FUNDAMENTAL CONTROLS ON PLANT ROOT EXUDATION UNDER CLIMATE CHANGE

A thesis submitted to The University of Manchester for the degree of

Doctor of Philosophy

in the Faculty of Science and Engineering

2021

REUBEN C.P. MARGERISON

School of Natural Sciences

Department of Earth and Environmental Sciences

List of Contents

Word count: 25,759

List of Figures

List of Tables

Abstract

Title: Fundamental Controls On Plant Root Exudation Under Climate Change

Reuben C.P. Margerison

Root exudates are increasingly recognised as a key driver of global carbon cycling and ecosystem function. The analysis of root exudates has traditionally taken place on plants that have been grown in hydroponic systems. However, this environment lacks the highly complex and heterogeneous structure, as well as the microbial community, of soil, and collected root exudates might not be meaningful for predicting their function in a soil environment. There is no general consensus on the best way to collect and analyse root exudates, because of methodological issues with plant growth in both hydroponics and soil. Here we show that a hybrid system of soil-based growth with a hydroponic root repair stage causes consistent differences in 1) root traits, 2) root metabolites, and 3) root exudates, when compared with growth in purely hydroponic systems. We additionally show that this method was capable of detecting a distinct metabolomic signature of drought in whole root tissue and in exudates, even after a hydroponic root-recovery period, validating its use for abiotic stress-type environments. This work also advances the use of Fourier Transform Infra-Red spectroscopy as an appropriate and inexpensive high-throughput method for analysis of exudates as a sample type, particularly when combined with mass spectrometric techniques. Our results demonstrate the utility of infra-red spectroscopy in investigations of exudates and soil chemistry and show that this inexpensive technique can offer an insight into belowground systems. Future work concerning rhizosphere responses to abiotic stresses should focus on soil-based systems and should consider spectroscopic analyses as an alternative or additive to spectrometric techniques.

Declaration

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Copyright statement

I. The author of this thesis (including any appendices and /or schedules to this thesis) owns certain copyright or related rights in it (the "Copyright") and he has given The University of Manchester certain rights to use such Copyright, including for administrative purposes.

II. Copies of this thesis, either in full or in extracts and whether in hard or electronic copy, may be made only in accordance with the Copyright, Designs and Patents Act 1988 (as amended) and regulations issued under it or, when appropriate, in accordance with licensing agreements which the University has from time to time. This page must form part of any such copies made.

III. The ownership of certain Copyright, patents, designs, trademarks and other intellectual property (the "Intellectual Property") and any reproductions of copyright works in the thesis, for example graphs and tables ("Reproductions"), which may be owned by third parties. Such Intellectual Property and Reproductions cannot and must not be made available for use without the prior written permission of the owner(s) of the relevant Intellectual Property and/or Reproductions.

IV. Further information on the conditions under which disclosure, publication and commercialisation of this thesis, the Copyright and any Intellectual Property and/or Reproductions described in it may take place is available in the University IP Policy (see http://documents.manchester.ac.uk/DocuInfo.aspx?DocID=24420), in any relevant Thesis restriction declarations deposited in the University Library, The University Library's regulations (see http://www.library.manchester.ac.uk/about/regulations/) and in The University's policy on Presentation of Theses.

Acknowledgements

This thesis would not have been possible without the expertise of my supervisory team, and the resources of the Universities of Manchester, Liverpool, and Amsterdam. My deepest gratitude to all involved for your support and advice throughout. I am sure it was not easy, and your patience is appreciated. Franciska, you said I would be a coffee drinker by the end, and you were not wrong. A sincere thank you to my parents, for always being there for me in the three decades leading to this point. Finally, a heartfelt thank you to all of those below, without whom I would not be here.

Alex, for unceasing support and rekindling my joy in my research.

Cassio, to whom I will always be deeply in the red.

Giles, who was always there and helped me over the line.

Howbeer, for all the time, advice, and cheer.

Patrick, for tolerating my obsessions with ‘soil juice’.

Debbie, for keeping the lab running and still making time for my awkward requests.

Neelam, for bringing life to my dark corner - and to my plants whilst I gallivanted.

Ian, for teaching me which end of a mass spec was which.

Kat, for sneaking my samples in.

Jake, not just a good friend, but a good man, who gave me a home when I needed it most.

Chris, for keeping me company throughout, especially as we circled the end.

Becky, for always being there when I was in need of some catharsis.

Richie, for the friendship and the enthusiasm.

John, who gave me company in the dark times.

Megan, for putting up with me on all the walks.

Sam, for always being there on those days.

Rhys, for showing me fun again.

Em, for quite a few pieces of assistance.

Frankie, the one friend I can be quiet with.

Emma, for company and tales from the antipode.

Naomi, for friendship and reminders of the other side.

Eric, for being a friend despite the tendency to invisibilia.

Jack, for consistent psychological senzu.

Rich, for showing me another world, and believing in me.

Elle, who is quite simply - in her words and mine - amazing.

The Author

Reuben Margerison graduated with a BSc ARCS in Biology from Imperial College London in 2014, where his research project looked at root organ regeneration. He later graduated from the University of Liverpool in 2016 with an MRes in Advanced Biological Sciences, specialising in Plant Sciences, after completing a project looking at the evolution of plant-mutualistic microbes in response to simulated climate change. He has also worked at the University of Tokyo on a long-term plant demographics project focussing on mycoheterotrophy in plants in rural Fukushima prefecture since 2017, and has run a field project on the metabolomics of plant-soil interactions in Chinese subtropical tree species at Sun Yat-sen University in Guangzhou.

Reuben is also Chair of the Agri-Food Early Career Committee at the Society of Chemical Industry. During his five-year tenure he has set up a number of programmes, including a science photography competition to enable people working in the sector to share the beauty in their work in the hope of inspiring future scientists to pursue a career in plant research, and a mentoring scheme for students from historically under-represented backgrounds who are considering applying for a research degree.

1. Introduction

Plants interact with their environment in many complex ways, using morphology, chemistry and highly adapted signalling with other organisms to manipulate their surroundings and improve their performance. Because they are limited to only exploring their immediate vicinity during their lifetime, their ability to respond appropriately to abiotic or biotic changes is key to their ability to survive, grow and reproduce. While much attention has been focussed on morphological, architectural and chemical characteristics, known as plant functional traits, little has been afforded root exudation. Increasingly, researchers have come to view exudation as a trait in its own right, recognising that controlled exudation of low molecular weight compounds is a crucial plant-microbial signalling tool. While the field has moved on from the idea that exudation is merely unconstrained leakage of plant cell contents into the soil, difficulties in measurement can often lead to misapplication and misidentification of collected sample as root exudates (Williams et al. 2021a). For example, if root exudates are indeed finely tuned signals to attract a specific rhizosphere microbial community, then artificial growth methods, such as hydroponics or agar, could induce a completely different metabolic signature from that of natural soil conditions, partly as a result of different root trait expression in these media. Further, immediate collection after root washing could capture cell contents rather than true exudates. These general issues with realism of root exudate samples have given rise to a number of methodologies and innovations, such as Rhizobox (Oburger 2013), split root experiments (Vierhielig, Lerat & Piché 2003), and excavation of natural root systems (commonly used for mature trees; Jakoby et al. 2020). However, all these approaches have been shown to alter the expression and volume of exudates, in particular amino and organic acids (Oburger et al. 2013). It is clear that the collection of exudates is highly complex and subject to shifts of plant exudation in response to changes in their environment, which makes accurate characterisation very difficult. Perhaps as a result, there is much unexplained variance in experiments that seek to link plant traits with microbial driven functions. Therefore the creation of a robust and accurate method to analyse pure exudates (excluding, for instance, contamination from damaged cells), is a starting point to examine plant responses to various stresses and stimuli, with the ultimate

goal of being able to build a body of research that can be used to understand plant-microbial interactions and resulting functions over a range of scenarios.

1.1 Root Exudation: The State of the Art

Root exudation is common to all land plants and is the end destination of up to forty percent of the carbon fixed through photosynthesis (Whipps 1990). Given this enormous proportion of primary productivity that plants devote to exudation, while currently measurement methodology is being debated, the process must be essential and therefore understanding it is crucial. The purpose of exudation is to modify the root environment to create a 'rhizosphere' that is of benefit to the growth of the plant, primarily through the selection of a specific rhizosphere microbial community. Root exudates are carbon-based low molecular weight compounds such as organic acids, sugars and amino acids (Uren, 2000) that microbes use to fuel their growth (Bais et al*.,* 2006) (Fig. 1), and high molecular weight compounds, such as extensively branched polysaccharides, that through their physical properties are thought to adhere soil particles to roots (Galloway et al. 2020). Exudates can influence nutrient availability by direct release of hydrogen ions and organic acids to alter soil chemistry and pH and change the balance of anion and cation uptake (Shen et al, 2005). Exudates can therefore have strong localised effects on soil properties and the soil food web, making plants ecosystem engineers. The chemical constituents of root exudates exhibit high variability; as well as plant species identity, a major factor in this variability is the local environment of an individual plant. Whilst there are gaps in knowledge around plant root exudate responses to biotic and abiotic contexts, it is known that root exudates change in response to factors such as neighbour competition (Semchenko et al. 2014), nutrient availability (Meier et al. 2019), and drought (de Vries et al. 2019). These differences in exudates could be due to different environmental contexts requiring different modifications to achieve the same rhizosphere conditions, or that different environmental contexts require a different rhizosphere and thus a different exudate response is required to drive the change in local conditions; either of these would have to be integrated with the resources the plant is able to produce, which may be limited by factors such as incident sunlight.

Since root identity, development, morphogenesis, and surface formation have genetic controls conserved across all land plants (Sarkar et al., 2007; Willmann and Poethig, 2007; Wang et al., 1997; Honkanen et al., 2016 respectively) it is reasonable to hypothesise that there is at least a partially conserved genetic pathway for control of root exudation; however it has been found that root exudates are often not reconstructable phylogenetically due to high variability (Mönchgesang et al., 2016; although see Williams et al., 2021b). A complicating factor in the understanding of root exudates is that their composition can be highly variable in time and space. Additionally, because of the many different methods used to date to collect exudates, there are few robust, generalised principles that have been determined so far regarding species or environmental effects on root exudates.

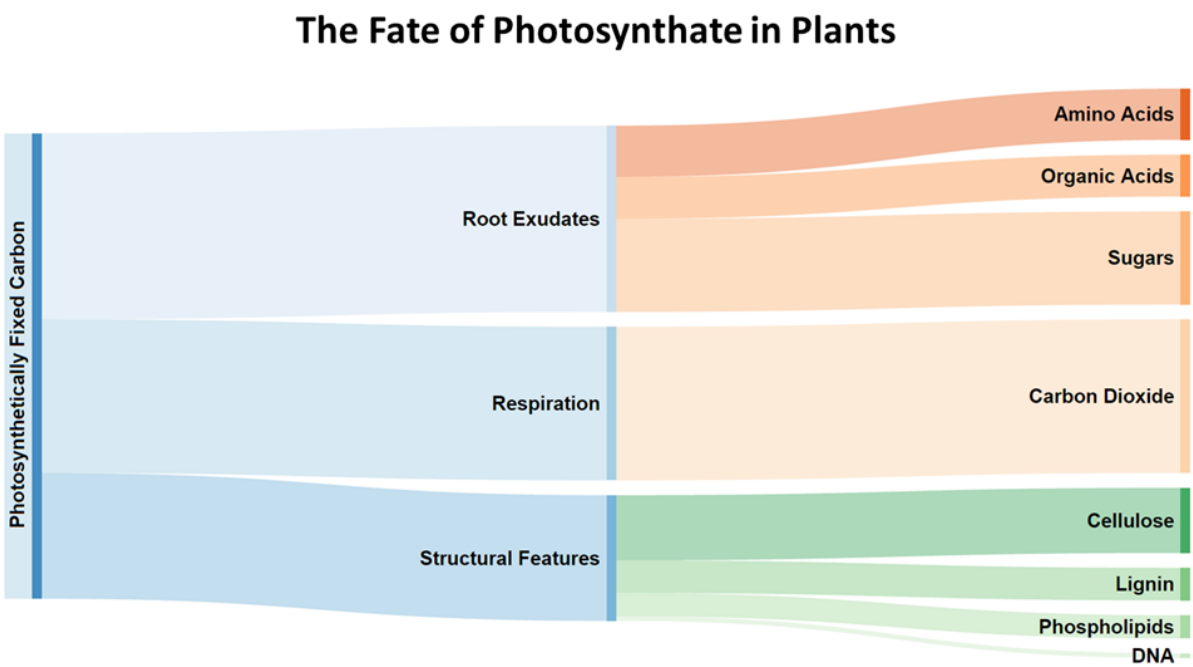

Figure 1: The estimated end destination of photosynthetically fixed carbon in plants, assuming a plant that does not make mycorrhizal associations. Constructed using maximum exudation information from Whipps (1990), Marschner (1995), and Uren (2000). In plants that make direct root symbioses exact proportions of carbon allocation would differ; based on current evidence it is likely that up to 20% of carbon fixed (up to half of what is listed here as 'Root Exudates') would instead be allocated to mycorrhizal or N-fixing symbionts (Smith and Read, 2008; Valentine et al., 2013), and such associations may change the rate of photosynthesis (e.g. Wright et al., 1998).

1.1.1 Methods

Until recently root exudation studies have been constrained by the use of hydroponic systems, which have been criticised because there are suspicions that the resulting exudates do not accurately represent the exudate metabolite signature in true soil (Oburger and Jones, 2018). Some of the problems inherent with purely hydroponic systems include: a lack of substrate resistance, affecting root traits; the microbial community being different or absent due to immersion and lack of niche space; root symbionts not being present in medium; or symbionts not recruited due to the lack of need to forage. A drought treatment that bears any resemblance to reality is also challenging to produce in an aqueous medium.

However, soil as a substrate for exudation studies poses its own problems: it is difficult to remove from roots without physical damage and disruption of links with root associated symbiots, and the chemical heterogeneity of soil makes a reliable signal difficult to detect and interpret. There are multiple ways to approach this problem. To help to limit the signal from the soil overwhelming the low absolute volume of exudates, studies such as that in Petriacq et al. (2017) have looked at using systems such as a 1:9 soil:sand mixture, to give structure and ecological relevance to the substrate. These systems have an enormous advantage in that they enable collection longitudinally - but are still highly artificial substrates, and caution must therefore be used when attempting to draw conclusions about the findings of such studies to broader systems. Many of the issues with a soil sand mix are similar to those of hydroponics: the homogenous distribution of nutrients, the potential dilution or loss of microbial symbionts, growth promoting microbes, and even an absence of antagonists.

There is a genuine need for a system based in a real soil environment, and there has been increasing interest in developing such hybrid soil-based methods. Many studies have washed roots free of soil before collecting putative exudates (Ström et al., 1994; Aulakh et al., 2001; Lucas García et al., 2001; Canarini et al., 2016) but there are concerns that this could be damaging to the plant (Oburger and Jones, 2018). A key recent finding in the development of hybrid exudate collection mechanisms is that intentional damage to root systems has broadly similar chemistry to putative root exudates collected immediately after washing off soil (Williams et al., 2021a). The use of a hydroponic recovery period in that study enabled movement of the metabolomic profile of putative exudates away from that of simply cell contents, and the method has also recently been used to examine exudates across a range of

grassland species (Williams et al., 2021b). The central issues that I aim to tackle in this thesis are that it is not known whether such a soil-hydroponic hybrid method produces a different exudate profile to that of purely hydroponic systems, or indeed whether it is possible to use such methods to detect the effects of abiotic stresses such as drought - a concern given the hydroponic root repair stage. This thesis aims to clarify these uncertainties.

1.1.2 Exudates in plant-microbial interactions

Plants with direct fungal and bacterial symbioses, where symbionts live in contact with or partly inside the roots, exchange carbon-containing photosynthate for increased nutrient uptake (Lagunas, Schafer and Gifford, 2015). Compared to roots, fungal hyphae have a larger surface area and can penetrate smaller spaces within soil aggregates. Ectomycorrhizal fungi (EMF) provide organic nitrogen for trees and shrubs, in addition to decomposing organic matter, yet require deep soil. Arbuscular mycorrhizal fungi (AMF) provide other plants with phosphates and some ammonium from more fertile soils. Plants cannot produce nitrogenase, the enzyme that fixes nitrogen, due to its oxygen sensitivity (Gallon, 1981), therefore several plant families form symbioses with nitrogen-fixing bacteria, often in nitrogen poor environments. The vast majority of plant species make direct symbiotic associations with one or more symbionts in their root system, and components of root exudates are used to attract symbiotic partners. However, this thesis considers such direct trades with symbiotic partners as separate from root exudation.

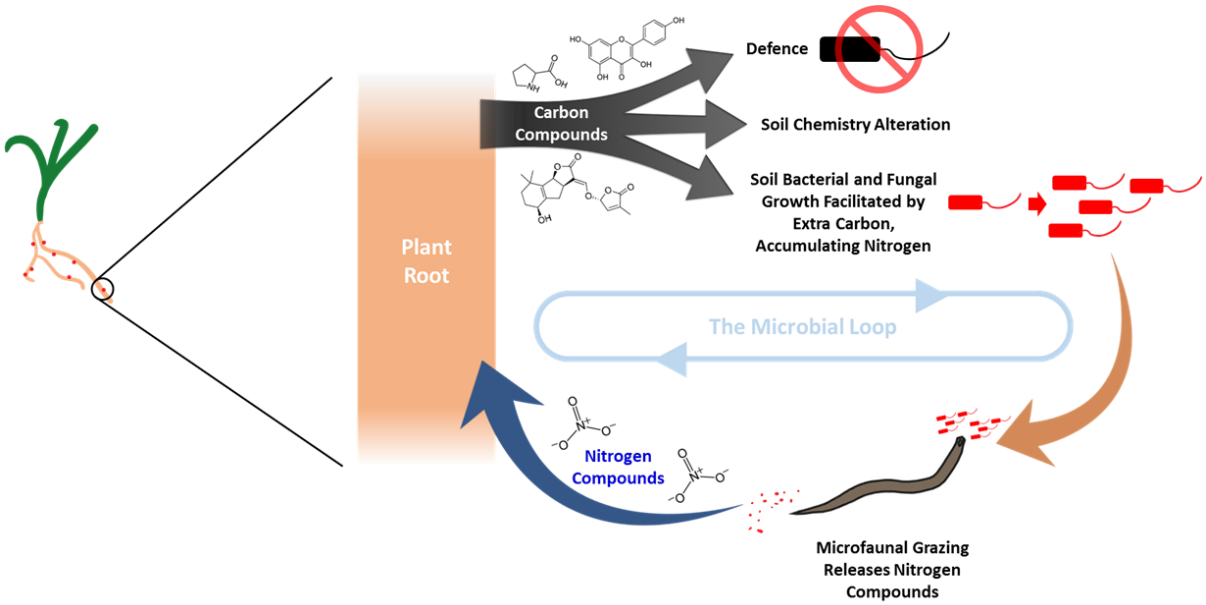

Figure 2: Rhizosphere environmental manipulation through plant root exudation. Exudates can help to defend the plant from microbial attack, alter soil chemical properties, or promote the growth of nitrogen accumulating microbial species. Nitrogen is released upon grazing by microfauna (made using information from Bais et al. (2006) and Bonkowski (2004)).

Plants have complex indirect impacts on soil microbiota through root exudation, and exudates are a major source of organic carbon in the soil (Hutsch, Augustin & Merbach 2002; Nguyen 2003). One example is the "microbial loop" (Clarholm, 1985; Figure 2): diazotrophic bacteria such as *Azotobacter vinelandii* use plant-derived carbon compounds in exudates to fix nitrogen; microfauna, including bacterivorous nematodes, graze on the bacteria and excrete ammonium, which nitrifying bacteria convert into nitrites then nitrates. These knock-on impacts within soil communities then feed back to the plant, as nitrates can be absorbed by plant roots without the need for direct symbioses, compensating the initial carbon cost of exudation (Marschner 1995). Due to the intimate relationships between plants and soil microbes, it is important to understand the genetic and molecular mechanisms behind root exudation both as a regulator of climate change (Drigo, Kowalchuk, and van Veen, 2008), and to inform applied projects in ecosystem functioning and crop productivity. Given that one of the key roles proposed for root exudates is that of driving soil microbial community assembly within the rhizosphere, there is therefore a possibility of engineering plants to recruit a microbial community that offers growth promotion effects, such as drought protection (de Vries et al., 2020).

This thesis aims to show that the hybrid method detailed in Williams et al. (2021a) is distinct from lifetime hydroponic growth, and is an appropriate methodology for detecting abiotic stresses. It also aims to use this method to explore the effects of simulated climate change on root exudation.

