triangles = high soil strength-control root drench. Each symbol is an individual plant, with trendlines fitted to highlight significant predictors remaining in multiple linear regression models. A& D: solid lines correspond to low bulk density, and dashed lines correspond to high bulk density; C: solid line corresponds to GA_3 treatment, and dashed line corresponds to control root drench.

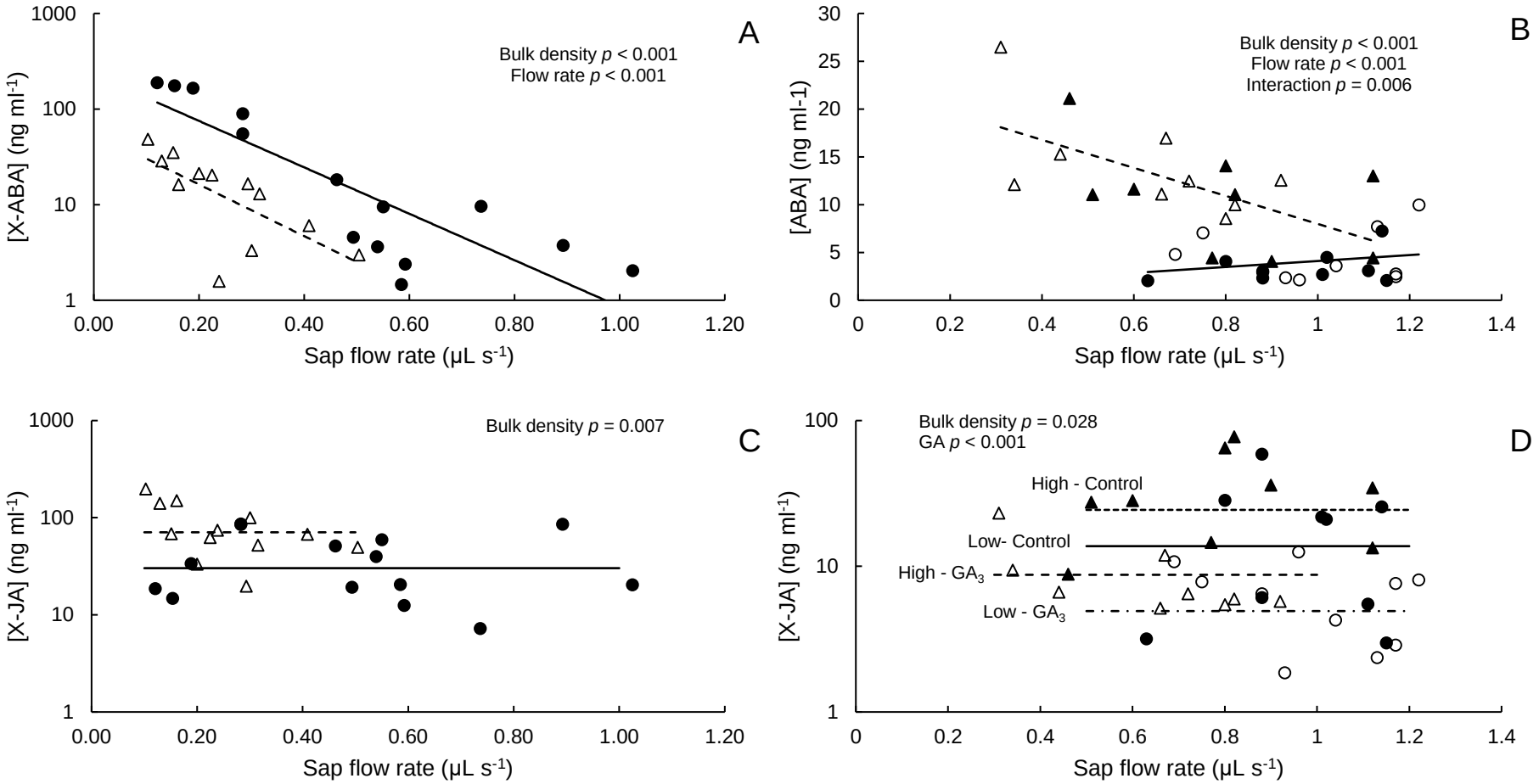


Figure 5-3: Relationship between [X-ABA] and [X-JA] and sap flow rate in tomatoes growing in low and high bulk density soils. A & C: (Chapter 2) Circles = Low bulk density; hollow triangles = high bulk density. B & D: (Chapter 3) Filled circles = Low bulk density-control root drench; hollow circles: low bulk density-GA_3 root drench; filled triangles = high bulk density-control root drench, hollow triangles = high bulk density-GA_3 root drench. Eachsymbol is an individual plant, with trendlines fitted to highlight significant predictors remaining in multiple linear regression models, with solid lines corresponding to low bulk density, and dashed lines corresponding to high bulk density, unless otherwise indicated.