1.2 Climate change: why might it impact exudation?

The Intergovernmental Panel on Climate Change (IPCC) predicts a rise in global mean surface temperature of up to 0.7^{o}C by 2035, leading to an increase in drought both globally and in north-western Europe (Kirtman et al., IPCC 2013). This change is expected to present an evolutionary and ecological challenge to many organisms, especially plants, as primary producers, and their interaction with soil microbes. Plant carbon inputs to the soil, in the form of root exudates, play an important role in shaping the composition and activity of soil microbial communities – which then feed back into plant growth by releasing nutrients. It is little known how and to what extent the soil microbiota is buffering these above-ground responses. The large uncertainties illustrate that establishing the interactions between plant exudation and microbial nutrient cycling under differing environmental conditions is necessary to understand soil greenhouse gas emission. Soil microbes contribute enormously to global nutrient cycling (de Vries et al., 2013). The greenhouse gases that cause the most radiative forcing are water vapour and compounds containing carbon (carbon dioxide, CO_2; methane, CH_4) or nitrogen (nitrous oxide, N_2O; nitric oxide, NO). The top metre of soil is a massive potential carbon source, containing double the amount of carbon as the atmosphere (Jobbagy and Jackson, 2000). A change in soil microbial community composition could dramatically increase greenhouse gas emission via nutrient cycling (Allison, Wallenstein and Bradford, 2010), exacerbating climate change and risks forming feedback loops, leading to further greenhouse gas release (Cox et al., 2000). It is vital that both direct and indirect effects on the mechanisms of exchange between atmosphere, plants and soil microbiota are understood; a better understanding the chemical composition and soil- and microbial-fate of

root exudates would enable improved modelling of nutrient cycling and greenhouse gas emissions (Fry et al., 2018).

On a large scale, anthropogenic climate change mainly impacts terrestrial organisms through abiotic factors (Nijp et al. 2014). Resultant changes in plant community structure and functional composition (Wookey et al. 2009) cause a cascade of changes within microbial community structure, and activity (Fenner & Freeman 2011; Ward et al. 2013). The missing link in this system is likely to be root exudates, which are plant-species specific, and highly tailored to the needs of the plant. Higher temperatures typically increase microbial enzyme production (Ernakovich et al. 2014), which results in higher rates of microbial respiration, and decomposition (Karhu et al. 2014). When drought-stressed, the fitness response of individual plants is more due to rhizosphere microbial community composition and response than genetic differences in the plants (Lau and Lennon 2012). This indicates that observed tolerance and adaptation of plants in response to climate change may not be due to plant genomic shifts, but determined by interactions with soil microbiota.

Historically, plants and microbes have been considered as separate systems. Since the millennium, however, an increasing body of work is showing their extensive interactions, (e.g. Bardgett and Wardle, 2003), with the synergistic relationships and cycles of positive and negative feedback (Wardle et al., 2004) showing this division to be entirely artificial. The vast majority of plant species exhibit complex, mutualistic interactions with soil microbiota (Bonkowski, 2004), which can be so intimately linked that genotypic variations in one community affect the other (Johnson et al., 2010) and microbial community structure can influence selection on plant genetic traits (Lau and Lennon, 2011). Microbiota are therefore both dependent on, and key regulators of plant growth, diversity and community composition (van der Heijden, Bardgett, and Straalen, 2008; van der Putten et al., 2013). As a consequence, any abiotic stress on one component of the system will naturally affect the other. The effects of these on the plant-microbial relationship have been extensively studied, but there is so far no consensus on how nutrient and carbon exchange between plants and microbes is affected. A few studies have proposed that microbial communities eventually acclimate to warmer temperatures (Atkin & Tjoelker 2003; Bradford et al. 2008, although this is disputed, see Hartley et al., 2009), but it is known that altered precipitation, also associated with climate change, impacts microbial communities (de Vries and Shade, 2013). Temperature sensitivity

in plant exudate production may drive or dampen microbial response and tip the system between acting as a carbon sink or source. In addition, while wild plant species adapt both physiologically and genetically well to increasing severity and frequency of extreme weather (Ravenscroft, Fridley, and Grime, 2014; Ravenscroft, Whitlock, and Fridley, 2015), soil microbes dependent on plant exudates have shown both heightened and lessened responses to extreme weather (Abeli, Jäkäläniemi & Gentili 2014).

In this thesis I will use the pioneering soil-hydroponic hybrid method first detailed in Williams et al. (2021a) to investigate the response of grass root exudation to single and multiple climate change stresses. This will be the first work, to the author's knowledge, to combine a soil-hydroponic growth system, with cutting edge metabolomic techniques to determine the effect of climate change on exudate expression.

1.3 Plant traits and their impact on exudation

Plant functional traits are often divided into morphological, architectural and chemical groups, with the distinction being loosely aligned with the root economics spectrum as presented by Bergmann et al. (2020). Simply, morphological traits such as root diameter and specific root length (SRL) are associated with 'collaboration' with soil microbes, especially mycorrhizal fungi, while architectural traits include root nitrogen content, number of root tips and branches and root length, all traits that are associated with resource economy ('conservation' in Bergmann's analysis). Plants create a finite amount of photosynthate with which to build structures and organs, attract pollinators and exude low molecular weight compounds in order to build a rhizosphere community. They must be judicious with this resource, and their trait expression will therefore generally reflect the most important resource limitations of their environment. For example, species with acquisitive traits are generally found in areas with high nutrient levels, such as fertilised intensively managed grasslands (Leuschner et al., 2013). These traits include high tissue nitrogen content, many root tips and branches, and high root surface area, all traits that aid foraging into new areas and favour rapid growth (Wright et al. 2004).

The architecture (RSA), morphology, and chemical processes within a plant's root system is heavily dependent on the local micro- and macro-distribution of nutrients (Bardgett,

Mommer and De Vries, 2014). It is therefore a developmentally plastic set of traits that respond to abiotic environmental cues, such as water availability (Deak & Malamy 2005), and biotic cues, such as the presence of mutualistic *Pseudomonas* bacteria (Zamioudis *et al.,* 2013). The impact of soil presence on root physiology may explain why previous attempts to significantly reduce root exudate variability using model species in controlled laboratory environments were unsuccessful (Mönchgesang et al., 2016). Determining whether laboratory-based results are similar to natural processes is crucial to informing future research and mitigation and management schemes; climate change, particularly drought, directly affects root traits and can induce significant developmental delay, and it is logical that this will also impact root exudation. Root morphological and architectural traits should therefore be considered in conjunction with root exudation, and as exudates of plants within natural habitats are likely to be different to those grown hydroponically, there is therefore a need to develop new ways of understanding the constituents of root exudates and how these impact the rhizosphere environment, and it is currently an area of active research.

1.4 Modern Metabolomics Methods

Metabolomics is a set of techniques that aim to identify all of the chemistry of a biological system. It is unique in that it allows the direct measurement of a phenotype; where genomics allows you to see the possibility, and transcriptomics shows what genes are being expressed, metabolomics allows the direct and highly sensitive measurement of the end result of these processes, the chemical phenotype, and thus infers function. This is of particular interest for plant-soil interactions, as the only possible mediators of this intra-kingdom signalling would be molecular in scale. Metabolomics theoretically allows us to characterise these signals and nutrient flows in a system. This thesis will consider one of the most common mass spectral techniques, Gas Chromatography - Mass Spectrometry (GC-MS). GC-MS allows for the separation, detection, and annotation of single molecules within a sample, which is useful for understanding pathways. While GC-MS has been used in root exudate studies (e.g Williams et al., 2021a; Williams et al., 2021b) it has several drawbacks. It is known to require large sample sizes, and it is not always possible to identify molecules definitively.

Here this thesis aims to pioneer the use of infra-red spectroscopy for the analysis of root exudates as a sample type, and to show that in controlled studies this is an appropriate and effective technique which in time can be applied to larger-scale and field-based studies. Infra-red spectroscopy involves the detection of the absorption of infra-red (IR) radiation by bonds within a sample that are active in this region. This has long been used to identify pure molecules (e.g. Bellamy, 1968) but can also be used to infer broad molecular classes in complex mixtures, such as biological samples. Biological samples produce a highly characteristic infra-red absorption spectrum with areas of absorbance peaks typical to common classes of biomolecules occurring in particular regions of the IR spectrum (Figure 3): a fatty acid region from 3050-2800cm^{-1}; the 1750-1500 cm^{-1} amide region in which proteins and peptides absorb strongly; a so called 'mixed' region from 1500-1250 cm^{-1}, in which carboxylic acid functional groups, unbonded amino acids, and polysaccharides absorb; and the (poly)saccharide region at 1200-900 cm^{-1} (Schmidt and Flemming, 1998). Whilst IR spectroscopy is not always able to specifically identify a particular molecule, if there are bonds that are infra-red active within that metabolite, they will appear in spectroscopic measurements to contribute to the spectrum recorded. Sample types have a highly specific metabolic fingerprint measured using this method, and this can be used to identify differences between groups in everything from food additives to fungal genera (Mohsin et al., 2019; Salman et al., 2010). This means that infra-red spectroscopy can offer key insight into broad chemical shifts between experimental treatments even if GC-MS is unable to identify metabolites. This thesis will use three implementations of the technology of IR spectroscopy: Transmission Fourier Transform IR Spectroscopy (FTIR), Attenuated Total Reflectance-FTIR (ATR-FTIR), and Optical-Photothermal IR Spectroscopy (O-PTIR).

The IR source in Transmission FTIR and ATR-FTIR is a heat lamp coupled with a Michaelson Interferometer. This interferometer causes constructive and destructive interference of the IR radiation produced by the lamp, causing a rage of wavelengths to be incident on a sample, and the IR absorbance of the sample is recorded as an interferogram. The data must then be transformed by use of the Fourier Transform in order to return them to spectral form for further analyses. In Transmission FTIR the IR source is shone through the sample, which is dried onto an IR transparent material (e.g. silicon), and the amount of decrease in radiation compared with a blank is recorded as the absorption. In ATR-FTIR, by contrast, the sample is

placed on a crystal with a high refractive index, such as a diamond. The radiation is shone through the crystal, and is totally internally reflected. However, due to the antenna effect, a small fraction of this radiation is absorbed by the sample on the crystal instead of being internally reflected, and this absorbance can be recorded (Hollas, 2004). O-PTIR uses a different physical phenomenon, the photothermal effect, wherein an incident IR beam that is absorbed by the surface of a sample will induce a temperature increase, changing the refractive index of the sample. The magnitude of the photothermal effect may then be detected by a visible light laser focussed on the same point as the IR beam, with the magnitude of the diffraction being proportional to the IR radiation absorbed by the sample. As wavelength is a key determinant of resolution, the use of a single-wavelength detector allows for constant special resolution at varied IR wavelengths, enabling measurement from precise areas and cell-scale structures (Reffner, 2018). In this thesis I will demonstrate a range of experimental scenarios where IR spectroscopy is capable of offering insight into the underlying processes of root exudation.

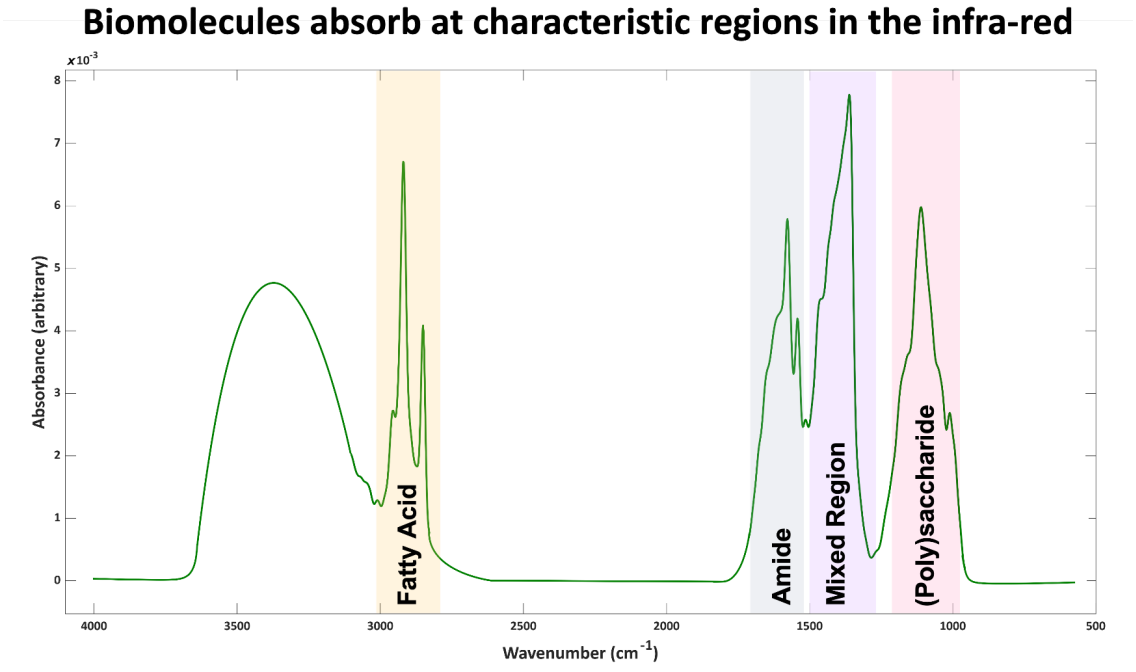

Figure 3: Idealised Fourier-Transform Infra-Red spectrum constructed from absorbance data of root exudates from *Arabidopsis thaliana* ecotype Col-0, collected and processed using the methods detailed in Chapter 2. Typical absorbance regions of categories of biomolecules are highlighted; fatty acid in yellow, amide in blue, the mixed region in purple, and (poly)saccharide in pink. Overlay information taken from Schmidt and Flemming (1998).

1.5 Aims and Thesis Summary

Here, the overarching hypothesis is that the soil-hydroponic hybrid method can be used a scaffold to collect realistic exudation profiles for analysis, and enable study of the effect of climate-change related stresses on plant root exudates. This ensures that any results shown experimentally under changing climatic conditions may be fairly applied to real-world scenarios. Given that all other aspects of plant trait responses change under a changing climate, altered climate affects soil microbial functioning and nutrient cycling, and that the primary purpose of exudates may be to recruit microbial communities to respond to a specific set of environmental circumstances, my expectation is that **climate change affects the quantity and quality of plant root exudation**.

Specific aims are as follows:

1. Validate the use of a hybrid soil-hydroponic growth system against commonly used hydroponics systems.
2. Demonstrate the use of the hybrid method to create a drought effect which is detectable even after a recovery period.
3. Compare the effects of two interacting climate stresses on two plant species with contrasting root economic resource use strategies.

In order to test the hypothesis and meet these aims, I conducted three studies for the work in this thesis using three contrasting Graminoid species that are of agricultural or ecological importance: barley, *Hordeum vulgare,* a crop plant; sweet vernal grass, *Anthoxanthum odoratum,* a slow-growing grass with conservative traits; and cock's foot grass, *Dactylis glomerata,* a fast-growing species with exploitative traits. These were chosen as each has been extensively studied with respect to their carbon economy and root functional traits, and are all grasses in order to look at commonalities and differences from quite closely related species that nevertheless have different life strategies.

In **Chapter 2** a modern hybrid exudate collection method is compared with a classical hydroponic collection system, from a root trait, exudate, and whole-root metabolomics perspective in *Hordeum vulgare*. Lifetime hydroponic growth was found to be fundamentally different from plants gown initially in soil in every aspect measured, showing that hydroponics are not appropriate for exudate analysis.

Chapter 3 utilises the sample collection methods and data analysis techniques explored in Chapter 2 on an ecologically relevant grassland species, *Anthoxanthum odoratum,* to examine whether these methods are appropriate for detection of abiotic stress responses. The hybrid method discussed in this thesis is found to detect a strong drought effect despite a hydroponic recovery period, opening the way for further studies.

Chapter 4 then uses these analytical techniques in a large, multifactorial experiment examining the effect of both drought and warming on exudates and on soil leachates from *Anthoxanthum odoratum* and *Dactylis glomerata*, finding that warming alone is significantly more disruptive to the metabolic footprint of exudates and soil than drought and warming together, or drought alone.

The final chapter, **Chapter 5**, discusses the findings of this thesis in the context of the wider field.

1.6 Statements of Contribution

Chapter 2

Preliminary work by RM with advice from FdV, RG, PG, GJ. Experimental design by RM with assistance from FdV, RG, and HM. Root trait analyses by RM with advice from FdV and AW. Mass Spectrometry preparation and analysis by RM with assistance and training from AW, HM, KH, and YX. Spectroscopic sample and data analyses by RM with training and assistance from CL and HM. Manuscript drafting by RM with advice and assistance from AW, FdV, GJ, and EF.

Chapter 3

Preliminary work by RM, SG, AH, and AW with advice from FdV. Experimental design by RM and AW. Root trait analyses by RM with advice from FdV and AW. Mass Spectrometry preparation and analysis by RM with assistance and training from JB and AW. Spectroscopic sample and data analyses by RM with training and assistance from CL, AW and HM. Manuscript drafting by RM with advice and assistance from AW, FdV, GJ, and EF.

Chapter 4

Preliminary work by AW with advice from FdV. Spectroscopic sample and data analyses by RM with training and assistance from AW, CL, and EF. Manuscript drafting by RM with advice and assistance from AW, FdV, GJ, and EF.

1.7 References

Allison, Steven D., Matthew D. Wallenstein, and Mark A. Bradford. 2010. “Soil-Carbon Response to Warming Dependent on Microbial Physiology.” *Nature Geoscience 2010 3:5* 3 (5). Nature Publishing Group:336–40. https://doi.org/10.1038/ngeo846.

Atkin, Owen K., and Mark G. Tjoelker. 2003. “Thermal Acclimation and the Dynamic Response of Plant Respiration to Temperature.” *Trends in Plant Science* 8 (7). Elsevier Current Trends:343–51. https://doi.org/10.1016/S1360-1385(03)00136-5.

Aulakh, M. S., R. Wassmann, C. Bueno, J. Kreuzwieser, and H. Rennenberg. 2001. “Characterization of Root Exudates at Different Growth Stages of Ten Rice (Oryza Sativa L.) Cultivars.” *Plant Biology* 3 (2). Georg Thieme Verlag Stuttgart ·New York:139–48. https://doi.org/10.1055/s-2001-12905.

Bais, Harsh P., Tiffany L. Weir, Laura G. Perry, Simon Gilroy, and Jorge M. Vivanco. 2006. “The Role of Root Exudates in Rhizosphere Interactions With Plants and Other Organisms.” *Annual Review of Plant Biology* 57 (1):233–66. https://doi.org/10.1146/annurev.arplant.57.032905.105159.

Bardgett, Richard D., and David A. Wardle. 2010. *Aboveground-Belowground Linkages: Biotic Interactions, Ecosystem Processes ... - Richard D. Bardgett, David A. Wardle*

Bardgett, Richard D., Liesje Mommer, and Franciska T. De Vries. 2014. “Going Underground: Root Traits as Drivers of Ecosystem Processes.” *Trends in Ecology & Evolution* 29 (12). Elsevier Current Trends:692–99. https://doi.org/10.1016/J.TREE.2014.10.006.

Bellamy, L. J. 1968. “Advances in Infrared Group Frequencies”. Chapman and Hall.

Bonkowski, Michael. 2004. “Protozoa and Plant Growth: The Microbial Loop in Soil Revisited.” *New Phytologist* 162 (3):617–31. https://doi.org/10.1111/j.1469-8137.2004.01066.x.

Bonkowski, Michael. 2004. “Protozoa and Plant Growth: The Microbial Loop in Soil Revisited.” *New Phytologist* 162 (3):617–31. https://doi.org/10.1111/j.1469-8137.2004.01066.x.

Bradford, Mark A., Christian A. Davies, Serita D. Frey, Thomas R. Maddox, Jerry M. Melillo, Jacqueline E. Mohan, James F. Reynolds, Kathleen K. Treseder, and Matthew D. Wallenstein. 2008. “Thermal Adaptation of Soil Microbial Respiration to Elevated Temperature.” *Ecology Letters* 11 (12). John Wiley & Sons, Ltd:1316–27. https://doi.org/10.1111/J.1461-0248.2008.01251.X.

Canarini, Alberto, Andrew Merchant, and Feike A. Dijkstra. 2016. “Drought Effects on Helianthus Annuus and Glycine Max Metabolites: From Phloem to Root Exudates.” *Rhizosphere* 2 (December). Elsevier:85–97. https://doi.org/10.1016/j.rhisph.2016.06.003.

Cox, Peter M., Richard A. Betts, Chris D. Jones, Steven A. Spall, and Ian J. Totterdell. 2000. “Acceleration of Global Warming Due to Carbon-Cycle Feedbacks in a Coupled Climate Model.” *Nature 2000 408:6809* 408 (6809). Nature Publishing Group:184–87. https://doi.org/10.1038/35041539.

De Vries, Franciska T, and Ashley Shade. 2013. “Controls on Soil Microbial Community Stability under Climate Change.” *Frontiers in Microbiology* 0 (SEP). Frontiers:265. https://doi.org/10.3389/FMICB.2013.00265.

De Vries, Franciska T, Elisa Thébault, Mira Liiri, Klaus Birkhofer, Maria a Tsiafouli, Lisa Bjørnlund, Helene Bracht Jørgensen, et al. 2013. “Soil Food Web Properties Explain Ecosystem Services across European Land Use Systems.” *Proceedings of the National Academy of Sciences* 110 (35):14296–301. https://doi.org/10.1073/pnas.1305198110.

De Vries, Franciska T., Alex Williams, Fiona Stringer, Robert Willcocks, Rosie McEwing, Holly Langridge, and Angela L. Straathof. 2019. “Changes in Root-Exudate-Induced Respiration Reveal a Novel Mechanism through Which Drought Affects Ecosystem

Carbon Cycling." *New Phytologist* 224 (1). John Wiley & Sons, Ltd:132–45. https://doi.org/10.1111/nph.16001.

De Vries, Franciska T. , Rob I. Griffiths, Christopher G. Knight, Oceane Nicolitch, and Alex Williams. 2020. "Harnessing Rhizosphere Microbiomes for Drought-Resilient Crop Production." *Science* 368 (6488). American Association for the Advancement of Science:270–74. https://doi.org/10.1126/SCIENCE.AAZ5192.

Deak, Karen I., and Jocelyn Malamy. 2005. "Osmotic Regulation of Root System Architecture." *The Plant Journal* 43 (1). John Wiley & Sons, Ltd:17–28. https://doi.org/10.1111/J.1365-313X.2005.02425.X.

Drigo, Barbara, George A. Kowalchuk, and Johannes A. van Veen. 2008. "Climate Change Goes Underground: Effects of Elevated Atmospheric CO2 on Microbial Community Structure and Activities in the Rhizosphere." *Biology and Fertility of Soils 2008 44:5* 44 (5). Springer:667–79. https://doi.org/10.1007/S00374-008-0277-3.

Ernakovich, Jessica G, Kelly A. Hopping, Aaron B. Berdanier, Rodney T. Simpson, Emily J. Kachergis, Heidi Steltzer, and Matthew D. Wallenstein. 2014. "Predicted Responses of Arctic and Alpine Ecosystems to Altered Seasonality under Climate Change." *Global Change Biology* 20 (10). John Wiley & Sons, Ltd:3256–69. https://doi.org/10.1111/GCB.12568.

Gallon, J. R. 1981. "The Oxygen Sensitivity of Nitrogenase: A Problem for Biochemists and Micro-Organisms." *Trends in Biochemical Sciences* 6 (C). Elsevier Current Trends:19–23. https://doi.org/10.1016/0968-0004(81)90008-6.

García, J. A. Lucas, C. Barbas, A. Probanza, M. L. Barrientos, and F. J. Gutierrez Mañero. 2001. "Low Molecular Weight Organic Acids and Fatty Acids in Root Exudates of Two Lupinus Cultivars at Flowering and Fruiting Stages." *Phytochemical Analysis* 12 (5). John Wiley & Sons, Ltd:305–11. https://doi.org/10.1002/PCA.596.

Hartley, Iain P., David W. Hopkins, Mark H. Garnett, Martin Sommerkorn, and Philip A. Wookey. 2009. "No Evidence for Compensatory Thermal Adaptation of Soil

Microbial Respiration in the Study of Bradford et Al. (2008).” *Ecology Letters* 12 (7). John Wiley & Sons, Ltd:E12–14. https://doi.org/10.1111/J.1461-0248.2009.01300.X.

Heijden, Marcel G A Van Der, Richard D. Bardgett, and Nico M. Van Straalen. 2008. “The Unseen Majority: Soil Microbes as Drivers of Plant Diversity and Productivity in Terrestrial Ecosystems.” *Ecology Letters* 11 (3):296–310. https://doi.org/10.1111/j.1461-0248.2007.01139.x.

Honkanen, Suvi, Victor A.S. Jones, Giulia Morieri, Clement Champion, Alexander J. Hetherington, Steve Kelly, Hélène Proust, Denis Saint-Marcoux, Helen Prescott, and Liam Dolan. 2016. “The Mechanism Forming the Cell Surface of Tip-Growing Rooting Cells Is Conserved among Land Plants.” *Current Biology*, 1–7. https://doi.org/10.1016/j.cub.2016.09.062.

Hütsch, Birgit W, Jürgen Augustin, and Wolfgang Merbach. 2002. “Plant Rhizodeposition ± an Important Source for Carbon Turnover in Soils.” *J. Plant Nutr. Soil Sci* 165:397–407. https://doi.org/10.1002/1522-2624.

J. Michael Hollas. 2004. “Modern Spectroccopy”. John Wiley & Sons.

Jakoby, Gilad, Ido Rog, Shacham Megidish, and Tamir Klein. 2020. “Enhanced Root Exudation of Mature Broadleaf and Conifer Trees in a Mediterranean Forest during the Dry Season.” *Tree Physiology* 40 (11). Oxford Academic:1595–1605.

Jobbágy, Esteban G, and Robert B Jackson. 2000. “The Vertical Distribution of Soil Organic Carbon and Its.” *Ecological Applications* 10 (2):423–36. https://doi.org/10.1890/1051-0761(2000)010[0423:TVDOSO]2.0.CO;2.

Johnson, David, Ian C. Anderson, Alison Williams, Raj Whitlock, and J. Philip Grime. 2010. “Plant Genotypic Diversity Does Not Beget Root-Fungal Species Diversity.” *Plant and Soil* 336 (1):107–11. https://doi.org/10.1007/s11104-010-0452-9.

Karhu, Kristiina, Marc D. Auffret, Jennifer A. J. Dungait, David W. Hopkins, James I. Prosser, Brajesh K. Singh, Jens-Arne Subke, et al. 2014. “Temperature Sensitivity of

Soil Respiration Rates Enhanced by Microbial Community Response." *Nature 2014 513:7516* 513 (7516). Nature Publishing Group:81–84. https://doi.org/10.1038/nature13604.

Kirtman, B., S.B. Power, J.a. Adedoyin, G.J. Boer, R. Bojariu, I. Camilloni, F.J. Doblas-Reyes, et al. 2013. "Near-Term Climate Change: Projections and Predictability." *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*, 953–1028. https://doi.org/10.1017/CBO9781107415324.023.

Lagunas, B., P. Schafer, and M. L. Gifford. 2015. "Housing Helpful Invaders: The Evolutionary and Molecular Architecture Underlying Plant Root-Mutualist Microbe Interactions." *Journal of Experimental Botany* 66 (8):2177–86. https://doi.org/10.1093/jxb/erv038.

Lau, J, and J Lennon. 2011. "Evolutionary Ecology of Plant-Microbe Interactions: Soil Microbial Structure Alters Selection on Plant Traits." *New Phytologist* 192 (1):215–24. https://doi.org/10.1111/j.1469-8137.2011.03790.x.

Lau, Jennifer A, and Jay T Lennon. 2012. "Rapid Responses of Soil Microorganisms Improve Plant Fitness in Novel Environments." *Proceedings of the National Academy of Sciences of the United States of America* 109 (35):14058–62. https://doi.org/10.1073/pnas.1202319109.

Leuschner, Christoph, Stefanie Gebel, and Laura Rose. 2013. "Root Trait Responses of Six Temperate Grassland Species to Intensive Mowing and NPK Fertilisation: A Field Study in a Temperate Grassland." *Plant and Soil 2013 373:1* 373 (1). Springer:687–98. https://doi.org/10.1007/S11104-013-1836-4.

Marschner, H., and V. Römheld. 1995. "Strategies of Plants for Acquisition of Iron." *Iron Nutrition in Soils and Plants*. Springer, Dordrecht, 375–88. https://doi.org/10.1007/978-94-011-0503-3_52.

Marschner, H., and V. Römheld. 1995. “Strategies of Plants for Acquisition of Iron.” *Iron Nutrition in Soils and Plants*. Springer, Dordrecht, 375–88. https://doi.org/10.1007/978-94-011-0503-3_52.

Meier, Ina C., Timo Tückmantel, Julian Heitkötter, Karolin Müller, Sebastian Preusser, Thomas J. Wrobel, Ellen Kandeler, Bernd Marschner, and Christoph Leuschner. 2020. “Root Exudation of Mature Beech Forests across a Nutrient Availability Gradient: The Role of Root Morphology and Fungal Activity.” *New Phytologist* 226 (2). John Wiley & Sons, Ltd:583–94. https://doi.org/10.1111/nph.16389.

Mohsin, Ghassan Faisal, Franz Josef Schmitt, Clemens Kanzler, Arne Hoehl, and Andrea Hornemann. 2019. “PCA-Based Identification and Differentiation of FTIR Data from Model Melanoidins with Specific Molecular Compositions.” *Food Chemistry* 281 (May). Elsevier:106–13. https://doi.org/10.1016/J.FOODCHEM.2018.12.054.

Mönchgesang, Susann, Nadine Strehmel, Stephan Schmidt, Lore Westphal, Franziska Taruttis, Erik Müller, Siska Herklotz, Steffen Neumann, and Dierk Scheel. 2016. “Natural Variation of Root Exudates in Arabidopsis Thaliana-Linking Metabolomic and Genomic Data.” *Scientific Reports* 6 (February). Nature Publishing Group:29033. https://doi.org/10.1038/srep29033.

Mönchgesang, Susann, Nadine Strehmel, Stephan Schmidt, Lore Westphal, Franziska Taruttis, Erik Müller, Siska Herklotz, Steffen Neumann, and Dierk Scheel. 2016. “Natural Variation of Root Exudates in Arabidopsis Thaliana-Linking Metabolomic and Genomic Data.” *Scientific Reports* 6 (February). Nature Publishing Group:29033. https://doi.org/10.1038/srep29033.

Nguyen, Christophe. 2003. “Rhizodeposition of Organic C by Plants: Mechanisms and Controls.” *Agronomie* 23 (5–6). EDP Sciences:375–96. https://doi.org/10.1051/AGRO:2003011.

Nijp, Jelmer J., Juul Limpens, Klaas Metselaar, Sjoerd E. A. T. M. van der Zee, Frank Berendse, and Bjorn J. M. Robroek. 2014. “Can Frequent Precipitation Moderate the Impact of Drought on Peatmoss Carbon Uptake in Northern Peatlands?” *New*

Phytologist 203 (1). John Wiley & Sons, Ltd:70–80. https://doi.org/10.1111/NPH.12792.

Oburger, E., Dell'mour, M., Hann, S, Wieshammer, G., Puschenreiter, M. & Wenzel, W. (2013), Evaluation of a novel tool for sampling root exudates from soil-growm plants compared to conventional techniques. Environmental and Experimental Botany, 87, 235-247.

Oburger, Eva, and David L. Jones. 2018. "Sampling Root Exudates – Mission Impossible?" *Rhizosphere* 6 (June). Elsevier:116–33. https://doi.org/10.1016/j.rhisph.2018.06.004.

Pétriacq, Pierre, Alex Williams, Anne Cotton, Alexander E. McFarlane, Stephen A. Rolfe, and Jurriaan Ton. 2017. "Metabolite Profiling of Non-Sterile Rhizosphere Soil." *The Plant Journal* 92 (1). John Wiley & Sons, Ltd (10.1111):147–62. https://doi.org/10.1111/tpj.13639.

Putten, Wim H. Van der, Richard D. Bardgett, James D. Bever, T. Martijn Bezemer, Brenda B. Casper, Tadashi Fukami, Paul Kardol, et al. 2013. "Plant-Soil Feedbacks: The Past, the Present and Future Challenges." *Journal of Ecology* 101 (2):265–76. https://doi.org/10.1111/1365-2745.12054.

Ravenscroft, Catherine H., Jason D. Fridley, and J. Philip Grime. 2014. "Intraspecific Functional Differentiation Suggests Local Adaptation to Long-Term Climate Change in a Calcareous Grassland." *Journal of Ecology* 102 (1):65–73. https://doi.org/10.1111/1365-2745.12168.

Ravenscroft, Catherine H., Raj Whitlock, and Jason D. Fridley. 2015. "Rapid Genetic Divergence in Response to 15 Years of Simulated Climate Change." *Global Change Biology* 21 (11):4165–76. https://doi.org/10.1111/gcb.12966.

Reffner, John A. 2018. "Advances in Infrared Microspectroscopy and Mapping Molecular Chemical Composition at Submicrometer Spatial Resolution." *Spectroscopy* 33 (9).

Salman, A., L. Tsror, A. Pomerantz, R. Moreh, S. Mordechai, and M. Huleihel. 2010. “FTIR Spectroscopy for Detection and Identification of Fungal Phytopathogenes.” *Spectroscopy* 24 (3–4):261–67. https://doi.org/10.3233/SPE-2010-0448.

Sarkar, Ananda K., Marijn Luijten, Shunsuke Miyashima, Michael Lenhard, Takashi Hashimoto, Keiji Nakajima, Ben Scheres, Renze Heidstra, and Thomas Laux. 2007. “Conserved Factors Regulate Signalling in Arabidopsis Thaliana Shoot and Root Stem Cell Organizers.” *Nature 2007 446:7137* 446 (7137). Nature Publishing Group:811–14. https://doi.org/10.1038/nature05703.

Schmitt, Jürgen, and Hans Curt Flemming. 1998. “FTIR-Spectroscopy in Microbial and Material Analysis.” *International Biodeterioration & Biodegradation* 41 (1). Elsevier:1–11. https://doi.org/10.1016/S0964-8305(98)80002-4.

Semchenko, Marina, Sirgi Saar, and Anu Lepik. 2014. “Plant Root Exudates Mediate Neighbour Recognition and Trigger Complex Behavioural Changes.” *New Phytologist* 204 (3). https://doi.org/10.1111/nph.12930.

Shen, J., H. Li, G. Neumann, and F. Zhang. 2005. “Nutrient Uptake, Cluster Root Formation and Exudation of Protons and Citrate in Lupinus Albus as Affected by Localized Supply of Phosphorus in a Split-Root System.” *Plant Science* 168 (3). Elsevier:837–45. https://doi.org/10.1016/J.PLANTSCI.2004.10.017.

Ström, L., T. Olsson, and G. Tyler. 1994. “Differences between Calcifuge and Acidifuge Plants in Root Exudation of Low-Molecular Organic Acids.” *Plant and Soil 1994 167:2* 167 (2). Springer:239–45. https://doi.org/10.1007/BF00007950.

Vierheiler, H., Lerat, S. & Piché, Y. (2003) Systemic inhibition of arbuscular mycorrhiza development by root exudates of cucumber plants colonised by *Glomus mosseae*. Mycorrhiza, 13, 167-170.

Wang, H, S K Lockwood, M F Hoeltzel, and J W Schiefelbein. 1997. “The ROOT HAIR DEFECTIVE3 Gene Encodes an Evolutionarily Conserved Protein with GTP-Binding Motifs and Is Required for Regulated Cell Enlargement in Arabidopsis.” *Genes &*

Development 11 (6). Cold Spring Harbor Laboratory Press:799–811. https://doi.org/10.1101/GAD.11.6.799.

Whipps, JM. 1990. “Carbon Economy.” *The Rhizosphere* 59. Wiley & Son. http://ci.nii.ac.jp/naid/10025228691/en/.

Williams, Alex, Holly Langridge, Angela L. Straathof, Graeme Fox, Howbeer Muhammadali, Katherine A. Hollywood, Yun Xu, Royston Goodacre, and Franciska T. de Vries. 2021a. “Comparing Root Exudate Collection Techniques: An Improved Hybrid Method.” *Soil Biology and Biochemistry* 161 (October). Pergamon:108391. https://doi.org/10.1016/J.SOILBIO.2021.108391.

Williams, Alex, Holly Langridge, Angela L. Straathof, Howbeer Muhamadali, Katherine A. Hollywood, Royston Goodacre, and Franciska T. de Vries. 2021b. “Root Functional Traits Explain Root Exudation Rate and Composition across a Range of Grassland Species.” *Journal of Ecology* 00. John Wiley & Sons, Ltd:1–13. https://doi.org/10.1111/1365-2745.13630.

Willig, Sidney, Zeno Varanini, and Paolo Nannipieri. 2000. “Types, Amounts, and Possible Functions of Compounds Released into the Rhizosphere by Soil-Grown Plants.” *The Rhizosphere*, November. CRC Press, 35–56. https://doi.org/10.1201/9780849384974-8.

Willmann, Matthew R., and R. Scott Poethig. 2007. “Conservation and Evolution of MiRNA Regulatory Programs in Plant Development.” *Current Opinion in Plant Biology* 10 (5):503–11. https://doi.org/10.1016/j.pbi.2007.07.004.

Wookey, Philip A., Rien Aerts, Richard D. Bardgett, Florence Baptist, Kari Anne Bråthen, Johannes H. C. Cornelissen, Laura Gough, Et Al. 2009. “Ecosystem Feedbacks and Cascade Processes: Understanding Their Role in the Responses of Arctic and Alpine Ecosystems to Environmental Change.” *Global Change Biology* 15 (5). John Wiley & Sons, Ltd:1153–72. https://doi.org/10.1111/J.1365-2486.2008.01801.X.

Wright, Ian J., Peter B. Reich, Mark Westoby, David D. Ackerly, Zdravko Baruch, Frans Bongers, Jeannine Cavender-Bares, et al. 2004. “The Worldwide Leaf Economics

Spectrum." *Nature 2004 428:6985* 428 (6985). Nature Publishing Group:821–27. https://doi.org/10.1038/nature02403.

Zamioudis, Christos, Parthena Mastranesti, Pankaj Dhonukshe, Ikram Blilou, and Corné M J Pieterse. 2013. "Unraveling Root Developmental Programs Initiated by Beneficial Pseudomonas Spp. Bacteria." *Plant Physiology* 162 (1):304–18. https://doi.org/10.1104/pp.112.212597.

2. Hydroponics are not soil: detailing the differences in exudate composition, root system architecture, and root metabolic fingerprint in a commercial barley strain grown in hydroponic- and soil-based systems

Reuben Margerison[1,2], Alex Williams[3], Howbeer Muhammadali[2], Cassio Lima[2], Katherine A. Hollywood[4], Yun Xu[2], Giles Johnson[1], Patrick Gallois[5], Ellen L. Fry[6], Royston Goodacre[2,4] and Franciska T. de Vries[1,7]

[1]School of Natural Sciences, The University of Manchester, Oxford Road, M13 9PT, Manchester, UK

[2]Department of Biochemistry and Systems Biology, Institute of Systems, Molecular and Integrative Biology (ISMIB), University of Liverpool, Biosciences Building, Crown Street, L69 16 7ZB, Liverpool, UK

[3]School Biological Sciences, The University of Manchester, Oxford Road, M13 9PT, Manchester, UK

[4]Manchester Institute of Biotechnology, The University of Manchester, Princess Road, M1 7DN, Manchester UK

[5]School Biological Sciences, The University of Manchester, Oxford Road, M13 9PT, Manchester, UK

[6]Department of Biology, Edge Hill University, St Helens Road, Ormskirk, L39 4AP, Lancashire UK

[7]Institute of Biodiversity and Ecosystem Dynamics, University of Amsterdam, PO Box 94240, 20 1090 GE Amsterdam, The Netherlands

2.1 Key words

Root exudates; GC-MS; FTIR; root traits; mIRage; metabolomics; hydroponics; exudate collection

2.2 Abstract

Root exudates are increasingly recognised as a key driver of global carbon cycling and ecosystem function. The analysis of root exudates has traditionally taken place on plants that have been grown in hydroponic systems. However, this environment lacks the highly complex and heterogeneous structure, as well as the microbial community, of soil, and collected root exudates might not be meaningful for predicting their function in a soil environment. There is no general consensus on the best way to collect and analyse root exudates, because of methodological issues with both hydroponics and soil. Here we show that soil-based growth with a hydroponic repair stage causes consistent differences in 1) root traits, 2) root metabolites, and 3) root exudates, when compared with growth in purely hydroponic systems. Root traits showed a consistent change in expression towards a more acquisitive set of architectural features in plants with a soil life history. Root metabolites measured using ATR-FTIR, and for the first time, mIRage, contrasted between groups, even when grown in a nutritionally similar environment. Metabolic fingerprinting using FTIR on root exudates also revealed differences in the kinds of molecules present in root exudates between soil and hydroponic systems, with shifts in the saccharide and amide groups between treatments, and GC-MS also shows exudates from plants grown in soil have a completely different profile to plants grown in lifetime hydroponics, even after soil-grown plants have been transferred to a hydroponic system to recover washing damage. Our results demonstrate that hydroponics are not soil, cannot be treated as such, and this may have important repercussions for future studies. We show that a soil-hydroponic hybrid system, with a repair phase, could offer a useful alternative that may offer more realistic results. Future work within soil ecology must consider soil-hydroponic differences and should focus on soil-based systems.

2.3 Introduction

Plant-microbe interactions are central to the functioning of terrestrial ecosystems, but the intricacies of these relationships are only recently beginning to be understood (Bardgett and Wardle, 2010). Most plant species exhibit complex, mutualistic and/or antagonistic interactions with soil microbes (Bonkowski, 2004). These interactions are often so intimately linked that microbial community structure can influence selection on plant genetic traits (Lau and Lennon, 2011) and reproducibly induce specific plant phenotypes (Herrera Paredes et al., 2018). Conversely, plant phenotypic variation can impact microbial community structure and function (Johnson et al. 2010). An important mechanism through which plants influence their belowground environment is through the constant transport of primary and secondary metabolites into the rhizosphere, a process known as root exudation. Root exudation is common to all land plants and forms a major source of organic carbon (C) in the soil – plants can allocate up to 40% of the carbon fixed to root exudation (Whipps, 1990). Exudates contain released hydrogen ions and inorganic acids to alter soil chemistry and pH to influence nutrient availability, but are mostly carbon-based compounds such as organic acids, sugars and amino acids that microbes use to fuel their growth and activity (Uren, 2000; Bais et al., 2006), as well as branched polysaccharides thought to be used for construction of the physical rhizosphere environment (Galloway et al. 2020). Evidence also suggests that root exudation is an important conduit for communication between plants and microbes, but while this appears to be altered by developmental growth stage and phenotype, little is known about the genetic and molecular mechanisms driving root exudation (Mavrodi et al., 2021; Hannula et al., 2010). Additional understanding of these mechanisms will therefore offer increased precision in understanding soil food web dynamics under a range of scenarios, which in turn will help fill the knowledge gaps in the carbon cycle (Fry et al. 2018). By characterising the volume and molecular makeup of the carbon entering the rhizosphere, we may begin to understand how plants and microbes interact at the root-tip scale, and to shed light on the role of climate change in plant-microbial relations (Drigo, Kowalchuk, and van Veen, 2008).