JA inhibits many aspects of plant growth (reviewed Huang et al., 2017), including root and stem elongation and leaf expansion, and its biosynthesis is often associated with plant defences against herbivory and pathogens. Recent literature implicates JA in mechanoresponse pathways, particularly of shoots to touch (Chehab et al., 2012) and roots to external pressure (Larrieu et al., 2015). Reciprocal grafting of JA biosynthesis (spr-2) and response (jai-12) tomato mutants have already demonstrated the systemic action of JA signalling in response to plant wounding (Li et al., 2002). Similar experiments are warranted to investigate the precise role of changes in JA concentration *in planta* in regulating plant growth in compact or strong soils, as such experiments would be particularly useful to determine its physiological significance as a root-to-shoot signal. Elevated JA in xylem sap of plants with both WT rootstocks and scions does not necessarily mean that the wounding/compression of roots is the origin. Grafting of JA biosynthesis mutant scions (e.g. spr-2, def-1) to WT rootstocks and subsequent determination of [X-JA] in response to increased soil strength would inform our understanding of this signalling system further. Furthermore, while this work utilised the multi-hormone analysis of Albacete et al. (2008) to ensure consistency between experiments, future work (informed by JA biosynthesis pathways) should investigate the potential roles of precursors, conjugates and secondary metabolites associated with JA and so requires dedicated analyses of jasmonate species. This is required especially because foliar [JA] tended to decrease in plants grown in strong/compact soils (Figure 2-11; Figure 4-12), contrary to expectations of a root-to-shoot signal.

While GA_3 improved shoot growth in compact soil, it was also apparent from Chapter 3 (Figure 3-8) that application of GA_3 to the soil reduced the X-JA signal. Despite an

inverse relationship between [X-JA] and Ψ_{leaf} in control drench plants (Figure 5-2C), this increase in concentration was independent of the flow rate (Figure 5-3D). Possible antagonistic interactions occur between JA and GA via JAZ and DELLA proteins, as both hormones exert opposing effects on plant growth (Figure 5-4). GA is not known to depress JA biosynthesis, as the mechanism of cross-talk suggests it is the mode of action that is affected. Further work is necessary to determine how exogenous GA_3 effects stress perception, and how this may alter JA biosynthesis.

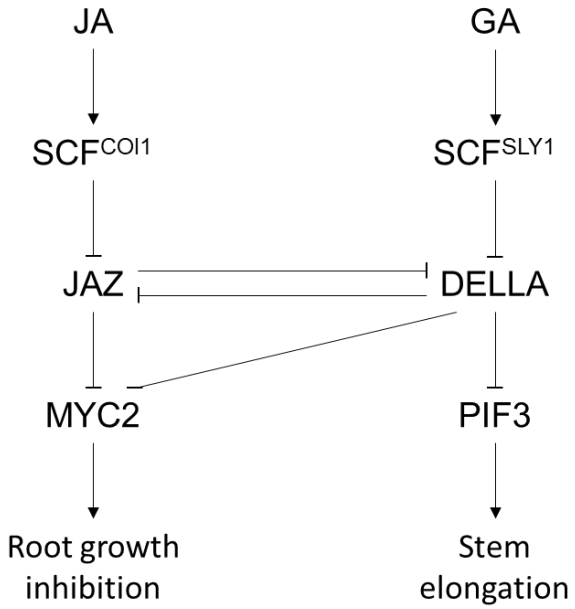


Figure 5-4: Antagonistic interactions between JA and GA regulate plant growth. Redrawn from Song et al. (2014).

5.5 Multi-hormone analyses create a complex picture of soil strength signalling and GA_3-mediated cross-talk

Multi-hormone analyses were employed here to obtain a wider picture of phytohormonal responses and relationships in plants growing in compact or strong

soils, and/or receiving GA_3 drenches. Across all experiments, foliar [tZ] tended to increase when exogenous GA_3 was supplied (Figure 3-9; Figure 4-12). This was conserved across systems, even when nitrogen % of the shoot was decreased in compact soil, which would suggest nutrient limitation (Figure 3-2E). GA and CK exert reciprocal interactions upon each other, regulating growth and developmental responses, and CKs are known to repress GA biosynthesis (Weiss and Ori, 2007). However, further work seems necessary to assess the decoupling of [CK] from nitrogen status by exogenous GA_3, particularly whether GA upregulates *IPT*.

Additionally, xylem sap [tZ] decreased in plants grown in strong sand (Figure 3-8). Reduced [X-CK] has been associated with both soil drying (Kudoyarova et al., 2007) and reduced nitrogen status (Rahayu et al., 2005), but this response occurred in a non-water/nutrient-limiting sand culture system (Figure 4-8; Figure 4-11). Further work should aim to investigate why [X-CK] of plants growing in strong soils is decoupled from nutrient status, in aiming to ascertain whether cytokinins provide a "measure" of soil strength, perhaps because they can be root synthesised. Establishing cytokinin export per unit of root biomass seems an important priority.

Increased [ACC] was observed in root tissues of plants growing in strong substrate (Figure 4-13), but no consistent changes were observed in xylem sap or foliar tissues. Evidence for increased foliar ethylene evolution exists in the work of Hussain et al. (1999; 2000), but these studies did not measure transport of ACC from the roots and so it is unclear whether ACC is transported as a long-distance signal. Furthermore, exerting external pressure upon plants increased ACC concentrations and ethylene evolution from the whole plant, both roots and shoots (Sarquis et al., 1992).

However, other work has failed to find an ethylene response (Moss et al., 1988). Further work should assess ethylene evolution from the sand culture system, to determine whether high root [ACC] increases localised rootzone ethylene evolution, or if in fact the lack of changes in foliar [ACC] is due to increased foliar ACC metabolism.