There is an increasing body of evidence that suggests root exudates are highly sensitive to the growth environment of the plant, and therefore artificial growth environments, including hydroponics, can result in unrealistic exudation patterns that tell us little about exudation in natural communities (Williams et al. 2021a). In a similar vein, there can be profound plasticity in exudation composition as a response to abiotic stress; for example, exudates collected from plants recovering from drought, when reapplied to soil, promote higher microbial respiration per unit C than those of control plants (de Vries et al., 2019). This is attributed to a carbon priming effect, i.e., when fresh labile inputs are added to soil, this triggers the microbial community to metabolise older stored C, as well as the newer input (Blagodatskaya & Kuzyakov 2008). As well as the direct change in exudation quality in response to stress, root traits relating to root system architecture (RSA), morphology and chemical processes are likely to impact the location, volume and effect of exudation (Bardgett, Mommer and De Vries, 2014). Root exudation has been linked to root traits such as root dry matter content (RDMC; Guyonet et al., 2018), and specific root length (SRL; Roumet et al, 2006), and plant functions such as root respiration (Sun et al. 2011). Root traits are a developmentally plastic set of characters that respond to abiotic environmental cues, such as water availability (Deak & Malamy 2005); and biotic cues, such as responding to the presence of mutualistic bacteria (e.g. *Pseudomonas,* Zamioudis et al., 2013). Taken together, there is a challenge in maintaining a realistic growth environment while disentangling the role of root architectural and morphological traits from exudation quantity and quality. Which are the best ways to measure exudation? How can the effect of the soil and soil chemical composition be removed from the exudates (Mönchgesang et al*.,* 2016).

Despite the implication of altered root physiology driving a change in root exudate expression (Petriacq et al., 2017), hydroponic collection was long the *de facto* method to directly assess the chemical composition of exudates (van Dam and Bouwmeester 2016). The only alternative was viewed to be the indirect quantification of exudates in the context of soil-systems, i.e. collection of 'leachates', wherein solvents are dripped through a planted soil column (e.g. Hagan et al., 2013). The main issue with this approach is that leachates are highly impure, consisting of lysed cell contents of both microbes and plants, dissolved soil carbon and an almost infinite range of other molecules (Williams et al. 2021a). Hydroponics are by no means a perfect solution, and as the analytical tools used to characterise exudates become

more cost-effective, there is a need for the growth method of plants for exudation studies to be the best that they can be. Understanding the limitations of different methods of root exudate collection, and how they relate to root trait expression and the composition of the chemical constituents within root exudates, is crucial for understanding the role of root exudation in plant-microbial interactions. To attempt to minimise these limitations, there has been much interest in the use of hybrid methods, wherein plants are grown in soil, and then are washed to remove remaining soil before collecting exudates (Ström et al., 1994; Aulakh et al., 2001; Lucas García et al., 2001; Canarini et al., 2016). However, as this washing process is suggested to be stressful and damaging to the plant (Oburger and Jones, 2018), recent work has focussed on the potential use of a recovery period in hybrid collection methods, which involves removing soil from living plants by repeated suspension and gentle shaking, and manual removal of soil particles with forceps. After the recovery period in this solution, the plants are transferred to an aerated hydroponic collection solution, where the samples are taken. Williams et al. (2021a) presented a study comparing metabolic profiles of exudates from 0-, 3- and 7-day recovery periods with profiles of mashed roots. They concluded that a longer recovery time was appropriate, based on the differences in metabolites. It seems probable that any damage from removal from soil, expressed by similarity with damaged cell contents, would be diluted and/or re-metabolised by the end of the 7 days. To confirm this, a root-soil system was included in the study comparing leachates to the exudates, although this suffers from the issues with leachates expressed above. As root trait expression is dependent on the growth system, purely hydroponic systems may not be informative models of soil-based growth. It is therefore essential to compare lifetime hydroponic growth with soil-based hybrid growth systems using a hydroponic recovery period to identify differences between the root system architecture, plant biochemistry, and root exudate composition in these growth modes.

Here, we aimed to consider the impact of purely hydroponic (aerated and non-aerated) systems on root trait expression, root biochemistry, and root exudate composition by comparing with a soil-hydroponic hybrid system. We hypothesise that 1) the morphological and architectural traits will be impacted by growth system, being less branched and less acquisitive in plants with a purely hydroponic life history compared with a soil-hydroponic hybrid with repair phase, 2) the metabolic fingerprint of the root tissue after the recovery

period will be significantly more similar between the two hydroponic treatments than the soil treatment, indicating that root traits are closely linked with tissue chemistry and impacted strongly by the growth medium, 3) we hypothesise that purely hydroponic systems give a different profile of root exudates compared with soil, and therefore may be inappropriate for the purposes of the study of root exudates. Specifically, we hypothesise that due to the lack of need for foraging and difficulties recruiting a microbial community in lifetime hydroponic solution, hybrid soil-hydroponic systems with a repair phase will show substantially different metabolomic profiles from hydroponic systems, with quantifiable changes in chemical composition and bond type. By comparing the three treatments, we aim to demonstrate that soil-hydroponic systems have plant traits associated with needing to forage through a heterogenous medium, which in turn has consequences for the tissue and exudate chemistry. We tested these hypotheses in a controlled experiment with the model crop barley (*Hordeum vulgare*), which provides an excellent system for the study of root exudation: it has an extensively documented genome (The International Barley Genome Sequencing Consortium et al., 2012); is a valuable and agroecologically important crop species (Langridge, 2018); is tolerant of lifetime hydroponic growth (El-Deeba et al., 2009); and has a large root system (Hackett, 1969). We also aim to advance new methods of analysis of roots and exudates.

2.4 Materials and Methods

2.4.1 Growth conditions

Using *Hordeum vulgare* var. Oddessy (Nickerson Seeds, Rothwell, UK), seeds were sown onto a commercially available soaked sandy soil ('top dressing', Interhort Ltd, Congleton, UK) and stratified in darkness for seven days at 4°C. They were then transferred to a growth cabinet (MobyLux GroBank, CLF Plant Climatics, Wertingen, Germany) set to 20^{o}C for an eight-hour day, and 16^{o}C at night. One-week post-germination, all seedlings were washed clean of soil, and then 'replanted' in either soil, in 10cm square pots with a total volume of 100cm^{3}, or in a hydroponic treatment.

For the hydroponic treatments, a soil solution was used to retain similar osmotic and microbiological background to the soil. This solution consisted of the fluid fraction of a 1:1 v/v

soil/water mix that has been refrigerated for three days to allow it to settle following initial mixing. The soil solution was poured into 100ml Schott Duran® bottles (SCHOTT UK Ltd., Stafford, UK), and the washed plants were suspended with roots immersed in the solution leaving the aboveground biomass clear of the fluid and are held in place with Azpack non-absorbent cotton wool (Fisher Scientific Ltd, Loughborough, UK). The bottles were covered with aluminium foil to retard algal growth, and the plants returned to the growth chamber. Plants were checked daily to ensure the roots remained immersed in soil solution and topped up with hydroponic solution when the level fell.

For the Hydroponics - Air treatment, the soil solution and root mass in each bottle was oxygenated with air using a HiDOM HD-603 aquarium pump (Shenzhen Hidom Electric Co., Ltd, Shenzhen, China) set to a flow rate of approximately 1ml s^{-1}. These stages and treatment groups are summarised in Figure 1.

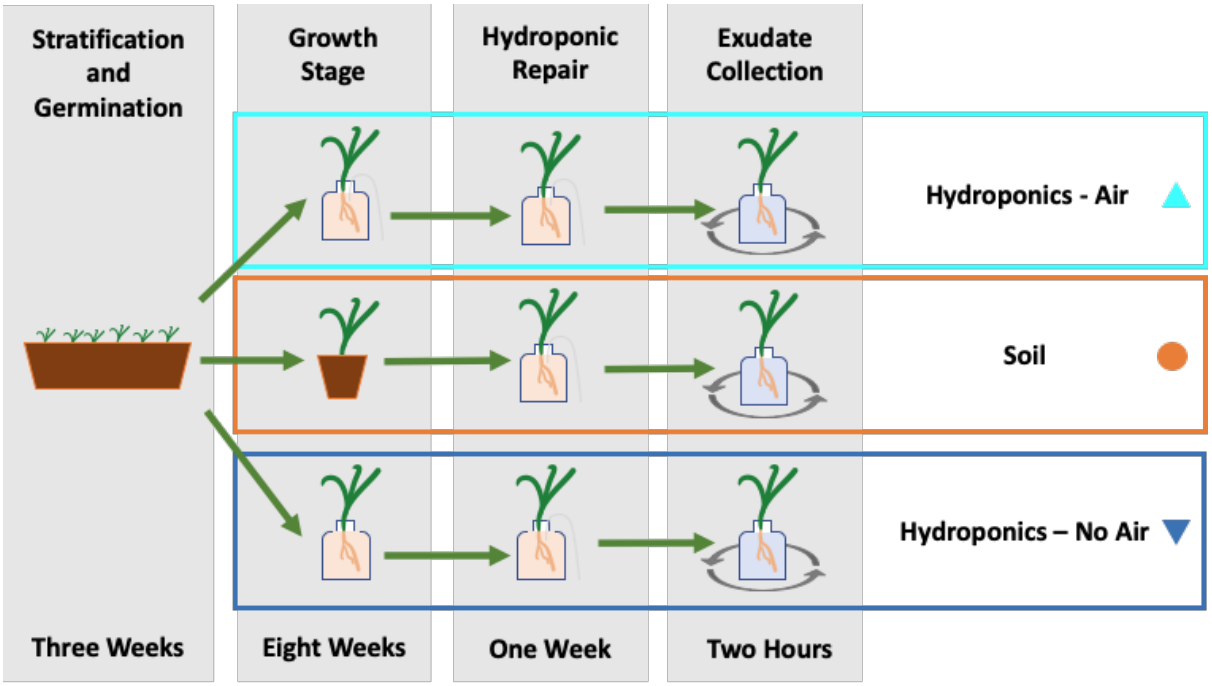

Figure 1: Timeline of experiment showing three experimental treatments; Germination stage lasted for one week, growth stage lasted for eight weeks, and the hydroponic repair stage lasted for one week. Whole length of plant growth in this experiment, including two weeks stratification, was sixteen weeks.

2.4.2 Exudate Collection

Barley plants were removed from pots and bottles and had their roots washed in lukewarm water to remove soil, with any remaining fibres resistant to washing removed with forceps. To allow plants time for recovery from root washing (in line with methods presented in Williams et al. 2021a and Williams et al. 2021b), the plants were transferred to a hydroponic growth environment, as above, with all treatments receiving air (Figure 2.2). After seven days, these roots were submerged and gently shaken in two 1L beakers of distilled water and one 1L beaker of milliQ water to remove any remaining soil solution. Plants were transferred to a new, milliQ-water rinsed, 100mL glass bottle containing 100 mL milliQ water. Roots were suspended with the above-ground biomass clear of the water with Parafilm M (Bemis Company, Inc. Neenah, WI, USA). Bottles with the plants were placed on ice in a Styrofoam cooler on a rotary shaker at 60 rpm for two hours (Fig. 2.3). Plants were removed from the bottle and set aside for downstream analysis, leaving an exudate solution. This was immediately filtered using a milliQ-washed 0.22µm filter (Merck Millipore (U.K.) Limited, Watford, SLGP033RS) to remove microbes and debris, and transferred in 3 30ml fractions into 50 ml Falcon Tubes (Greiner Bio-one CellStar 227261). The exudate solution was then frozen at -80^{o}C before being freeze-dried for 48 hours using a Scavac CoolSafe 55-9 Pro (LaboGene, Lynge, Denmark) to concentrate the solution for subsequent analyses.

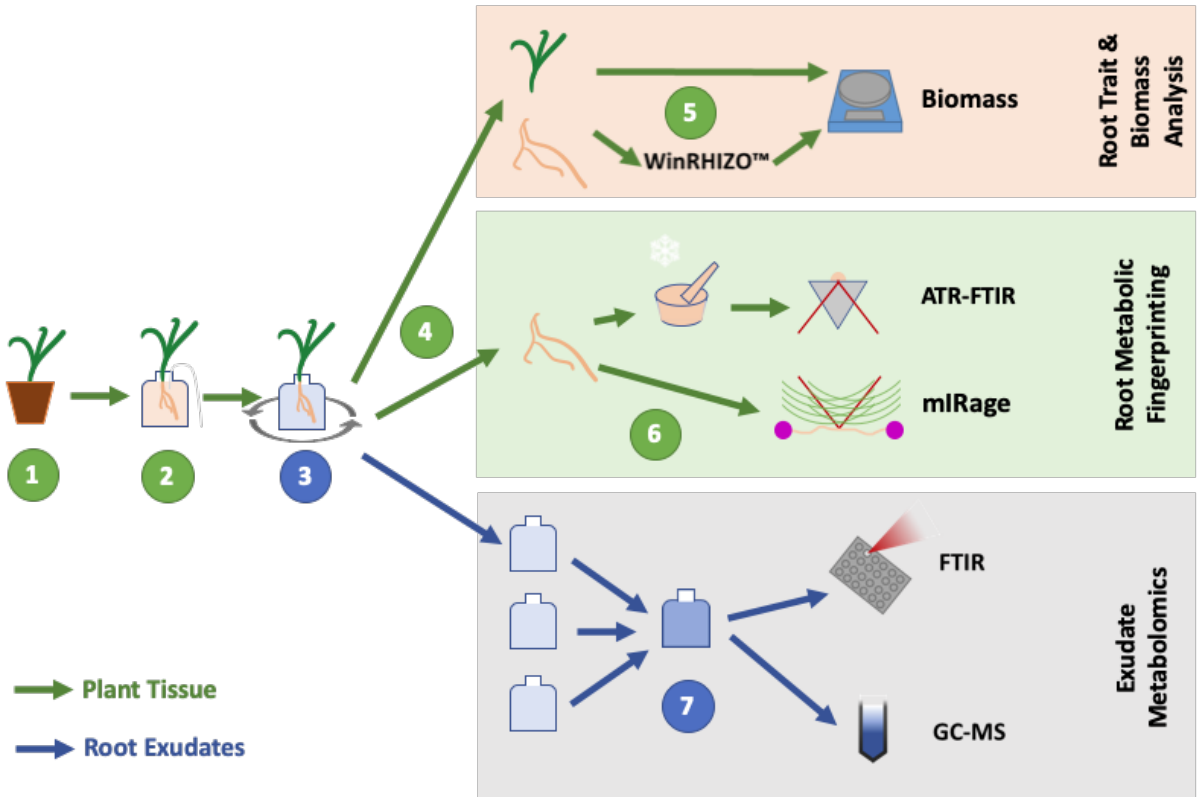

Figure 2: Schematic of downstream processing from growth phase onwards. 1) Growth Phase, here shown using the Soil treatment; 2) Hydroponic Repair Phase; 3) Exudate Collection; 4) Root samples are split into two groups, with two thirds of the individuals going for Root Trait Analysis and one third to Root Metabolic Fingerprinting; 5) aboveground and belowground biomass is separated, with the belowground biomass being root scanned before both are weighed, dried, and weighed; 6) root systems are microdissected to remove lateral roots; these are mounted on slides for mIRage while the rest of the root system is freeze-dried and ground to a powder before being placed on a diamond for ATR-FTIR (which is suitable for powder samples); 7) Four samples of collected exudates are pooled into one exudate sample (Table S2.1), and half of each pooled sample was used for GC-MS, with the other half used for FTIR.

2.4.3 Plant Root Traits

Root and shoots were separated. Shoots were weighed, dried at 60° C for 48h in paper bags and weighed again to have dry mass determined on a laboratory balance (Mettler Toledo). Roots were transferred to a 50 ml falcon tube containing 20% ethanol solution for storage until downstream analysis. Three root systems from each pooled exudate sample were used for root trait analysis (Table S2.1). Stored roots were briefly placed in tap water to remove ethanol and ease root mass separation. Roots were then immersed in deionised water in a clear Perspex 30cmx40cm tray coupled to an Epson Expression 11000XL flatbed scanner. Root strands were carefully separated using plastic tweezers to limit overlapping. Root images were captured in grayscale at a resolution of 800 dpi and root properties including root length, area, and number of tips as a function of root diameter were calculated using the WinRHIZO® pro software (Regent Instruments Inc., Canada). To retain all root biomass, the contents of the scanning tray were poured through a sieve and gently dried with a paper towel before being weighed. Roots were transferred to a paper bag and dried at 60° C for 48h. Following this period of dehydration, above- and below-ground biomass was determined using a balance. Root traits were calculated from measured data: Root Dry Matter Content (DMC), the dry mass per fresh mass; Root Tissue Mass Density (TMD), the ratio of dry mass to fresh volume; and Specific Root Length (SRL), the ratio of root length (correlated with resource acquisition) to dry mass (investment).

2.4.4 Root Metabolic Fingerprinting

Root O-PTIR (mIRage) - The remaining seven root systems from each treatment (those not used for root traits, see Table S2.1) were dissected to remove lateral roots. Lateral roots were fixed on to a glass slide with a commercially available nitrocellulose varnish and dried for six hours at room temperature. The prepared slide was placed on the stage of a mIRage optical photothermal microscope (Photothermal Spectroscopy Corp., Santa Barbara, CA, USA), with instrument control performed with the manufacturer-supplied *PTIR* software. The continuous wave 532nm diffraction laser was focussed on a point on the wall of an epidermal cell of the lateral root using the optical microscope under a 30X objective under phase contrast, and an

infra-red spectrum collected in the 800-1800cm^{-1} wavenumber range, with 50 scans per spectrum at 2cm^{-1} resolution. Seven lateral roots from seven plants from each of the three treatments had spectra collected from them, for a total of 147 spectra.

Root ATR-FTIR - The preserved root systems from the mIRage analysis were drained of ethanol, washed in deionised water and frozen at -80°C before being freeze-dried for 48 hours. The roots were cut with surgical scissors and placed in a Tissuelyser for one minute to break down the cells. The steel balls and casings for the Tissuelyser had been pre-prepared by chilling at -80°C for 30 minutes. The powdered samples were placed on the diamond Attenuated Total Reflectance (ATR) accessory of a Bruker Invenio S FTIR machine (Bruker, Billerica, MA, USA), and pressed onto the diamond surface with a pressure applicator, ensuring even pressure was applied across samples. A background measurement of the environment around the crystal was taken before every sample, and the crystal was cleaned with 70% ethanol between samples.

2.4.5 Root Exudate Analysis

Freeze-dried root exudates were reconstituted in 1ml of liquid chromatography grade water (Sigma-Aldrich, Gillingham, UK) for metabolomic analysis. Due to preliminary work finding that exudates collected from a single root system were not detectable with our analytical instruments, four replicates from each treatment were pooled to make a composite sample, resulting in seven pooled samples of each treatment (Fig 2.7). Gas Chromatography-Mass Spectrometry (GC-MS) was chosen due to the low proportion of lipids extracted by our water-based collection technique, with Transmission FTIR used to give insight into broad chemical shifts between treatments should metabolites not be identifiable following the GC-MS.

GC-MS – Reconstituted exudates were pipetted (using 200 - 1000 µL tips Fisher Brand Blue FB34611) into a 2 mL Safe-Lock microcentrifuge tube (Eppendorf 0030 120.094). An internal standard 10X stock solution composed of 30 mg each of the deuterated compounds Succinic-d4 and Glycine-d5 (Sigma-Aldrich) dissolved in HPLC grade water was prepared, and further diluted one in ten with water to produce an internal standard working solution. 100 µL of this standard was added (using 1 - 200 µL tips Fisher Brand Yellow FB34531) to each sample, in order to correct for possible drift over a machine run. Samples were vortexed for 10 s and

placed on a SpeedVac concentrator overnight to remove solvent. Samples were derivatized in order to open cyclic molecules and reduce boiling points to GC-operational temperatures with the addition of methoxy and trimethylsilyl groups. To do this, 50 µL of a 20 $mgmL^{-1}$ O-methoxyamine•HCl in pyridine solution was added to each sample. Samples were vortexed for 10 s and then placed in a heating block at 65°C for 40 min. 50 µL of N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA, Acros 221580250) was added to each sample, and samples were vortexed for 10 s before being returned to the heating block for 40 minutes. 1 mL of a retention index composed of 40 µL Decane, 40 µL Dodecane, 40 µL Pentadecane, 30 mg Docosane, and 30 mg Nonadecane dissolved in 10mL of Hexane was diluted in 9mL of anhydrous pyridine (all from Sigma-Aldrich) to form a working solution, 20 µL of which was added to each sample in order to aid with analyte identification and statistical analysis. The tubes were then vortexed for 10s and centrifuged at 17,000g for fifteen minutes. 100 µL of each sample was moved to a GC vial and sealed with a cap with a silicone septum (Chromacol Brand, Thermo-Fisher Scientific, Manchester, UK). Aliquots of all samples were mixed to form a QC sample to be sampled throughout the run. The GC vials containing the samples were loaded onto a Gerstel MPS-2 autosampler (Gerstel, Baltimore, USA), which directly injected the samples into an Agilent 6890N GC oven (Wokingham, UK) containing an Agilent VF5-ms CP8943 GC Column. Samples exit the column into a Leco Pegasus III Time-of-Flight (TOF) mass spectrometer (Leco, St Joseph, USA). The collected data were deconvolved and annotated with the *erah* R package (Domingo-Almenara et al., 2016), with calls individually checked against mass fragmentation patterns.

Transmission FTIR - A silicon 96-well IR plate (Bruker, Coventry, UK) was washed with 5% Sodium Dodecyl Sulfate (SDS) solution and rinsed with ethanol and deionised water to ensure an optically clean surface with no residue before 20µL of resuspended root exudate was directly loaded into individual wells. The plate was dried at 65^{o}C for one hour until completely dry, and loaded onto a motorized high-throughput cassette linked to a Bruker Invenio S FTIR machine (Bruker, Billerica, MA, USA). The FTIR machine was run in transmission mode in the 4000–600 cm^{-1} range at a resolution of 4 cm^{-1} using the protocol described in Winder et al. (2004). One process and one silicon blank was included on the plate (5% of total samples), to ensure contamination had not occurred at any point in the process.

2.4.6 Statistical analyses

To address our first hypothesis regarding the effect of growth system on root traits, we carried out an ordination using Principal Component Analysis (PCA) to visualise the root trait data across treatments. All data were log10-transformed and scaled, and projections were applied to the biplot to indicate which root traits drove differences between treatments. We also carried out a pairwise multilevel comparison of PERMANOVA (Martinez Arbizu, 2020) using the adonis function of the *vegan* package in R to search for significant differences between treatments. We followed this with Analysis of Variance to show significant differences between individual traits.

To address our second hypothesis regarding the effect of growth system on root chemistry, the ATR-FTIR root data was processed in MATLAB using Savitzky-Golay filtering to reduce noise, before removal of the interference from CO_2 in the 2400-2275cm^{-1} wavenumber region and trimmed to the 4000-800 cm^{-1} wavenumber range before undergoing baseline correction and normalisation using the in-house cluster toolbox in MATLAB (Mathwork, MA; toolbox available freely at https://github.com/Biospec/cluster-toolbox-v2.0; method after Timmins et al., 1998). We then visualised these data using PCA. The mIRage data were processed as for the ATR-FTIR.

To address our third hypothesis regarding the effect of growth system on root exudates, we pre-processed the GC-MS data using MassHunter (Agilent MassHunter) to convert raw output folders to mzXML format. MassHunter uses MSConvert software, with peak identification enabled with the Vendor algorithm (Adusumilli & Mallick 2017). The mzXML files were deconvolved and aligned (R package *erah*, Domingo-Almenara et al. 2016). We then completed a missing compound recovery step. This step ensured that all metabolites that were present in at least 10 samples appeared in the dataset. We implemented QC corrections for each dataset to correct for drift, batch and GC-MS injection order (after Dunn et al. 2011) using the cluster toolbox as before. The transmission FTIR was processed as the ATR-FTIR listed above, with further analysis on changes in wavenumbers between groups. As biological samples produce a characteristic infra-red absorption spectrum with areas of absorbance peaks typical to common classes of biomolecules occurring in particular regions of the IR spectrum, changes in functional groups in a sample may be inferred by examining changes in

absorbance in these regions. A fatty acid region (3050-2800cm^{-1}); the region in which proteins and peptides absorb strongly (1750-1500 cm^{-1}); the 'mixed' region in which carboxylic acid functional groups, unbonded amino acids, and polysaccharides absorb (1500-1250 cm^{-1}); and the (poly)saccharide region (1200-900 cm^{-1}) (Schmidt and Flemming, 1998). Significant differences between groups were modelled in Partial Least Squares Discriminant Analysis (PLSDA), and significant differences in wavenumbers were calculated using Sparse PLSDA (SPLSDA) using the *mixOmics* package in R (Rohart et al., 2017).

2.5 Results

2.5.1 Root Traits

The experimental treatments led to a distinct difference in the root system of the barley, with a visible difference in depth and branching between the hydroponics and the soil systems (Figure 3). The two hydroponics treatments (aerated and non-aerated) had lower biomass but higher root length.

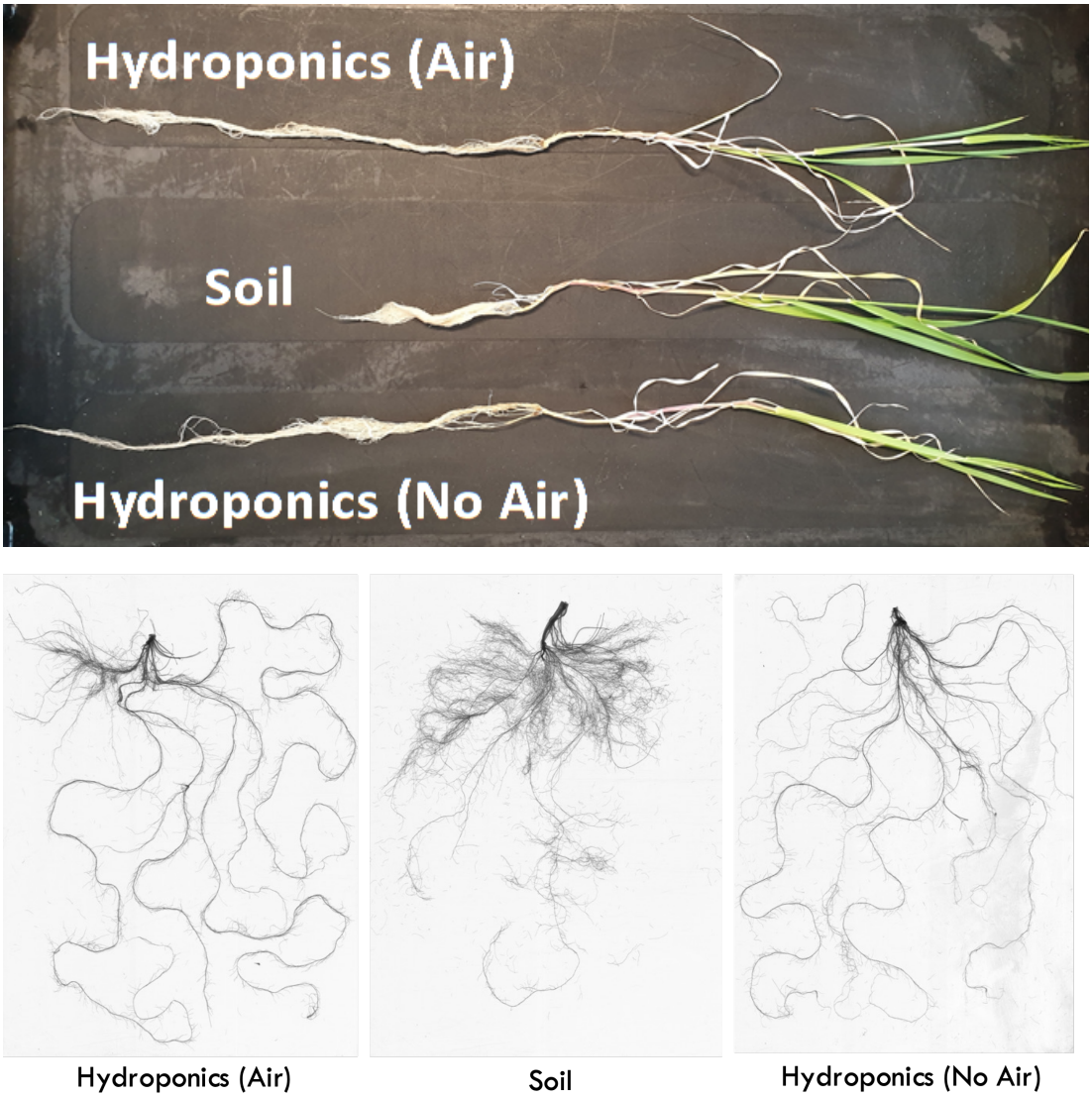

Figure 3: Root traits after the repair stage and root washing, a) whole plants immediately after removal from hydroponic repair solution, b-d) root systems arranged for analysis in WinRhizo software.

One-way ANOVA with post-hoc Tukey's Honest Significant Difference test revealed a general shift towards a more acquisitive root system in soil-based systems, with an increase in above and belowground biomass, root length density, volume, root tips, branching and aboveground dry matter content relative to the hydroponic treatments (Table 1, Figure S1). Of these traits, above and belowground biomass and root volume also responded to the hydroponic treatments in contrasting ways- all three had higher values for hydroponics grown with Air than No Air (Water; Figure S1). In addition, two traits responded very differently to

the treatments compared with the other traits: specific root length and surface area both had the highest values in the Air treatment, followed by the soil treatment, with No Air (Water) treatment having the lowest values (Figure S1). Three traits were not impacted by the treatments, these were root tissue density, root surface area and root dry matter content.

Table 1: Effect of the two hydroponic treatments and soil hybrid treatment on plant traits, analysed using one-way ANOVA. Where the data did not conform to a Gaussian distribution, log transformation was employed, as denoted by superscript log. Significant results at the $p<0.05$ level are highlighted in bold.

Plant Section	Trait	df	F	p
Roots				
	Belowground Biomass^{log}	**2,48**	**15.32**	**<0.001**
	Root Length Density^{log}	**2,48**	**30.52**	**<0.001**
	Root volume^{log}	**2,48**	**56.56**	**<0.001**
	Specific Root Length^{log}	**2,48**	**5.73**	**0.005**
	Root Tips^{log}	**2,48**	**31.34**	**<0.001**
	Root Branches^{log}	**2,48**	**86.03**	**<0.001**
	Root Tissue Density	2,48	1.58	0.216
	Root Surface Area by Mass^{log}	2,48	3.19	0.050
	Root Dry Matter Content	2,48	0.60	0.551
Shoots				
	Aboveground Biomass^{log}	**2,48**	**59.89**	**<0.001**
	Aboveground Dry Matter Content^{log}	**2,48**	**13.26**	**<0.001**

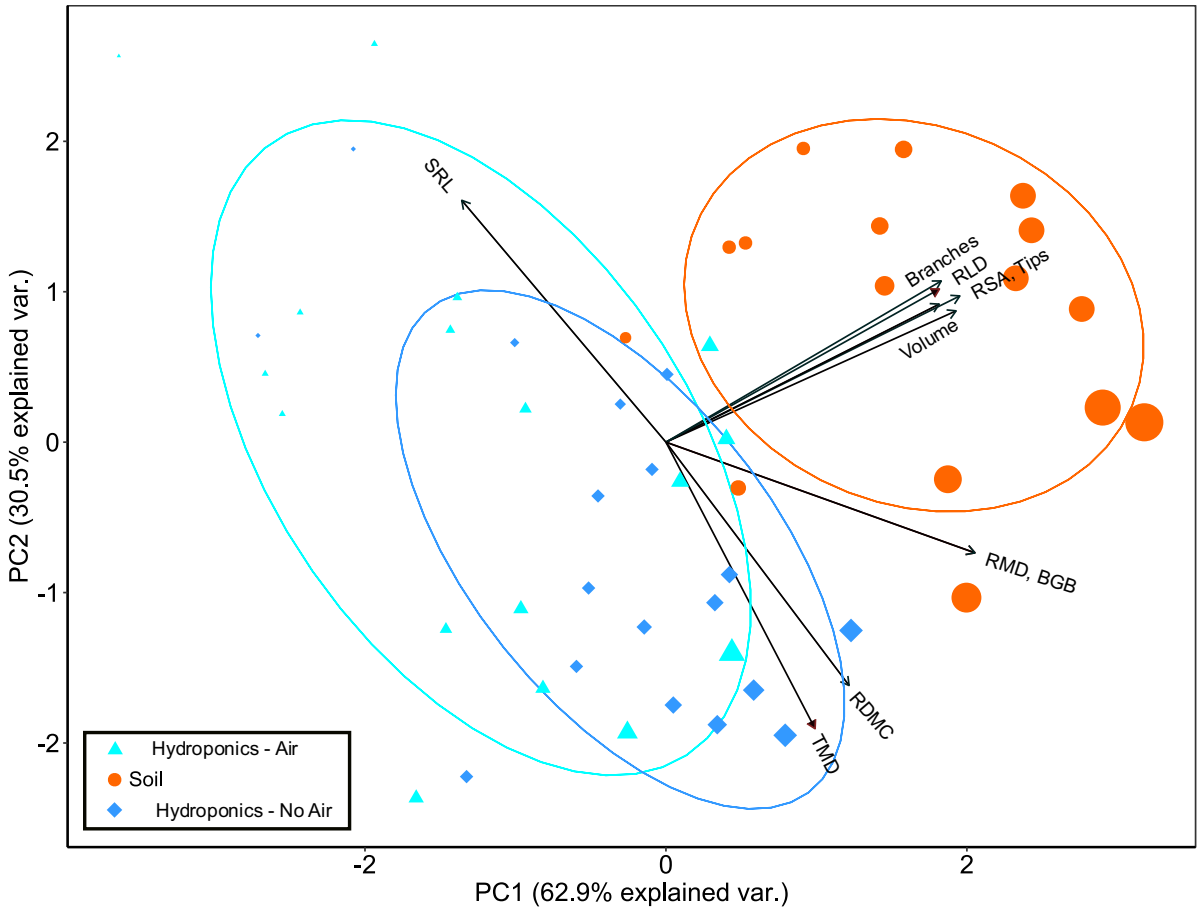

Figure 4: Biplot showing Principal Components Analysis of barley root traits in hydroponics with air, hydroponics without air, and soil. Points are scaled relative to the biomass of the root system. Projections show, clockwise from top left: SRL, Specific Root Length; Forks, the number of branching points in the root system; RLD, Root Length Density; Tips, the total number of root tips; SA, the surface area of the root system; Volume, the volume of the root system; Fresh Mass, the wet weight of the root tissue; BGB, the mass of a completely dehydrated root system; RDMC, the Root Dry Matter Content; and TMD, the Tissue Mass Density. Ellipses are set to 95% Confidence Intervals around the centroid.

When all traits were assessed using Principal Components Analysis (PCA), the data were primarily spread across two axes (Figure 4). The first two axes explain 93.4% of the total variance, and Horn's Parallel Analysis for Component Retention recommends using only these to explain the data. Axis 1, which explained 62.9% of the variance in the data, was primarily driven by traits associated with foraging: branches, tips, volume, RLD and RSA. The second axis, accounting for 30.5% of the variance, showed SRL at one extreme of the axis, and RDMC

and TMD at the opposite, indicating a more structural influence. When analysed using a pairwise multilevel comparison of PERMANOVA, the Soil treatment was significantly different from each of the hydroponic treatments, and this was primarily driven by the "foraging" axis (PC1; Soil vs No Air: F=20.90, R^2=0.40, p_{adj}=0.003; Soil vs Air: F=29.27, R^2=0.49, p_{adj}=0.003). The Soil treatment ellipse was associated with higher values in all foraging traits. There was also a significant difference between the two hydroponics treatments, which appeared to primarily be due to shifts along the "structural" axis (PC2; F=5.43, R^2=0.14, p=0.019).

2.5.2 Root Metabolic Fingerprinting

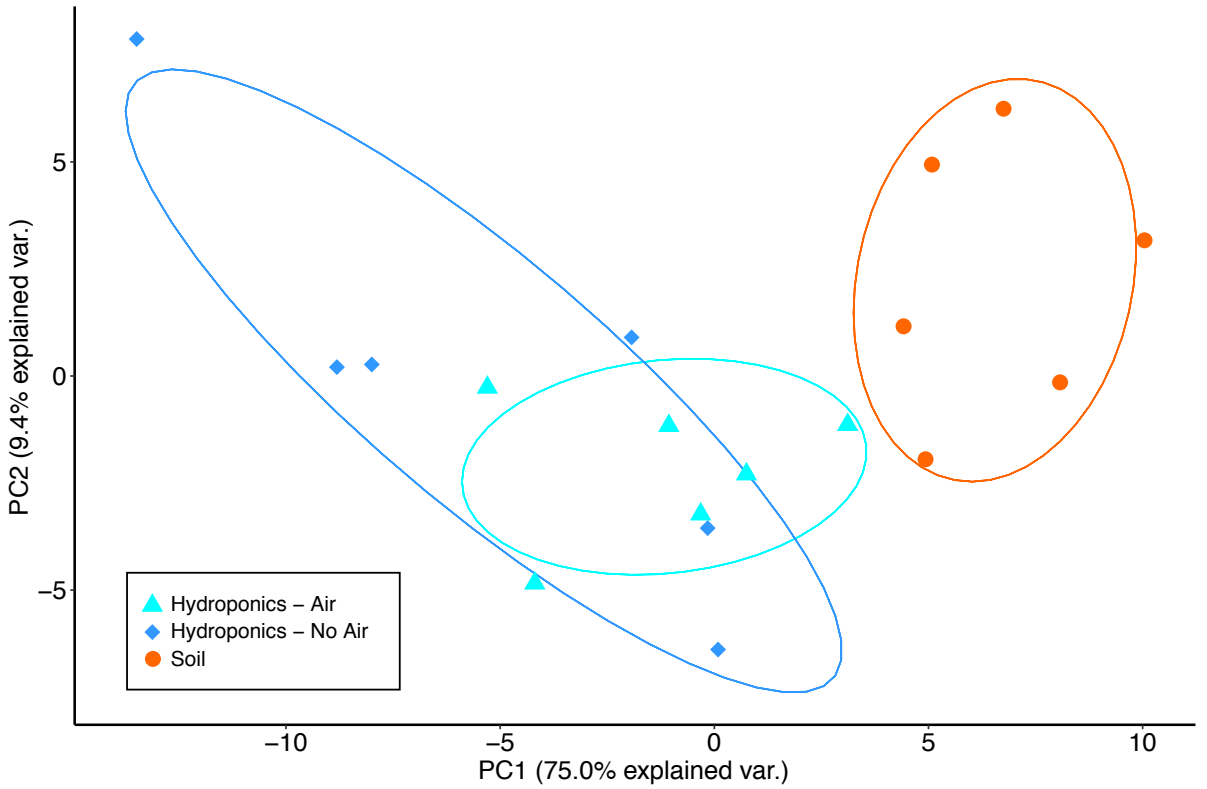

Figure 5: Ordination analysis using Principal Components of barley whole-root ATR-FTIR, showing the first two axes, explaining 82.4% of total variation within the ATR-FTIR data collected from samples of freeze-dried and ground barley whole root systems for the three treatments Hydroponics - Air, Soil, and Hydroponics - No Air. Ellipses denote 95% Confidence Intervals.

When examined using Principal Components Analysis the chemical composition of freeze-dried, powdered whole roots (measured using ATR-FTIR) shows a high value of explained variance on the first two axes, as determined by Horns Parallel Analysis for Component Retention (Figure 5). All three treatments were significantly different from one another, with the strongest difference being the chemical composition of roots grown in soil compared with the hydroponics treatments (Soil vs Hydroponics - No Air: F=25.43, R^2=0.72, p_{adj}=0.008; Soil vs Hydroponics - Air: F=18.68, R^2=0.65, p_{adj}=0.006). These differences appeared to be driven by PC1. The hydroponics treatments were not significantly different from one another, although there was higher variation in the No Air treatment than the other two treatments (F=2.40, R^2=0.19, p=0.139). A PLSDA model built after 999 permutations showed that Component 1 was able to distinguish Soil vs Others (AUC=1.000, p-value=0.0007) and Hydroponics - No Air vs Others (AUC=0.8611, p-value= 0.0149), but not Hydroponics - Air vs Others (AUC=0.6389 p-value=0.3490). Wavenumbers identified by Sparse PLSDA as driving this separation are every measurement taken in the 1028-958cm^{-1} region. Using previously defined labels from Schmidt and Flemming (1998) for the broad biological molecule categories that regions of the IR spectrum fall into shows that there is a clear shift in the type of saccharide present between whole-root tissue of soil systems when compared to the hydroponic treatments.

Optical Photothermal spectroscopy using mIRage revealed differences between groups that were slightly different to that of the traits and the ATR FTIR data (Figure 6). When evaluated using PCA and pairwise multilevel ANOVA, there was a significant separation between ellipses for the Soil and Hydroponics – No Air treatments along PC1 (F=5.66, R^2=0.32, p=0.003), but no difference between Soil and Hydroponics – Air. The hydroponics treatments were marginally significantly different from one another, again splitting along PC1 (F=4.49, R^2=0.27, p=0.026).