There is strong evidence that jasmonic acid act as a long-distance signal of root mechanical impedance. However, further work is necessary, particularly as this is the first work to characterise a possible role for [X-JA]. Since no effects on foliar [JA] were apparent, future work should look to investigate possible roles for JA conjugates or other metabolic products in growth restriction of plants in compact or strong soil.

5.6 Closing remarks

While much work remains to be done to further understand the physiological significance of many of the phytohormonal responses to soil compaction described in this thesis, a much more complex picture is emerging (Figure 5-5). Many of the putative signalling pathways investigated previously showed contradictory and/or equivocal evidence of their existence and/or physiological significance (Figure 1-4).

Moreover, this thesis has evaluated a possible phytohormonal strategy to overcome the effects of strong soil (Figure 5-2; Figure 5-3). In view of increasing concerns that soil compaction may be contributing to yield stagnation of many crops (Knight et al., 2012; Valentine et al., 2012), further work is needed to establish whether manipulating phytohormone signalling *in planta* represents a viable adaptation

strategy. While plant growth regulators such as GA_3 seem to alleviate shoot growth restriction, this thesis has shown that exogenous GA_3 applications affect multiple layers of plant growth and physiological regulation. Antagonistic relationships between GA_3 and xylem jasmonic acid were uncovered. The interactions between exogenous GA_3 and secondary products of JA warrants further investigation, particularly as the effects of GA_3 on plant physiology seem to vary between and within species. Such knowledge would may inform possible targets of either genetic manipulation or development of exogenous plant growth regulators to alleviate some of the physiological effects of strong soil.

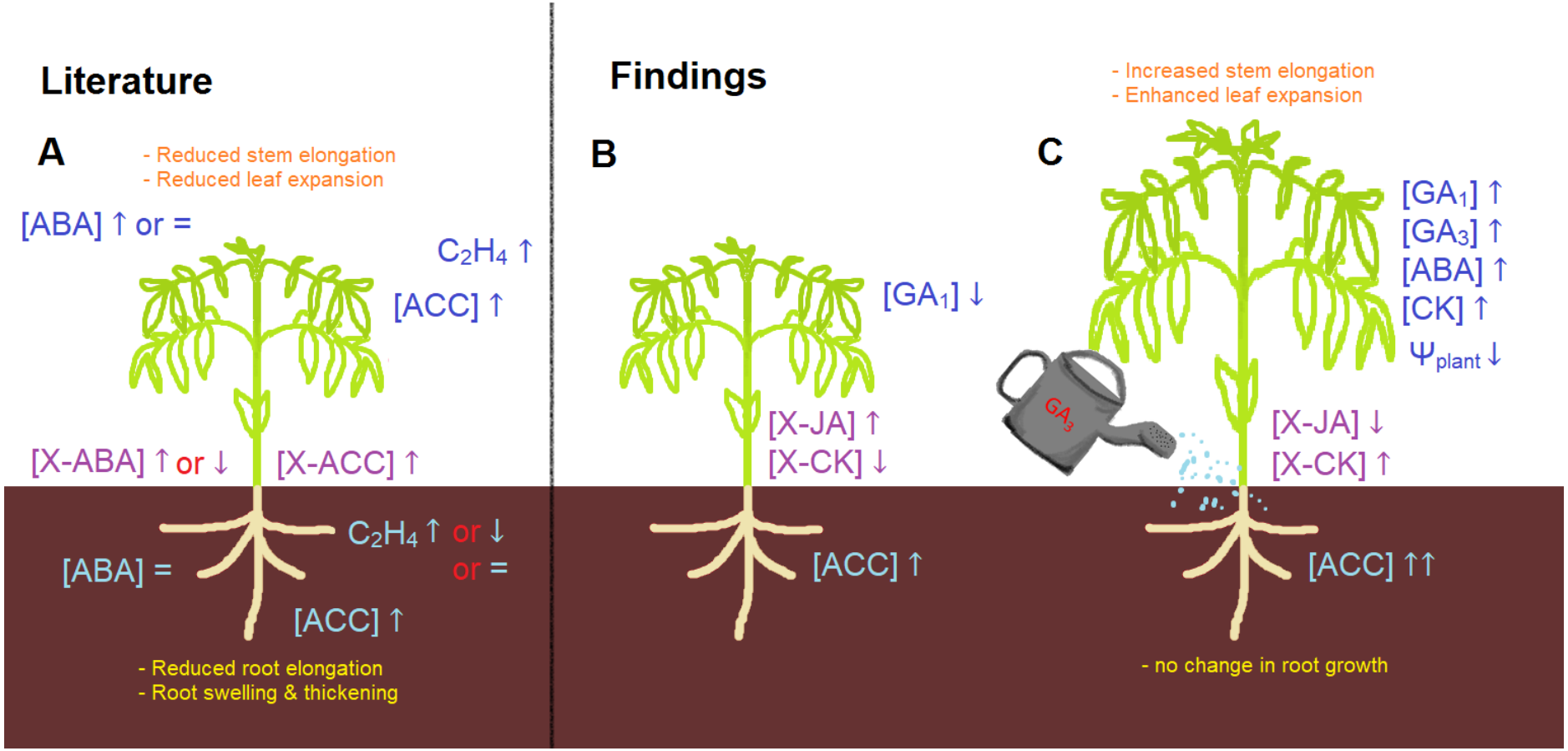


Figure 5-5: Possible phytohormonal responses of plants to compact or strong soil, from literature (A). The findings of this thesis are summarised: Compact/strong soils (B); Compact/strong soil + GA_3 root drench (C).