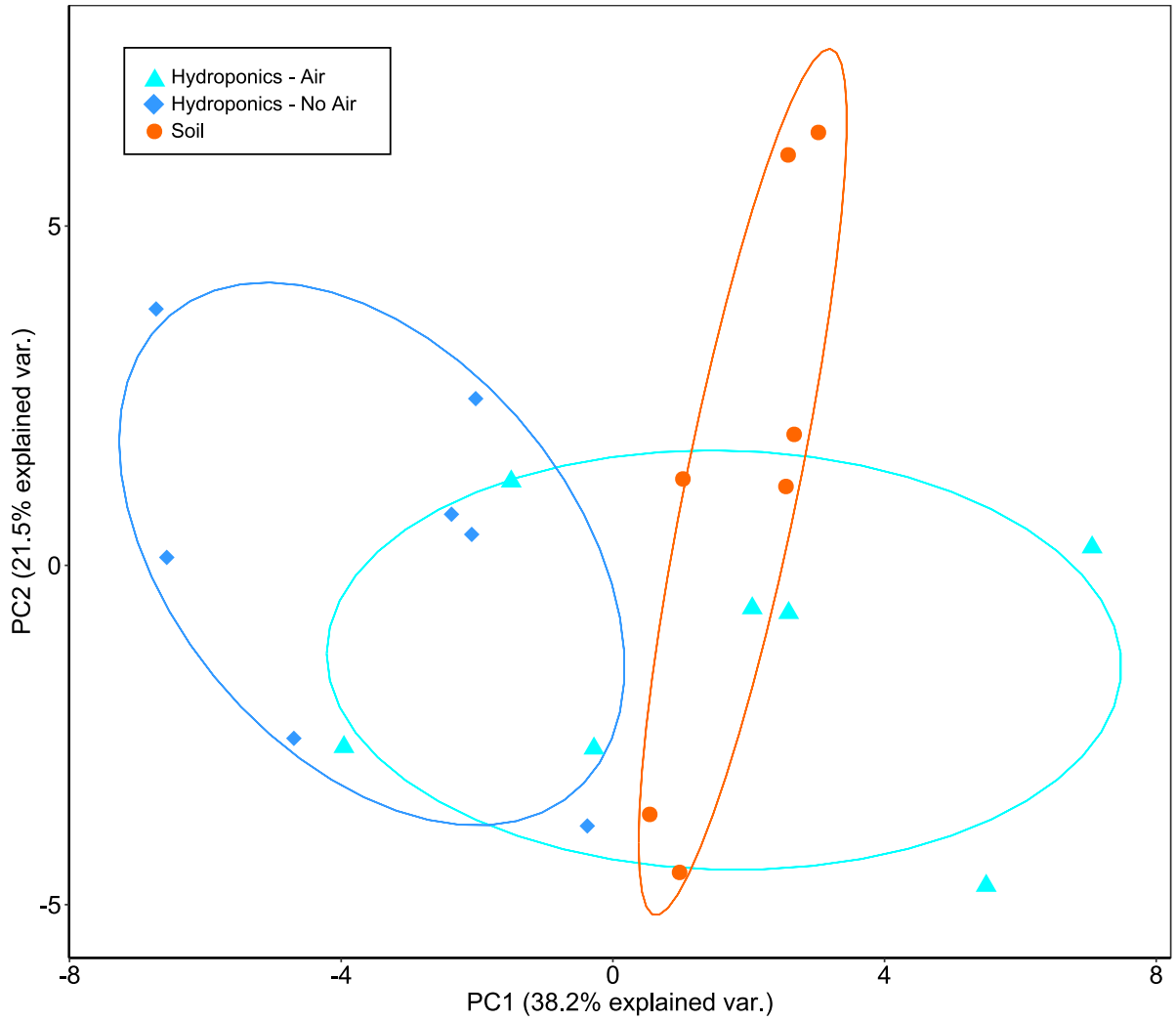

Figure 6: Principal Components Analysis of optical photothermal microscopy measurements on exterior cells of root systems. The first two axes account for 59.7% of total variation. Ellipses represent 95% confidence intervals.

2.5.3 Root Exudate Analysis

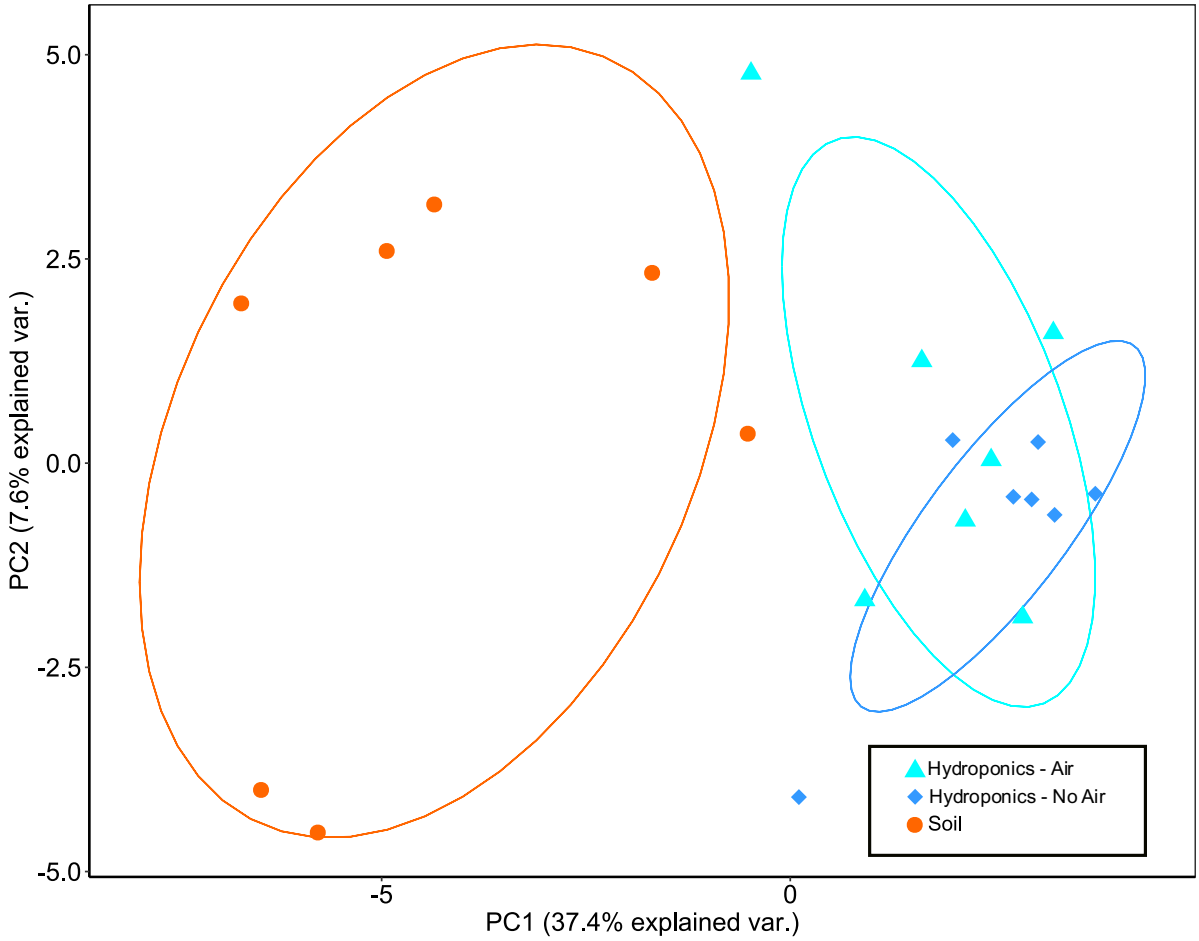

Figure 7: Principal Components Analysis of root exudates measured using gas chromatography- mass spectrometry (GC-MS). Ellipses denote 95% Confidence Intervals.

The root exudate exo-metabolome was significantly different between soil and hydroponic systems when measured with GC-MS, with few differences between Hydroponics - Air and Hydroponics - No Air. There was clear separation between soil and hydroponic growth (Fig. 7), and using pairwise multilevel ANOVA analysis showed that soil and the Hydroponics – Air and Hydroponics – No Air systems separate along PC1 ($F=5.61$, $R^2=0.32$, p_{adj} 0.006; $F=8.10$, $R^2=0.40$, $p_{adj}=0.006$;). The hydroponics treatments were not significantly different from one another ($F=0.54$, $R^2=0.04$, $p_{adj}=0.864$). Supplementary Figure 2 shows the clustering of QC samples prior to analysis.

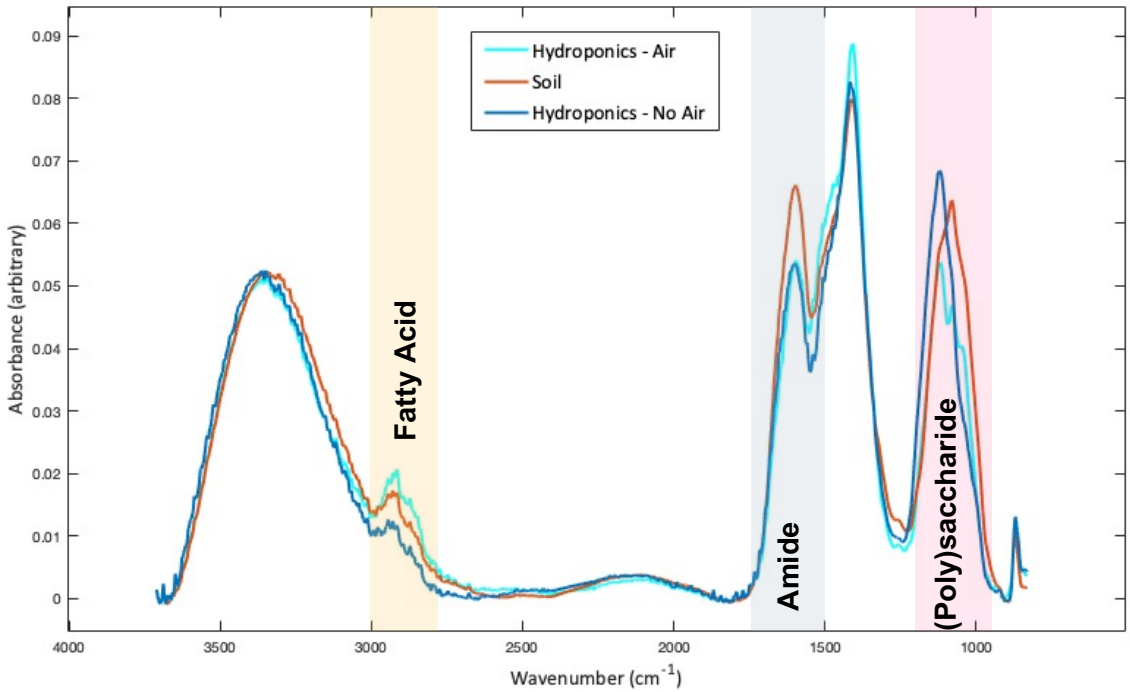

Figure 8: Spectra derived from FTIR analysis of root exudates from the three treatments from 4000-600nm. Each line represents the average spectra collected from each treatment. Overlays highlight regions containing bond types in the fatty acid, (poly)saccharide, amide, and mixed regions as defined by Schmidt and Flemming (1998).

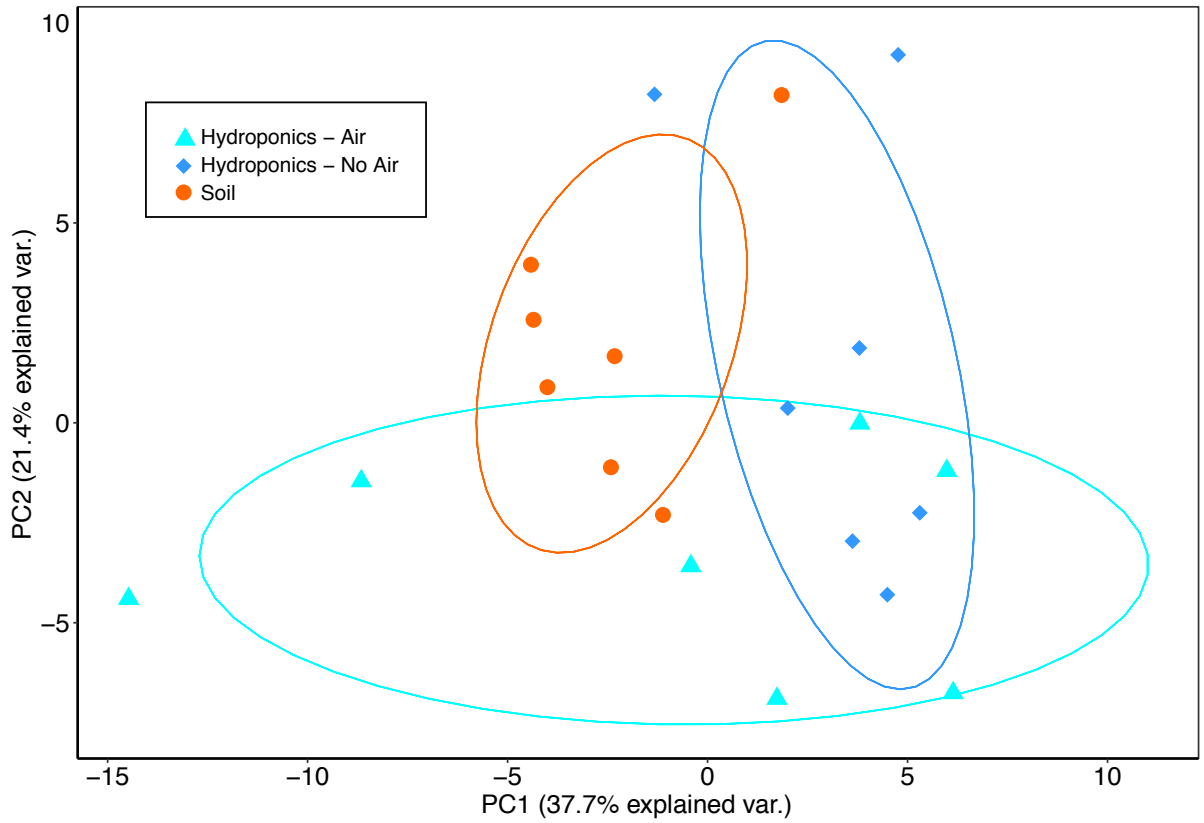

Figure 9: Principal Component Analysis of barley root exudate FTIR data. Each point represents one exudate sample, with four technical replicates averaged per individual sample to prevent analytical artifacts caused by the high-throughput measurement technique. Each exudate sample was a pooled sample of the exudates collected from four barley plants. Ellipses denote 95% Confidence Intervals.

Root exudate profile is also partially distinguishable by metabolic fingerprinting through FTIR. Processed average spectra are shown in Figure 8. PCA shows some separation between groups (Fig. 9), with pairwise multilevel ANOVA showing the Soil samples being significantly different from the Hydroponics – No Air treatment ($F=4.82$, $R^2=0.27$, $p_{adj}=0.006$), but the other pairs not differing significantly (Soil vs Hydroponics – Air $F=1.63$, $R^2=0.11$, $p_{adj}=0.408$; Hydroponics – Air vs Hydroponics – No Air $F=1.42$, $R^2=0.11$, $p_{adj}=0.408$). The PLSDA model that was built after 999 permutations showed that Component 1 was able to distinguish Soil vs Others (AUC=0.8980, p-value=0.003619) and Hydroponics - No Air vs Others (AUC=0.8571, p-value 0.009023), but not Hydroponics - Air vs Others (AUC=0.5408 p-value=0.765400). Sparse PLSDA identified a number of wavenumbers that were driving this separation, which

was every measurement taken in the 1015-968cm^{-1} region. Using previously defined labels for the broad biological molecule categories that regions of the IR spectrum fall into (Schmidt and Flemming, 1998; placed as an overlay on Figure 8), shows that there is a clear shift in the type of saccharide present in the exudates of soil systems when compared to the hydroponic treatments.

2.6 Discussion

This work aimed to illustrate that hydroponic and soil-based growth systems are not comparable, using barley as a model species. We hypothesised that these changes would be evident in root morphological and architectural traits, root tissue metabolites, and root exudates. Here we have shown that plants substantially reorganise their root systems under different growth systems, and have different root metabolic fingerprints, and also chemically distinct root exudates. While we cannot disentangle whether the effects seen on root metabolites and root exudates form a cascade of effects based on the shifts in architecture, or directly from the growth system itself, our results show that hydroponics are not soil, and do not form an adequate proxy for soil.

The first hypothesis considered the root architectural and morphological traits of the barley. We expected that there would be a significant difference in root traits between the plants grown in hydroponics alone, and those grown in the soil-hydroponic system, due to the absence of a physical substrate (e.g. Neumann et al., 2009; Petriacq et al., 2016; Kerstens et al., 2021; reviewed excellently in Chen et al*.,* 2015). We inferred that plants grown in hydroponics alone would show traits associated with less acquisitive strategies such as less branching. This expectation was based on the idea that there is less requirement for active foraging in an aqueous growth solution than soil; for example, root hairs, an acquisitive trait, have been shown to be redundant in hydroponics (Burke et al., 2021). Our findings supported those of Burke and colleagues, and our hypotheses: architectural traits were significantly more acquisitive in the soil treatment when compared with the two hydroponic solutions with an increase in branching, tips, volume, RLD, and RSA; all architectural traits associated with foraging and exploration. Therefore the primary differentiating factor between soil and

hydroponic treatments in our root trait dataset is along what we term a “foraging” axis. As being in hydroponics did not alter morphological traits in root systems, the results are not consistent with the second part of the trait hypothesis. However, the primary differentiators between the two hydroponics treatments were in an orthogonal direction to between hydroponics and soil, along a “structural” axis composed of morphological traits such as SRL and RDMC. Whilst architectural traits are different ways of assembling structures into a complete root system, morphological traits describe the individual building blocks of a root system and are stable enough that they have often been used as a differentiating factor and point of comparison between species (Albert et al, 2011; Mudrák et al., 2019), so to have significantly impacted them is illustrative of how hydroponic environments yield atypical results in root systems.

In our second hypothesis we expected a shift in the metabolic signature of the root tissue itself as a result of the growth mode. ATR-FTIR results showed a clear difference between Soil and Hydroponic treatments, with wavenumbers driving this difference mostly identified as being in the 1028-958cm^{-1} range. Using Schmidt and Flemming (1998) to define biological molecule categories that regions of the IR spectrum fall into shows that there is a clear shift in the type of (poly)saccharide present between whole-root tissue of soil systems when compared to the hydroponic treatments. The results of the optical photothermal spectroscopy using mIRage were less clear. Here, instead of a whole-root metabolic fingerprint, spectra were taken from a single epidermal cell from a lateral root. The treatments clustered differently to the ATR-FTIR data, with separation between Soil and Hydroponics - No Air, but not between Soil and Hydroponics – Air. In our study it is difficult to disentangle whether the results we have seen are a direct effect of the growth solution or of the shift in root traits. However, previous work has shown that tissue chemistry is related to root order; higher root orders are finer and more branched, and become more nitrogen rich (Pregitzer et al., 2002; Sasse et al., 2019) and lower in carbon and cellulose concentration (Guo et al., 2004). Given that this work shows that the Soil treatment has significantly more branching of the root system than the two hydroponics treatments, it is possible that the relative enrichment in the (poly)saccharide region in the hydroponics is due to the reduction in cellulose concentration as roots in the Soil treatment branch more. Given that the nutrient composition of the growth media should be roughly proportional between the soil and

hydroponic treatments as it is derived from the same source, it therefore could be hypothesised that this chemical difference is due to the plants making their choice through developmental and chemical plasticity.

Finally, we hypothesised that due to the lack of need for foraging and difficulties recruiting a microbial community in solution, hybrid soil-hydroponic systems with a repair phase would show substantially different exudate metabolomic profiles compared to lifetime hydroponic systems, with quantifiable changes in chemical composition and bond type. Hydroponic and soil-grown barley have different root exudation chemistries. Despite growing in media from the same source – either soil or a liquid extract of that soil - the metabolomic fingerprint of the root exudates is distinct between hydroponics and soil. Our GC-MS results show significant differences between the soil and two hydroponic treatments, but unfortunately the features driving these differences could not be identified or annotated. The FTIR absorbance data show a difference in the abundance of the types of bonds present in the exudates collected from soil, indicating that there is a substantial and material difference between the constituents of the root exudates of the different treatments. The particular wavenumbers highlighted as driving the differences between soil and the other treatments in our analyses in the poly(saccharide) section of a biological FTIR spectrum as defined by Schmidt and Flemming (1998) have been previously shown in pure chemical and plant-derived samples (Nikoneko et al., 2000, Nikoneko et al., 2005) to be associated with glycosidic linkages between sugar monomers. This indicates that Soil treatment exudate samples have different kinds of oligosaccharides. This is intriguing, because as well as being common plant starches, there are a number of cases where inter-kingdom plant-microbe signals have been shown to be oligosaccharides, such as elicitors of plant defence (Limpens et al., 2015). However, many oligosaccharides are microbe-derived (such as lipo-chito-oligosaccharides released by Arbuscular Mychorrizal Fungi; e.g. Maillet et al. (2011)) so it is plausible that this exudate collection method is capturing differences in microbial exudates rather than that of the plant – although it is likely that a lower proportion of our sample would be microbe-derived than in other methods such as soil column washing for the production of a leachate sample would contain.

Because our results show a significant chemical difference in root exudates between plants grown hydroponically or in soil, even when germinated in identical, soil-based conditions, this

indicates a degree of environment-dependent response and control in root exudation. These differences between soil and hydroponics cannot be explained simply by differences in root system aeration, as in most cases aerated and non-aerated hydroponics differed less from each other than from plants grown in soil. That differences in metabolome and metabolic fingerprint are robust to a hydroponic repair stage indicates that this method may be suitable for analysis of abiotic stress responses is plant-soil interactions. It also suggests that root exudation may be developmentally coded; since root identity, development, morphogenesis, and surface formation have genetic controls conserved across all land plants (Sarkar et al., 2007; Willmann and Poethig, 2007; Wang et al., 1997; Honkanen et al, 2016 respectively) it is reasonable to hypothesise that there is at least a partially conserved genetic pathway for control of root exudation. Further work could examine genetic controls on this trait, possibly by exploiting genetic variations between barley landraces.