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Appendices

Appendix 1

Soil bulk density impacts on root water potential and ABA export in drying soil

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ABSTRACT

Abscisic acid (ABA) is a phytohormone known to regulate leaf gas exchange and water loss by inducing stomatal closure. ABA is synthesised in response to a variety of abiotic stressors in soil, particularly water deficit. Previous work demonstrated that across a range of soil textures, root water potential better predicted xylem ABA concentration than soil matric potential. However, the impacts of soil management practices (e.g. cultivation, compaction, organic matter addition) on the relationship between root water potential and xylem ABA concentration, when texture is held constant, has not been investigated.

A loam-based growing substrate was compressed to three bulk densities (1.1, 1.3 and 1.4 g cm^{-3}) in pots designed to fit in a Scholander-type pressure chamber, allowing the water potential of the bulk root system to be measured. After measuring root water potential, additional pressure was applied to collect root xylem sap at flow rates that matched transpirational flow. This allowed accurate determination of ABA concentrations and delivery. Low bulk density enhanced the increase in xylem ABA concentration as root water potential declined. Increasing bulk density de-sensitised the relationship between root water potential and xylem ABA concentration. Further study is required to determine whether changes in soil structure due to field management regimes will alter the relationship between root and soil water potential.

Keywords: abscisic acid, bulk density, root water potential

INTRODUCTION

Soil structure refers to the arrangement of particles, aggregates and pores within a soil. This arrangement determines properties such as soil water holding capacity, movement of gases, liquids and nutrients, and consequently the support of plant growth (Bronick & Lal, 2005). The stability of a soil is influenced by a range of biotic and abiotic factors, including the basic soil texture, organic carbon content, and soil biota (Bronick & Lal, 2005). Maintaining soil structure and stability is of utmost importance in order to meet food security needs for the 21st century: global

climate change and resource depletion pose significant threats to crop yields (Lal, 2009).

Soil compaction, a process of soil structure degradation, has become increasingly widespread through the adoption of heavy agricultural machinery (Batey, 2009). Soil aggregate and pore spaces become deformed due to compressive forces at the surface of the soil, increasing soil bulk density and reducing available space for storage and movement of water, gases and nutrients (Hamza & Anderson, 2005). Compaction may restrict plant growth by increasing soil strength and creating anoxic layers within the soil, decreasing root penetration and resource acquisition (Batey, 2009). Soil compaction also contributes to soil erosion, leading to off-site effects including leaching, pollution and reduced flood mitigation (Batey, 2009). Susceptibility of a soil to compaction depends on its stability, and appropriate soil management is necessary to reduce compaction.

The relationship between soil water content and soil matric potential is referred to as the water release characteristic (WRC) (Gupta, Sharma & DeFranchi, 1989). As soil structure affects water retention and movement, changes to the WRC of a soil may indicate changes in soil structure (Gupta, Sharma & DeFranchi, 1989). Increasing bulk density may alter the WRC, and the magnitude of this change is dependent on soil texture (Box & Taylor, 1962, Stirzaker et al., 1996).

Plants transmit information on rootzone conditions to aerial tissues by synthesising and transporting chemical signals through the vascular system. The phytohormone abscisic acid (ABA) is produced in response to a wide variety of abiotic stresses, notably drought stress. Under soil water deficit, increased delivery of ABA from dehydrated roots to the leaves can cause stomatal closure even before changes in leaf turgor are detected (Wilkinson & Davies, 2002). Stomatal closure limits gas exchange and photosynthesis and may lead to decreased plant growth. Enhancing our understanding of the relationships between soil conditions and ABA production is important to minimise yield losses in unfavourable climates.

Across soils of different textures, root water potential (Ψ_{root}) is a good predictor of sap flow and ABA delivery from the roots, even as soil matric potential is varied (Dodd et al., 2010). However, Ψ_{root} is inaccessible in the field, and further work is required to fully understand the relationship between soil structure, Ψ_{root} and xylem sap ABA concentration ([X-ABA]), as other workers have suggested that the relationship between Ψ_{root} and Ψ_{soil} may be influenced not only by soil structure, but also root system architecture (Tardieu, Bruckler & Lafolie, 1992).

In this study, plants were grown at a range of bulk densities (1.1, 1.3 and 1.4 g cm^{-3}) and allowed to dry the soil. Plants were harvested on each day after withholding water, to investigate the impact of bulk density on the relationship between Ψ_{root} and [X-ABA].

MATERIAL AND METHODS

Preparation of soil

A loam-based growing substrate (John Innes No. 2, John Arthur Bowers, UK) was sieved to 10 mm and air-dried for 48 hours. The substrate was added in 3 cm

depth increments to PVC pots (inner diameter 6.4 cm, height 24 cm) designed to fit tightly inside a Scholander Pressure chamber (Soil Moisture Corp., USA) of the same dimensions, with each layer being compressed by a set weight.