2.6.4 Conclusion

Here we have shown that a hybrid growth system involving growth in soil followed by a hydroponic repair stage, similar to that described in Williams et al. (2021b), produces vastly different root system architectures and root- and exudate-chemistry compared to that of a purely hydroponic system. Given that realistic assessments of plant inputs to soil are critical in order to discern the role of specific metabolites in rhizosphere processes and the consequences on system carbon cycling, the most realistic growth systems feasible for understanding plant inputs to soil are required. Here we have shown that a homogenous, liquid environment, absent the microbial and physical context of the soil, does not give the same cues for exudate production as plants grown in soil, a difference robust to the hydroponic repair stage seen in a hybrid growth method. Hydroponic systems also remove the need for foraging, which results in a fundamentally altered root system architecture when compared to plants grown in soil. Applications of this new method could include using it to study abiotic stresses that are challenging to create in a hydroponic environment, such as drought. Future work within soil ecology must consider soil-hydroponic differences and focus on soil-based systems.

2.7 Acknowledgements

R.M. was supported by a Biotechnology and Biological Sciences (BBSRC) Doctoral Training Partnership (reference 1792130), which also funded this work. F.T.d.V. was funded by a BBSRC David Phillips Fellowship (BB/L02456X/1). Thank you to Neelam Notay for assistance with watering and root washing.

2.8 References

Adusumilli, Ravali, and Parag Mallick. n.d. “Chapter 23 Data Conversion with ProteoWizard MsConvert.” *Methods in Molecular Biology* 1550. Accessed October 31, 2021. https://doi.org/10.1007/978-1-4939-6747-6_23.

Albert, Cécile H., Fabrice Grassein, Frank M. Schurr, Ghislain Vieilledent, and Cyrille Violle. 2011. “When and How Should Intraspecific Variability Be Considered in Trait-Based Plant Ecology?” *Perspectives in Plant Ecology, Evolution and Systematics*. Urban & Fischer. https://doi.org/10.1016/j.ppees.2011.04.003.

Albert, Cécile H., Fabrice Grassein, Frank M. Schurr, Ghislain Vieilledent, and Cyrille Violle. 2011. “When and How Should Intraspecific Variability Be Considered in Trait-Based Plant Ecology?” *Perspectives in Plant Ecology, Evolution and Systematics* 13 (3). Urban & Fischer:217–25. https://doi.org/10.1016/J.PPEES.2011.04.003.

Aulakh, M. S., R. Wassmann, C. Bueno, J. Kreuzwieser, and H. Rennenberg. 2004. “Characterization of Root Exudates at Different Growth Stages of Ten Rice (Oryza Sativa L.) Cultivars.” *Plant Biology* 3 (02). Georg Thieme Verlag Stuttgart ·New York:139–48. https://doi.org/10.1055/S-2001-12905.

Bais, Harsh P., Tiffany L. Weir, Laura G. Perry, Simon Gilroy, and Jorge M. Vivanco. 2006. "The Role of Root Exudates in Rhizosphere Interactions With Plants and Other Organisms." *Annual Review of Plant Biology* 57 (1):233–66. https://doi.org/10.1146/annurev.arplant.57.032905.105159.

Bardgett, Richard D., and David A. Wardle. 2010. *Aboveground-Belowground Linkages: Biotic Interactions, Ecosystem Processes ... - Richard D. Bardgett, David A. Wardle – Oxford University Press*

Bardgett, Richard D., Liesje Mommer, and Franciska T. De Vries. 2014. "Going Underground: Root Traits as Drivers of Ecosystem Processes." *Trends in Ecology & Evolution* 29 (12). Elsevier Current Trends:692–99. https://doi.org/10.1016/J.TREE.2014.10.006.

Bergmann, Joana, Alexandra Weigelt, Fons van der Plas, Daniel C. Laughlin, Thom W. Kuyper, Nathaly Guerrero-Ramirez, Oscar J. Valverde-Barrantes, et al. 2020. "The Fungal Collaboration Gradient Dominates the Root Economics Space in Plants." *Science Advances* 6 (27). eaba3756. https://doi.org/10.1126/SCIADV.ABA3756.

Blagodatskaya E, Kuzyakov Y. 2008. Mechanisms of real and apparent priming effects and their dependence on soil microbial biomass and community structure: critical review. Biology and Fertility of Soils 45: 115–131.

Blagodatskaya, E., and Y. Kuzyakov. 2008. "Mechanisms of Real and Apparent Priming Effects and Their Dependence on Soil Microbial Biomass and Community Structure: Critical Review." *Biology and Fertility of Soils 2008 45:2* 45 (2). Springer:115–31. https://doi.org/10.1007/S00374-008-0334-Y.

Bonkowski, Michael. 2004. "Protozoa and Plant Growth: The Microbial Loop in Soil Revisited." *New Phytologist* 162 (3):617–31. https://doi.org/10.1111/j.1469-8137.2004.01066.x.

Burke, Sadaune E, Rognon L, Fontana A, Jourdrin M, and Fricke W. 2021. "A Redundant Hydraulic Function of Root Hairs in Barley Plants Grown in Hydroponics." *Functional*

Plant Biology : FPB 48 (4). Funct Plant Biol:448–59. https://doi.org/10.1071/FP20287.

Burke, Shannon, Emma Sadaune, Lisa Rognon, Alexane Fontana, Marianne Jourdrin, and Wieland Fricke. 2021. "A Redundant Hydraulic Function of Root Hairs in Barley Plants Grown in Hydroponics." *Functional Plant Biology* 48 (4). Funct Plant Biol:448–59. https://doi.org/10.1071/FP20287.

Canarini, Alberto, Andrew Merchant, and Feike A. Dijkstra. 2016. "Drought Effects on Helianthus Annuus and Glycine Max Metabolites: From Phloem to Root Exudates." *Rhizosphere* 2 (December). Elsevier:85–97. https://doi.org/10.1016/j.rhisph.2016.06.003.

Chen, Ying Long, Ivica Djalovic, and Zed Rengel. 2015. "Phenotyping for Root Traits." *Phenomics in Crop Plants: Trends, Options and Limitations*, January. Springer, New Delhi, 101–28. https://doi.org/10.1007/978-81-322-2226-2_8.

De Vries, Franciska T., Alex Williams, Fiona Stringer, Robert Willcocks, Rosie McEwing, Holly Langridge, and Angela L. Straathof. 2019. "Changes in Root-Exudate-Induced Respiration Reveal a Novel Mechanism through Which Drought Affects Ecosystem Carbon Cycling." *New Phytologist* 224 (1). John Wiley & Sons, Ltd:132–45. https://doi.org/10.1111/nph.16001.

Deak, Karen I., and Jocelyn Malamy. 2005. "Osmotic Regulation of Root System Architecture." *The Plant Journal* 43 (1). John Wiley & Sons, Ltd:17–28. https://doi.org/10.1111/J.1365-313X.2005.02425.X.

Domingo-Almenara, Xavier, Jesus Brezmes, Maria Vinaixa, Sara Samino, Noelia Ramirez, Marta Ramon-Krauel, Carles Lerin, et al. 2016. "ERah: A Computational Tool Integrating Spectral Deconvolution and Alignment with Quantification and Identification of Metabolites in GC/MS-Based Metabolomics." *Analytical Chemistry* 88 (19). American Chemical Society:9821–29. https://doi.org/10.1021/ACS.ANALCHEM.6B02927.

Drigo, Barbara, George A. Kowalchuk, and Johannes A. van Veen. 2008. “Climate Change Goes Underground: Effects of Elevated Atmospheric CO2 on Microbial Community Structure and Activities in the Rhizosphere.” *Biology and Fertility of Soils 2008 44:5* 44 (5). Springer:667–79. https://doi.org/10.1007/S00374-008-0277-3.

Dunn, Warwick B, David Broadhurst, Paul Begley, Eva Zelena, Sue Francis-McIntyre, Nadine Anderson, Marie Brown, et al. 2011. “Procedures for Large-Scale Metabolic Profiling of Serum and Plasma Using Gas Chromatography and Liquid Chromatography Coupled to Mass Spectrometry.” *Nature Protocols 2011 6:7* 6 (7). Nature Publishing Group:1060–83. https://doi.org/10.1038/nprot.2011.335.

El-Deeba, Mona. M., Mohamed N. El-Awady, Mahmoud M. Hegazi, Fathy A. Abdel-Azeem, and Mahmoud M. El-Bourdiny. 2009. “ENGINEERING FACTORS AFFECTING HYDROPONICS GRASS- FODDER PRODUCTION.” *Misr Journal of Agricultural Engineering* 26 (3). Misr Society of Agricultural Engineering (MSAE), Egypt:1647–66. https://doi.org/10.21608/MJAE.2009.108766.

Fry, Ellen L., Joanna Savage, Amy L. Hall, Simon Oakley, W. J. Pritchard, Nicholas J. Ostle, Richard F. Pywell, James M. Bullock, and Richard D. Bardgett. 2018. “Soil Multifunctionality and Drought Resistance Are Determined by Plant Structural Traits in Restoring Grassland.” *Ecology* 99 (10). John Wiley & Sons, Ltd:2260–71. https://doi.org/10.1002/ECY.2437.

Fry, Ellen L., Jonathan R. De Long, Lucía Álvarez Garrido, Nil Alvarez, Yolima Carrillo, Laura Castañeda-Gómez, Mathilde Chomel, et al. 2019. “Using Plant, Microbe, and Soil Fauna Traits to Improve the Predictive Power of Biogeochemical Models.” *Methods in Ecology and Evolution* 10 (1). John Wiley & Sons, Ltd:146–57. https://doi.org/10.1111/2041-210X.13092.

Galloway, Andrew F., Jumana Akhtar, Susan E. Marcus, Nathan Fletcher, Katie Field, and Paul Knox. 2020. “Cereal Root Exudates Contain Highly Structurally Complex Polysaccharides with Soil-Binding Properties.” *The Plant Journal* 103 (5). John Wiley & Sons, Ltd:1666–78. https://doi.org/10.1111/TPJ.14852.

García, J. A. Lucas, C. Barbas, A. Probanza, M. L. Barrientos, and F. J. Gutierrez Mañero. 2001. “Low Molecular Weight Organic Acids and Fatty Acids in Root Exudates of Two Lupinus Cultivars at Flowering and Fruiting Stages.” *Phytochemical Analysis* 12 (5). John Wiley & Sons, Ltd:305–11. https://doi.org/10.1002/PCA.596.

Guo, Dali L., Robert J. Mitchell, and Joseph J. Hendricks. 2004. “Fine Root Branch Orders Respond Differentially to Carbon Source-Sink Manipulations in a Longleaf Pine Forest.” *Oecologia* 140 (3). Springer Verlag:450–57. https://doi.org/10.1007/s00442-004-1596-1.

Guyonnet, Julien P., Amélie A. M. Cantarel, Laurent Simon, and Feth el Zahar Haichar. 2018. “Root Exudation Rate as Functional Trait Involved in Plant Nutrient-Use Strategy Classification.” *Ecology and Evolution* 8 (16). John Wiley & Sons, Ltd:8573–81. https://doi.org/10.1002/ECE3.4383.

Hackett, C 1969. “A study of the root system of barley.” *New Phytologist* 68 (4). John Wiley & Sons, Ltd:1023–30. https://doi.org/10.1111/J.1469-8137.1969.TB06502.X.

Hagan, Donald L., Shibu Jose, and Chung-Ho Lin. 2013. “Allelopathic Exudates of Cogongrass (Imperata Cylindrica): Implications for the Performance of Native Pine Savanna Plant Species in the Southeastern US.” *Journal of Chemical Ecology 2013 39:2* 39 (2). Springer:312–22. https://doi.org/10.1007/S10886-013-0241-Z.

Hannula, S. E., W. de Boer, and J. A. van Veen. 2010. “In Situ Dynamics of Soil Fungal Communities under Different Genotypes of Potato, Including a Genetically Modified Cultivar.” *Soil Biology and Biochemistry* 42 (12). Pergamon:2211–23. https://doi.org/10.1016/J.SOILBIO.2010.08.020.

Herrera Paredes, Sur, Tianxiang Gao, Theresa F. Law, Omri M. Finkel, Tatiana Mucyn, Paulo José Pereira Lima Teixeira, Isaí Salas González, et al. 2018. “Design of Synthetic Bacterial Communities for Predictable Plant Phenotypes.” *PLoS Biology* 16 (2). Public Library of Science. https://doi.org/10.1371/journal.pbio.2003962.

Honkanen, Suvi, Victor A.S. Jones, Giulia Morieri, Clement Champion, Alexander J. Hetherington, Steve Kelly, Hélène Proust, Denis Saint-Marcoux, Helen Prescott, and Liam Dolan. 2016. “The Mechanism Forming the Cell Surface of Tip-Growing Rooting Cells Is Conserved among Land Plants.” *Current Biology*, 1–7. https://doi.org/10.1016/j.cub.2016.09.062.

Johnson, David, Ian C. Anderson, Alison Williams, Raj Whitlock, and J. Philip Grime. 2010. “Plant Genotypic Diversity Does Not Beget Root-Fungal Species Diversity.” *Plant and Soil* 336 (1):107–11. https://doi.org/10.1007/s11104-010-0452-9.

Kerstens, Merijn, Vera Hesen, Kavya Yalamanchili, Andrea Bimbo, Stephen Grigg, Davy Opdenacker, Tom Beeckman, Renze Heidstra, and Viola Willemsen. 2021. “Nature and Nurture: Genotype-Dependent Differential Responses of Root Architecture to Agar and Soil Environments.” *Genes 2021, Vol. 12, Page 1028* 12 (7). Multidisciplinary Digital Publishing Institute:1028. https://doi.org/10.3390/GENES12071028.

Kover, Paula X., William Valdar, Joseph Trakalo, Nora Scarcelli, Ian M. Ehrenreich, Michael D. Purugganan, Caroline Durrant, and Richard Mott. 2009. “A Multiparent Advanced Generation Inter-Cross to Fine-Map Quantitative Traits in Arabidopsis Thaliana.” *PLOS Genetics* 5 (7). Public Library of Science:e1000551. https://doi.org/10.1371/JOURNAL.PGEN.1000551.

Kover, Paula X., William Valdar, Joseph Trakalo, Nora Scarcelli, Ian M. Ehrenreich, Michael D. Purugganan, Caroline Durrant, and Richard Mott. 2009. “A Multiparent Advanced Generation Inter-Cross to Fine-Map Quantitative Traits in Arabidopsis Thaliana.” *PLOS Genetics* 5 (7). Public Library of Science:e1000551. https://doi.org/10.1371/JOURNAL.PGEN.1000551.

Langridge, Peter. 2018. “Economic and Academic Importance of Barley.” Springer, Cham, 1–10. https://doi.org/10.1007/978-3-319-92528-8_1.

Lau, J, and J Lennon. 2011. “Evolutionary Ecology of Plant-Microbe Interactions: Soil Microbial Structure Alters Selection on Plant Traits.” *New Phytologist* 192 (1):215–24. https://doi.org/10.1111/j.1469-8137.2011.03790.x.

Maillet, Fabienne, Véréna Poinsot, Olivier André, Virginie Puech-Pagés, Alexandra Haouy, Monique Gueunier, Laurence Cromer, et al. 2011. “Fungal Lipochitooligosaccharide Symbiotic Signals in Arbuscular Mycorrhiza.” *Nature* 469 (7328). Nature Publishing Group:58–64. https://doi.org/10.1038/nature09622.

Martinez Arbizu, P.. pairwiseAdonis: Pairwise multilevel comparison using adonis. 2020 R package version 0.4

MATLAB, 2018. *9.7.0.1190202 (R2019b)*, Natick, Massachusetts: The MathWorks Inc.

Mavrodi, Olga V., Janiece R. McWilliams, Jacob O. Peter, Anna Berim, Karl A. Hassan, Liam D. H. Elbourne, Melissa K. LeTourneau, et al. 2021. “Root Exudates Alter the Expression of Diverse Metabolic, Transport, Regulatory, and Stress Response Genes in Rhizosphere Pseudomonas.” *Frontiers in Microbiology* 12 (April). Frontiers Media SA. https://doi.org/10.3389/FMICB.2021.651282.

Mönchgesang, Susann, Nadine Strehmel, Diana Trutschel, Lore Westphal, Steffen Neumann, and Dierk Scheel. 2016. “Plant-to-Plant Variability in Root Metabolite Profiles of 19 Arabidopsis Thaliana Accessions Is Substance-Class-Dependent.” *International Journal of Molecular Sciences* 17 (9):1565. https://doi.org/10.3390/ijms17091565.

Mönchgesang, Susann, Nadine Strehmel, Stephan Schmidt, Lore Westphal, Franziska Taruttis, Erik Müller, Siska Herklotz, Steffen Neumann, and Dierk Scheel. 2016. “Natural Variation of Root Exudates in Arabidopsis Thaliana-Linking Metabolomic and Genomic Data.” *Scientific Reports* 6 (February). Nature Publishing Group:29033. https://doi.org/10.1038/srep29033.

Mönchgesang, Susann, Nadine Strehmel, Stephan Schmidt, Lore Westphal, Franziska Taruttis, Erik Müller, Siska Herklotz, Steffen Neumann, and Dierk Scheel. 2016.

"Natural Variation of Root Exudates in Arabidopsis Thaliana-Linking Metabolomic and Genomic Data." *Scientific Reports* 6 (February). Nature Publishing Group:29033. https://doi.org/10.1038/srep29033.

Mudrák, Ondřej, Jiří Doležal, Alena Vítová, and Jan Lepš. 2019. "Variation in Plant Functional Traits Is Best Explained by the Species Identity: Stability of Trait-Based Species Ranking across Meadow Management Regimes." *Functional Ecology* 33 (4). John Wiley & Sons, Ltd:746–55. https://doi.org/10.1111/1365-2435.13287.

Mudrák, Ondřej, Jiří Doležal, Alena Vítová, and Jan Lepš. 2019. "Variation in Plant Functional Traits Is Best Explained by the Species Identity: Stability of Trait-Based Species Ranking across Meadow Management Regimes." *Functional Ecology* 33 (4). John Wiley & Sons, Ltd:746–55. https://doi.org/10.1111/1365-2435.13287.

Neumann, Günter, Timothy S. George, and Claude Plassard. 2009. "Strategies and Methods for Studying the Rhizosphere—the Plant Science Toolbox." *Plant and Soil 2009 321:1* 321 (1). Springer:431–56. https://doi.org/10.1007/S11104-009-9953-9.

Nikonenko, N. A., D. K. Buslov, N. I. Sushko, and R. G. Zhbankov. 2005. "Spectroscopic Manifestation of Stretching Vibrations of Glycosidic Linkage in Polysaccharides." In *Journal of Molecular Structure*, 752:20–24. Elsevier. https://doi.org/10.1016/j.molstruc.2005.05.015.

Oburger, Eva, and David L. Jones. 2018. "Sampling Root Exudates – Mission Impossible?" *Rhizosphere* 6 (June). Elsevier:116–33. https://doi.org/10.1016/j.rhisph.2018.06.004.

Pétriacq, Pierre, Alex Williams, Anne Cotton, Alexander E. McFarlane, Stephen A. Rolfe, and Jurriaan Ton. 2017. "Metabolite Profiling of Non-Sterile Rhizosphere Soil." *The Plant Journal* 92 (1). John Wiley & Sons, Ltd (10.1111):147–62. https://doi.org/10.1111/tpj.13639.

Pétriacq, Pierre, Alex Williams, Anne Cotton, Alexander E. McFarlane, Stephen A. Rolfe, and Jurriaan Ton. 2017. "Metabolite Profiling of Non-Sterile Rhizosphere Soil." *The*

Plant Journal 92 (1). John Wiley & Sons, Ltd (10.1111):147–62. https://doi.org/10.1111/tpj.13639.

Ploschuk, Rocío Antonella, Daniel Julio Miralles, Timothy David Colmer, Edmundo Leonardo Ploschuk, and Gustavo Gabriel Striker. 2018. "Waterlogging of Winter Crops at Early and Late Stages: Impacts on Leaf Physiology, Growth and Yield." *Frontiers in Plant Science* 0. Frontiers:1863. https://doi.org/10.3389/FPLS.2018.01863.

Pregitzer, Kurt S., Jared L. DeForest, Andrew J. Burton, Michael F. Allen, Roger W. Ruess, and Ronald L. Hendrick. 2002. "Fine Root Architecture of Nine North American Trees." *Ecological Monographs* 72 (2):293–309. https://doi.org/10.1890/0012-9615(2002)072[0293:FRAONN]2.0.CO;2.

Roumet, Catherine, Carlos Urcelay, and Sandra Díaz. 2006. "Suites of Root Traits Differ between Annual and Perennial Species Growing in the Field." *New Phytologist* 170 (2). John Wiley & Sons, Ltd:357–68. https://doi.org/10.1111/j.1469-8137.2006.01667.x.

Sarkar, Ananda K., Marijn Luijten, Shunsuke Miyashima, Michael Lenhard, Takashi Hashimoto, Keiji Nakajima, Ben Scheres, Renze Heidstra, and Thomas Laux. 2007. "Conserved Factors Regulate Signalling in Arabidopsis Thaliana Shoot and Root Stem Cell Organizers." *Nature 2007 446:7137* 446 (7137). Nature Publishing Group:811–14. https://doi.org/10.1038/nature05703.