For compacted treatments, a Universal Testing Machine (Alfred J. Amsler & Co., Schaffhausen, Switzerland) was used to compress a metal cylinder (diameter 62 cm, height) onto the surface of each layer. For the control treatment, bodyweight (55 kg) was exerted onto the cylinder. The two compacted treatments were compressed with 200 kg and 400 kg. The upper surface of the filled pots were then covered with tin foil, and the bottom halves were submerged in tap water and allowed to re-wet through capillary action until field capacity was reached (approximately 5 days).

Plant growth conditions

Helianthus annuus, cv. Tall Yellow, (sunflower) seeds were germinated on wet paper towels sealed in plastic bags, wrapped in foil and kept in the dark at 21°C. Seedlings were transplanted after 3 days, when the radicles had reached at least 15 mm. To improve establishment, a 3 cm deep hole was made at the surface of the soil for each of the 3 seedlings planted per pot. The plants were grown in a controlled-environment room (day/night temperature of 24ºC/19ºC, 16 h photoperiod). Plants were etiolated in darkness until the hypocotyls were approximately 60 – 80 mm long, to facilitate xylem sap sampling from the hypocotyl after de-topping. Pots were maintained at field capacity for 3 weeks by applying tap water slowly to the surface of the soil, minimising disturbance of the soil surface. After 3 weeks, the soil was allowed to dry by withholding water from all plants. After 3 days of soil drying, 2 plants per treatment were harvested each day (6 plants per day). Soil bulk density was quantified at the end of the experiment by drying soil at 80°C for two weeks, and calculating the mass per cm^3 volume of cylinders from the surface of the soil.

Measurements

Stomatal conductance (AP4 porometer: Delta-T Devices Ltd, Cambridge, UK) and leaf water potential (C52 thermocouple psychrometers: Wescor Inc., Logan, UT, USA) of the second leaf pair were measured at harvest. Plants were de-topped immediately below the cotyledons and the pot transferred to a Scholander Pressure chamber (Soil Moisture Corp., USA) to measure root water potential. By calculating water loss by transpiration in the hour prior to sampling, xylem sap was collected at the correct flow rate to accurately determine ABA concentration using radioimmunoassay (Quarrie, 1988). Leaf area was measured at harvest using a Leaf Area Meter (Licor 3100: Li-Cor Corporation, Lincoln, NE, USA). Shoot tissues were dried at 80ºC for two weeks to obtain dry weights.

RESULTS AND DISCUSSION

Increasing bulk density from 1.1 to 1.4 g cm^{-3} decreased total leaf area (Fig. 1) by 57%, in agreement with previous studies of sunflower growth in compacted soils. Similarly, by increasing bulk density from 1.3 to 1.7 g cm^{-3} in coarse soil, Andrade, Wolfe & Fereres (1993) decreased total leaf area of sunflowers by over 50%. Despite

these differences in plant size between treatments, there was no influence of plant size on Ψ_{root} (data not shown).

All bulk density treatments exhibited similar, negative relationships between gravimetric soil water content at harvest and Ψ_{root}. However, Ψ_{root} was significantly lower (more negative) in plants grown in compacted soils at the same soil water content (Fig. 2). Despite differences in Ψ_{root} at the same soil water content, the relationship between [X-ABA] and bulk soil gravimetric water content was conserved across the three bulk density treatments (Fig. 3).

[X-ABA] increased linearly as Ψ_{root} decreased (Fig. 4). The sensitivity of ABA production to declining Ψ_{root} significantly differed between compaction treatments. Higher bulk density attenuated the effect of decreasing Ψ_{root} on [X-ABA], as control plants (1.1 g cm^{-3}) had higher X-ABA as Ψ_{root} decreased (Fig. 4). Although it has been suggested that Ψ_{root} is the best predictor of [X-ABA] across soil textures and matric potentials (Dodd et al. 2010), the significant interaction between bulk density and Ψ_{root} found here implies that this relationship could be altered by soil structure.

Changes in bulk density influence soil structural properties and plant growth. Stirzaker et al. (1996) found that barley seedlings grew largest at an intermediate bulk density: this allowed optimal resource acquisition due to good root-to-soil contact and maximum soil volume. Root growth may be restricted in strong soils, accelerating the depletion of water and nutrients in soil zones closest to the roots. Clustering of root systems in strong soils may influence the relationship between bulk soil water content and Ψ_{root}. Tardieu, Bruckler & Lafolie (1992) modelled the impact of root architecture on Ψ_{root} and found that clumped root systems had lower Ψ_{root} at the same bulk Ψ_{soil} (compared to plants in which roots were evenly distributed in the soil profile) since water held in uncolonised zones of soil was not available for plant uptake. In the work presented here, plants grown in the highest bulk density treatment experienced root restriction, as all the roots were confined to the upper 10 cm of soil (Fig. 5). Treatment differences in Ψ_{root} at a given soil water content (Fig. 2) may reflect differences in local soil water availability, as opposed to bulk soil water content measured here gravimetrically, in agreement with the models of Tardieu, Bruckler & Lafolie (1992).

Leaf water potential and stomatal conductance decreased in all treatments as soil water content decreased (p<0.001). However, leaf water potential had no significant influence on stomatal conductance, and at high leaf water potentials significant differences in stomatal conductance were still observed between treatments (Fig. 6). Previous studies (Andrade, Wolfe & Fereres, 1993, Masle & Passioura, 1987) observed decreased leaf conductance in the absence of changes to leaf water potential, suggesting the action of a chemical signal (e.g. ABA), rather than hydraulic signalling.

The role of ABA in soil strength signalling remains controversial. Wild-type and ABA-deficient Az34 barley seedlings grown in compacted soil (1.6 cm^{-3} and above) showed increased [X-ABA] at 6 days after emergence, with a single relationship between [X-ABA] and leaf conductance (Mulholland et al., 1996). However, Whalley et al. (2006) did not find any significant changes in shoot [X-ABA]

in field-grown wheat under different compaction and irrigation regimes, leading them to dismiss the role of ABA in signalling of soil strength stresses. In both of these cases, xylem sap was not collected at transpirational flow rates, which most accurately estimates phytohormone concentrations (Netting et al., 2012).Since our work showed that bulk density did not affect [X-ABA] of well-watered plants or the increase in [X-ABA] with soil drying, it seems unlikely that ABA mediates the reduction in leaf area and stomatal conductance caused by soil compaction. Consequently, alternative root-to-shoot signals produced under high soil strength, such as ethylene (Hussain et al., 2000) and gibberellic acids (Coelho Filho et al., 2013) should be investigated to enhance our understanding of plant growth regulation in compacted soil.

ACKNOWLEDGEMENTS

This work was supported by funding from the Biotechnology and Biological Sciences Research Council (BBSRC).

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Figures

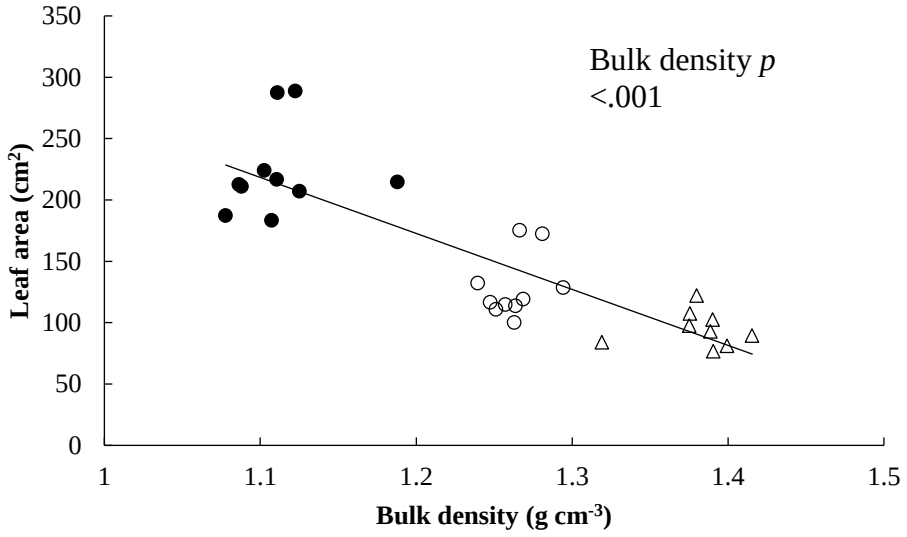

Figure 1: Relationship between soil bulk density and total leaf area at harvest. Relationship was conserved across plants grown in all bulk densities. Symbols indicate soil compaction treatments, with each point an individual plant: filled circles = control/55 Kg; hollow circles = 200 Kg; hollow triangles = 400 Kg. Linear regression fitted to data.

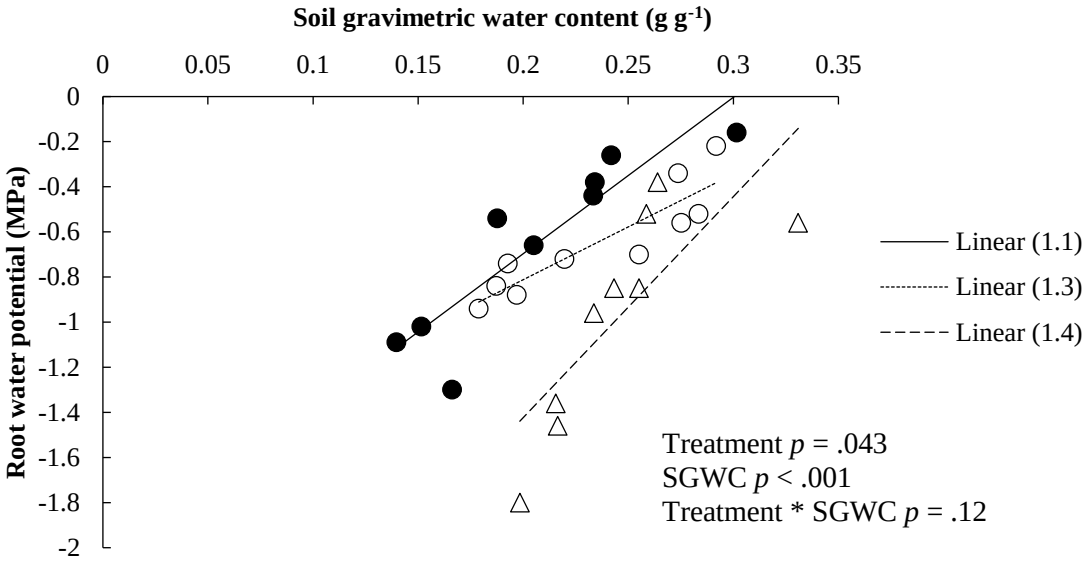

Figure 2: Relationship between root water potential and soil gravimetric water content in sunflowers grown at 3 soil bulk densities. Symbols as described in Fig. 1, regression lines were fitted when significant (P < 0.05). P-values for treatment, soil gravimetric water content (SGWC) and their interaction indicated.

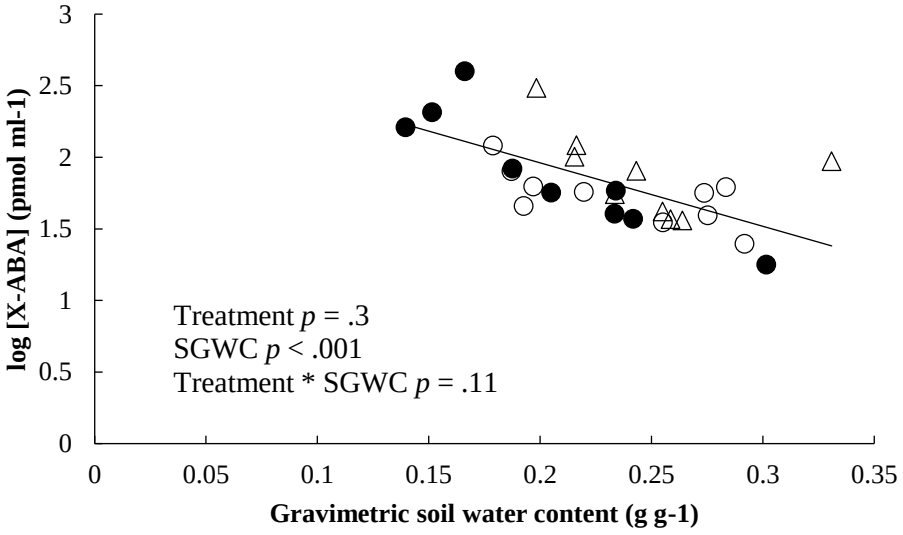

Figure 3: Relationship between log[X-ABA] and soil water content. Relationship was conserved across plants grown in all bulk densities. Symbols as described in Fig. 1. P-values for treatment, SGWC and their interaction indicated.

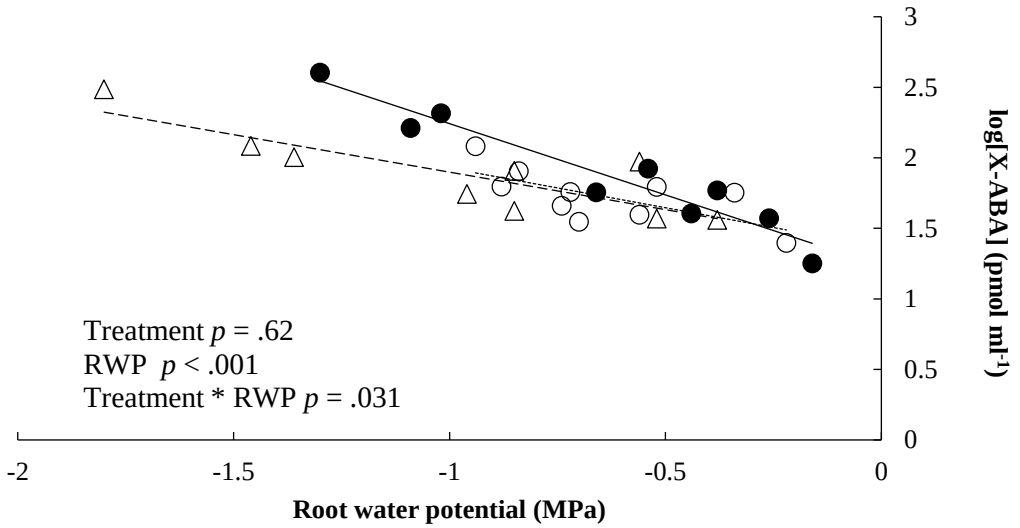

Figure 4: Relationship between root water potential and [X-ABA]. Symbols as described in Fig. 1, regression lines in Fig. 3. P-values for treatment, root water potential and their interaction indicated.

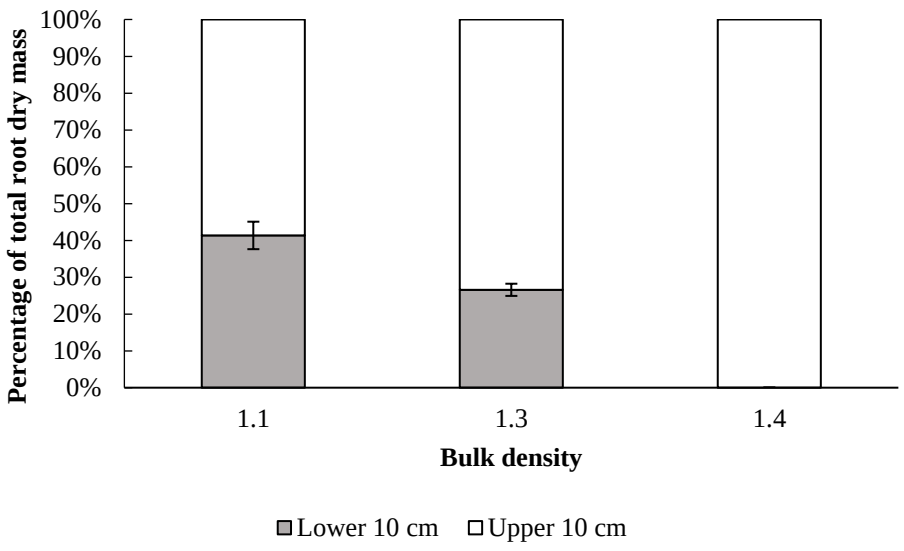

Figure 5: Distribution of dry root mass between the upper 10 cm and lower 10 cm of the pot. Error bars show S.E., *n* = 3 per treatment.

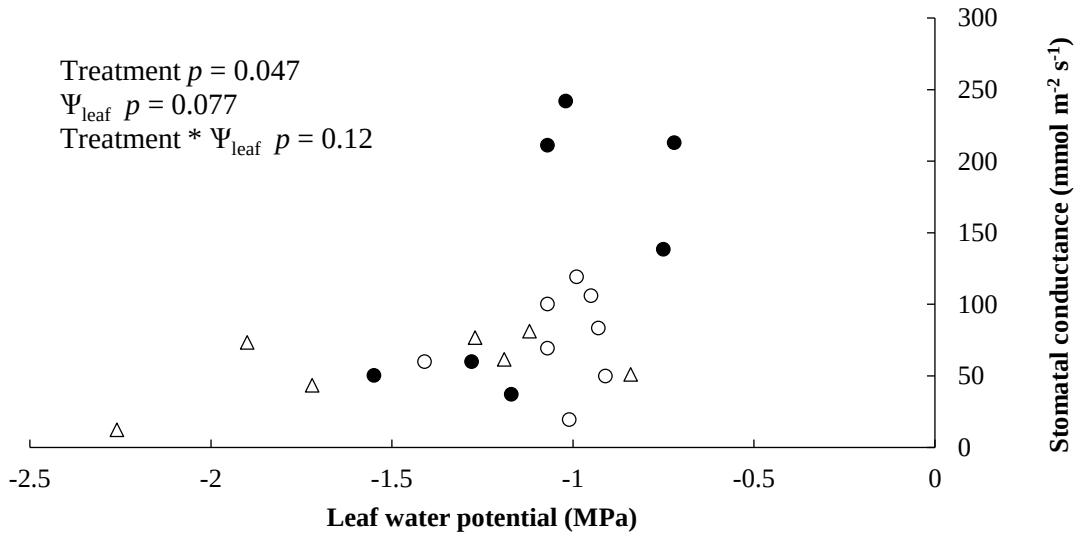

Figure 6: Relationship between leaf water potential and stomatal conductance. Symbols as described in Fig. 1. P-values for treatment, Ψ_{leaf} and their interaction indicated.

Appendix 2

Soil penetrometer resistance

Penetrometer resistance was measured to quantify soil strength. Soil was packed into pots as described in Chapter 2.2. An Instron 5944 Load Frame (Instron, Illinois Tool Works Inc., Glenview, IL, USA) fitted with a 100 N load cell measured force exerted by a needle penetrometer (2 mm diameter) to displace the soil. Penetrometer resistance rose significantly ($p < 0.001$) as bulk density was increased from 1.3 g cm^{-3} to 1.6 g cm^{-1} (Figure A-1).

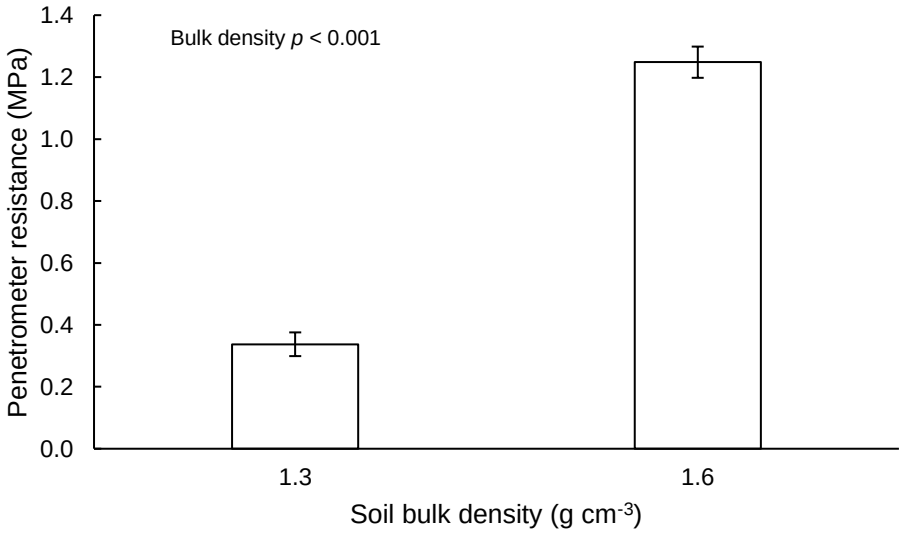

Figure A-1: Penetrometer resistance of Norfolk sandy loam soil packed to contrasting bulk densities. Bars are means of 4 replicates ± S.E.