Sasse, Joelle, Josefine Kant, Benjamin J. Cole, Andrew P. Klein, Borjana Arsova, Pascal Schlaepfer, Jian Gao, et al. 2019. "Multilab EcoFAB Study Shows Highly Reproducible Physiology and Depletion of Soil Metabolites by a Model Grass." *New Phytologist* 222 (2). John Wiley & Sons, Ltd:1149–60. https://doi.org/10.1111/NPH.15662.

Schmitt, Jürgen, and Hans Curt Flemming. 1998. "FTIR-Spectroscopy in Microbial and Material Analysis." *International Biodeterioration & Biodegradation* 41 (1). Elsevier:1–11. https://doi.org/10.1016/S0964-8305(98)80002-4.

Ström, L., T. Olsson, and G. Tyler. 1994. “Differences between Calcifuge and Acidifuge Plants in Root Exudation of Low-Molecular Organic Acids.” *Plant and Soil 1994 167:2* 167 (2). Springer:239–45. https://doi.org/10.1007/BF00007950.

Sun, Tao, and Zijun Mao. 2011. “Functional Relationships between Morphology and Respiration of Fine Roots in Two Chinese Temperate Tree Species.” *Plant and Soil 2011 346:1* 346 (1). Springer:375–84. https://doi.org/10.1007/S11104-011-0825-8.

Timmins, Éadaoin M., Susan A. Howell, Bjørn K. Alsberg, William C. Noble, and Royston Goodacre. 1998. “Rapid Differentiation of Closely Related Candida Species and Strains by Pyrolysis-Mass Spectrometry and Fourier Transform-Infrared Spectroscopy.” *Journal of Clinical Microbiology* 36 (2). American Society for Microbiology (ASM):367. /pmc/articles/PMC104544/.

Van Dam, Nicole M., and Harro J. Bouwmeester. 2016. “Metabolomics in the Rhizosphere: Tapping into Belowground Chemical Communication.” *Trends in Plant Science* 21 (3). Elsevier Current Trends:256–65. https://doi.org/10.1016/J.TPLANTS.2016.01.008.

Wang, Haiyang, Susan K Lockwood, Mark F Hoeltzel, and John W Schiefelbein. 1997. “The ROOT HAIR DEFECTIVE3 Gene Encodes an Evolutionarily Conserved Protein with GTP-Binding Motifs and Is Required for Regulated Cell Enlargement in Arabidopsis.” *Genes and Development* 11 (6). Cold Spring Harbor Laboratory Press:799–811. https://doi.org/10.1101/gad.11.6.799.

Whipps, JM. 1990. “Carbon Economy.” *The Rhizosphere* 59. Wiley & Son. http://ci.nii.ac.jp/naid/10025228691/en/.

Williams, Alex, Holly Langridge, Angela L. Straathof, Graeme Fox, Howbeer Muhammadali, Katherine A. Hollywood, Yun Xu, Royston Goodacre, and Franciska T. de Vries. 2021a. “Comparing Root Exudate Collection Techniques: An Improved Hybrid Method.” *Soil Biology and Biochemistry* 161 (October). Pergamon:108391. https://doi.org/10.1016/J.SOILBIO.2021.108391.

Williams, Alex, Holly Langridge, Angela L. Straathof, Howbeer Muhamadali, Katherine A. Hollywood, Royston Goodacre, and Franciska T. de Vries. 2021b. “Root Functional Traits Explain Root Exudation Rate and Composition across a Range of Grassland Species.” *Journal of Ecology* 00. John Wiley & Sons, Ltd:1–13. https://doi.org/10.1111/1365-2745.13630.

Willig, Sidney, Zeno Varanini, and Paolo Nannipieri. 2000. “Types, Amounts, and Possible Functions of Compounds Released into the Rhizosphere by Soil-Grown Plants.” *The Rhizosphere*, November. CRC Press, 35–56. https://doi.org/10.1201/9780849384974-8.

Willmann, Matthew R., and R. Scott Poethig. 2007. “Conservation and Evolution of MiRNA Regulatory Programs in Plant Development.” *Current Opinion in Plant Biology* 10 (5):503–11. https://doi.org/10.1016/j.pbi.2007.07.004.

Zamioudis, Christos, Parthena Mastranesti, Pankaj Dhonukshe, Ikram Blilou, and Corné M J Pieterse. 2013. “Unraveling Root Developmental Programs Initiated by Beneficial Pseudomonas Spp. Bacteria.” *Plant Physiology* 162 (1):304–18. https://doi.org/10.1104/pp.112.212597.

3. Detecting belowground drought effects in *Anthoxanthum odoratum* - a multi-trait approach

Reuben Margerison[1,2], Alex Williams[3] , Cassio Lima[2], Alistair Hanak[1] , Shannon Grimes[1] , Howbeer Muhammadali[2], Jurre Bleeker[5], Ellen L. Fry[6], Giles Johnson[1], Patrick Gallois[4], Royston Goodacre[2] and Franciska T. de Vries[1,6]

[1]School of Natural Sciences, The University of Manchester, Oxford Road, M13 9PT, Manchester, UK

[2]Department of Biochemistry and Systems Biology, Institute of Systems, Molecular and Integrative Biology (ISMIB), University of Liverpool, Biosciences Building, Crown Street, L69 16 7ZB, Liverpool, UK

[3]Biosciences, The University of Sheffield, Western Bank, S10 2TN

[4]School of Biological Sciences, The University of Manchester, Oxford Road, M13 9PT, Manchester, UK

[5]Institute of Biodiversity and Ecosystem Dynamics, University of Amsterdam, PO Box 94240, 20 1090 GE Amsterdam, The Netherlands

[6]Department of Biology, Edge Hill University, St Helens Road, Ormskirk, L39 4AP, Lancashire UK

3.1 Key words

Drought; root exudates; GC-MS; FTIR; root traits; mIRage; metabolomics; hydroponics; exudate collection

3.2 Abstract

Root exudates form the basis of plant communication with the rhizosphere, but historically collection and quantification has involved the use of hydroponic systems. These are problematic because they do not resemble real soil, and presenting abiotic stressors, such as drought, in such systems is challenging; creating a drought treatment in such systems often requires high salinity or chemicals to induce osmotic stress. There has been recent interest in analysis of root exudation responses in soil-based systems. Here we test whether a drought signal was evident in root tissue chemistry and root exudates in the ecologically relevant perennial grass *Anthoxanthum odoratum* using an analytical approach which combines lifetime soil-growth with a post-washing hydroponic repair stage, using established mass-spectrometric methods (GC-MS) to identify precise molecules associated with a drought effect, and a novel application of infra-red spectroscopic analyses (FTIR) to identify broad shifts in molecule type. We showed that architectural root traits were strongly affected by drought while morphological traits were not, indicating possible developmental delay caused by drought. A distinct metabolomic signature of drought was observed in whole root tissue and in exudates, even after a hydroponic root-recovery period. The signal of drought was not evident in total exudates when analysed using GC-MS. This work indicates that FTIR is an appropriate and inexpensive high-throughput method for identifying a drought effect. Future work concerning rhizosphere responses to abiotic stresses should focus on soil-based systems, and should consider spectroscopic analyses as an alternative or additive to spectrometric techniques.

3.3 Introduction

Drought has strong and severe effects on plant growth and development, resulting in smaller plants with reduced root systems and altered physiology and metabolism. This includes a reduction in photosynthetically fixed carbon (reviewed in Pinheiro and Chaves, 2011) and altered root system architecture (RSA; Fry et al., 2018a). Consequently, the primary method by which roots interact with the soil, root exudation, is also thought to change in quantity and quality under drought - but much of this work has been undertaken on partial root systems (Canarini et al., 2016; Gargallo-Garriga et al., 2018). Exudates comprise a range of low molecular weight compounds, released by the plant, that culture a specific rhizosphere microbial community (Briones et al., 2019). Through this, root exudation is a primary driver of soil food web assembly (de Vries and Caruso, 2016), and it is known that soil microbial communities respond to drought through both community reorganisation and shifts in activity (de Vries et al., 2018), which has the potential for cascading effects on ecosystem function and service delivery (Williams and de Vries, 2020). Microbial respiration is strongly influenced by inputs of fresh labile carbon in the form of root exudates. Studies have shown that there is a clear difference in exudate chemistry, demonstrated by the addition of exudates from droughted or non-droughted plants, which elicit contrasting respiration rates (de Vries et al., 2019). However, while these differences have been shown using biological assays, they have not been directly analysed using metabolomic approaches, and so the exact changes in exudate chemistry under drought are currently unknown.

Root trait changes under drought are comparatively well-characterised, but there is a question over whether observed changes towards more 'conservative' resource strategies, e.g. reduced surface area, branching, root tips and so on, constitute a change in resource economics strategy or whether it is merely developmental delay (de Vries et al., 2016). Because plants have a finite amount of carbon, and this is further reduced under drought when the need to conserve water necessitates stomatal closure and reduced photosynthesis, there is likely to be a change towards building and consolidating structures to resist drought, rather than continually foraging for more resources (de Vries et al., 2016; Balachowski & Volaire 2018). Further, creating associations with rhizosphere microbes is also likely to be of

less importance under drought, and lower biomass would mean a smaller sphere of influence for the root system (Preece & Penuelas 2016). Therefore linking traits to tissue chemistry and root exudation metabolomics offers the possibility of determining whether the plant has diverted resources into a different strategy in order to cope with the drought stress. As research continues in the field of root exudates, the need to apply treatments to mimic real world problems such as drought, and examine the metabolomic consequences, is intensifying (de Vries et al., 2019). However, many studies on metabolomics and exudation under drought take place on partial root systems (Delhaize et al., 1993; Gaume et al., 2001; Shi et al 2011), or in hydroponic systems with artificially induced osmotic stress e.g. polyethylene glycol (Puntase et al., 2004; Naveed et al., 2019), which due to highly artificial environment and the profound effect of hydroponics on root development, root chemistry, and exudates (Chapter 2), mean it may not be possible to extrapolate these findings to real-world scenarios. We therefore need to consider more realistic scenarios while also considering the issues that these may bring.

One solution is the introduction of a soil growth phase in drought experiments, which will allow the roots to develop in a physical medium with microbial context. One reason hydroponics is so popular is because collection of root exudates is difficult: in soil there is the likelihood of contamination by microbial signalling chemicals and other soil inorganic chemistry, plus the trauma of removal. However, we have pioneered in other studies (Williams et al., 2021a; 2021b; Chapter 2) the coupling of soil-hydroponic hybrid growth system with GC-MS metabolomics to demonstrate nuanced changes in root exudate metabolites, and the use of infra-red spectroscopy to identify broad shifts in metabolic fingerprint (Chapter 2). Here we aim to apply this method in a novel study that aims to use our exudate collection method to impose realistic drought in the soil phase and potentially detect a signal following a hydroponic repair, combining soil and hydroponic growth strategies to improve root exudate collection in droughted systems without artefacts of stress from lifetime hydroponics. We hypothesise that plant response to drought is reflected in 1) root functional effects traits, 2) whole root chemistry and 3) chemistry of root exudates, and that this is still detectable after a recovery period. Here we aim to show that a combined soil-hydroponic approach with downstream FTIR analysis is able to detect this effect. We tested this hypothesis in a greenhouse study using the perennial grass *Anthoxanthum odoratum*.

This species is ecologically relevant: it is a common constituent of semi-natural temperate grassland and is highly responsive to drought (De Vries et al., 2016).

3.4 Materials and Methods

3.4.1 Experimental design

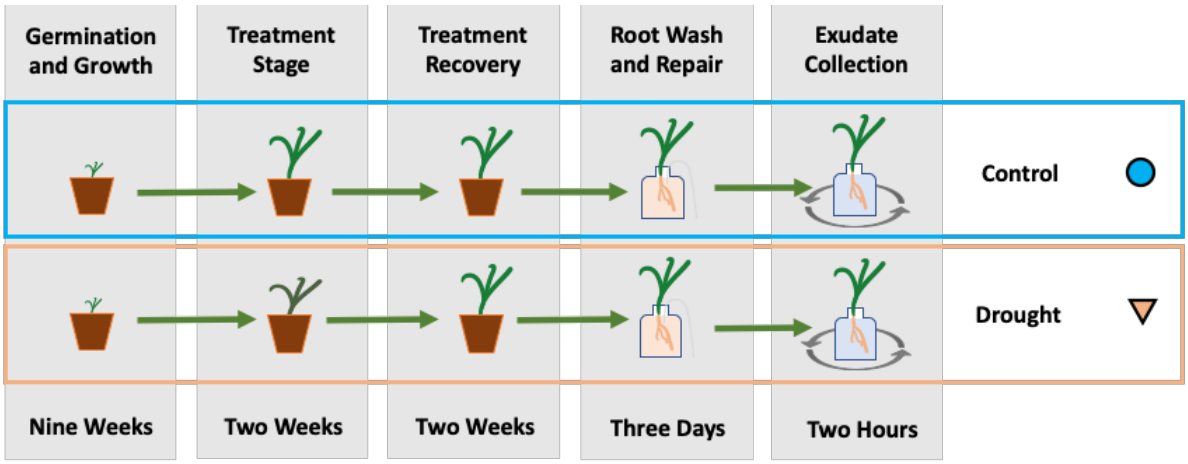

Figure 1: Timeline of experiment showing the two experimental treatments; germination stage lasted for one week, growth stage lasted for eight weeks. The experimental treatment was then applied, either a two week severe drought, or a continuation of the same watering regime. After this, there was a two week treatment recovery period consisting of a return to control conditions for the droughted plants, before the roots were washed and the plants were transferred to hydroponic repair for three days. Whole duration of growth and sample collection was 95 days.

We germinated seed of *Anthoxanthum odoratum* (Emorsgate Seeds, Somerset UK) on potting soil for one week in a growth chamber set to 20°C, 18/6h photoperiod until cotyledon leaves appeared, before being transferred to 10cm diameter pots containing 400g of sand mixed 50/50 v/v with field soil collected from Colt Park (54°11′ 37.1′′N 2°20′ 54.9′′W, altitude 348m), a mesotrophic grassland managed with light grazing and low use of fertiliser, in May 2019. Prior to use the soil was sieved at 4mm and stored at 4°C. Ten pots were maintained at field capacity throughout the experiment and hereafter referred to as the control treatment. The remaining ten would be the drought treatment. We grew seedlings for eight weeks before subjecting the drought treatment to two weeks of severe drought (dried to 30% water holding capacity (WHC)) followed by two weeks of recovery. After twelve weeks total growth, we prepared all plants for exudate collection. We carefully washed the roots clean of all soil

particles and placed the plants into 100ml Schott Duran® bottles (SCHOTT UK Ltd., Stafford, UK) with a soil solution. The plants were suspended with their roots submerged in the soil solution, and the aboveground fraction held clear of the fluid using Azpak non-absorbent cotton wool. The soil solution was created by mixing the same soil as earlier in water at a 1:1 v/v soil/water mix and leaving to settle at 4℃ for three days, before removing the settled soil. The hydroponics were aerated sing a HiDOM HD-603 aquarium pump (Shenzhen Hidom Electric Co., Ltd, Shenzhen, China) set to a flow rate of approximately 1ml s^{-1} .The plants were returned to the growth chamber under the same conditions for three days before harvest (Williams et al., 2021a and Williams et al., 2021b).

3.4.2 Root Analysis

All root systems were placed in a 20% ethanol storage solution at the point of harvest to prevent microbial activity until use. For root analyses, we used five replicates of each treatment for root traits, and five for root chemistry.

3.4.2.1 Root traits

Roots were removed from their storage solution and immersed in tap water to remove ethanol and ease root mass separation, before being transferred to a clear plastic tray on an Epson Expression 11000XL flatbed scanner. The root system was spread out using plastic tweezers to minimise overlap, and scanned at 600dpi. Root images were captured at a resolution of 600 dpi and the images were analysed using the WinRHIZO® pro software (Regent Instruments Inc., Canada). The contents of the scanning tray were filtered with a sieve, blotted dry, and weighed, before being dried in a paper bag at 60° C for 48h and weighed again. This allowed measurement and calculation of architectural root functional traits: root length, number of root tips, root forks and root volume, and morphological traits: specific root length, root tissue density, root dry matter content and root diameter, as well as root surface area.

3.4.1.2 Root chemistry

The preserved root systems used for mIRage analysis were drained of ethanol and rinsed in deionised water. These were then frozen at -80°C and freeze-dried, before being placed in

pre-cooled stainless steel Tissuelyser vessels, and shaken for one minute to break down the cells. The powdered samples were pressed onto the diamond of an Attenuated Total Reflectance (ATR) accessory for the Bruker Invenio S FTIR (Bruker, Billerica, MA, USA) with a pressure applicator to ensure reproducibility between samples. Data were collected in the 4000–400 cm^{-1} range at a resolution of 4 cm^{-1}. The crystal was cleaned with 70% ethanol between samples and a new background measurement was taken before every sample.

3.4.3 Exudate Analysis

3.4.3.1 Exudate Collection

All plants were removed from the hydroponic recovery system, and had their root systems gently immersed into a 1L beaker of deionised water, a further 1L beaker of deionised water, and finally into a 1L beaker of milliQ water to remove soil solution. Plants were suspended with Parafilm M (Bemis Company, Inc. Neenah, WI, USA) in a new, milliQ-rinsed 100mL glass Schott bottle, with their roots immersed in 100mL of milliQ water. These bottles were transferred to an iced cooler on a rotary shaker for two hours, set to 60rpm. Plants were removed from the bottles and roots stored in 20% ethanol for downstream analysis. The aboveground biomass was weighed, before being dried at 60°C for 72 hours, after which we reweighed them. The remaining exudate solution for each sample was filtered using a 0.22µm filter (Merck Millipore (U.K.) Limited, Watford, SLGP033RS) that had been pre-washed with milliQ water and decanted into three 50 ml Falcon Tubes (Greiner Bio-one CellStar 227261), and immediately frozen at -80°C. The frozen exudate solution was freeze-dried using a Scavac CoolSafe 55-9 Pro (LaboGene, Lynge, Denmark) for 48 hours.

3.4.3.2 Exudate Chemistry

For exudate chemistry, three replicates from each treatment were sent to the University of Amsterdam's Institute of Biodiversity and Ecosystem Dynamics for optimisation of the Gas-Chromatography Mass Spectrometer. This meant that seven replicates were available for testing exudate chemistry.

3.4.3.2.1 GC-MS

Samples were positioned randomly on the sample tray, with initial sample injection performed using a PAL3 Series II auto-sampler piloted by MassHunter software (Agilent, Technologies, UK). Pooled quality control samples were run every fifth injection, with blanks at start and end of run. 1 µL of derivatized sample was loaded into a J&W HP-5ms Intuvo column (30 m x 0.25 mm x 0.25 µm) in a 8860 GC (Agilent Technologies, UK), with inlet temperature at 280°C and the injector operated in single overlap mode. The flow rate of the helium carrier gas was 34.2 $mLmin^{-1}$. Injection temperature cycle was 4min at 70°C, before a ramp of 15°$Cmin^{-1}$. until 325°C, with a final 6min hold. In the 5977B series MSD quadrupole mass spectrometer ion generation occurs at the 70 eV electron beam with an ionization current of 35 µA. In the m/z scanning range of 50–550 amu spectra were recorded at 2.91 scans per second. EI ion source was kept at 230°C and the MS QUAD at 150°C for duration. Total run time per sample was 27 min, with a retention time correction applied using the retention index method described in Begley et al. (2009).

3.4.3.2.1 Transmission FTIR

We prepared a 96-well IR plate by washing with 5% Sodium Dodecyl Sulfate (SDS) and rinsing with ethanol and deionised water. We resuspended the freeze-dried root exudate solutions in ultrahigh purity HPLC-grade water, and loaded 20µL into individual wells. We completely dried the plate by heating it at 65°C for 60 minutes, then loaded it onto the motorised high-throughput cassette of a Bruker Invenio S FTIR machine (Bruker, Billerica, MA, USA). We followed the protocol of Winder et al. (2004) to set the correct transmission mode on the FTIR machine, briefly, 4000-600 cm^{-1} range at a resolution of $4cm^{-1}$.

3.4.4 Statistical analysis

To test the first section of my hypothesis, which was that drought response in *Anthoxanthum odoratum* was evident in the functional effects traits of the roots, we conducted a set of Welch's two-sample t-tests on the root traits measured with drought treatment as the explanatory variable. Welch's t-test is appropriate here because it does not assume equal variance for both of the groups tested. This was followed by a Principal Components Analysis, which compressed the variability in the root traits into a reduced set of dimensions, and determined the optimum number of axes to explain the data. (R version 3.6.0 "Planting of a Tree", The R Foundation for Statistical Computing, 2019).

The second part of the hypothesis concerned the effect of drought on the whole root chemistry of the *A. odoratum.* The ATR-FTIR data were processed using using MATLAB and the in-house cluster toolbox MATLAB scripts (Mathwork, MA; freely available at https://github.com/Biospec/cluster-toolbox-v2.0), using Savitz-Golay filtering to reduce noise, removal of the interference from CO_2 between the 2400-2275 wavenumber region and trimming to the 4000-800 wavenumber range, before being baseline corrected and normalised. These data were then visualised using PCA.

To test the third part of the hypothesis, which concerned the drought effect on root exudate chemistry, we processed the GC-MS data as per the method outlined in Chapter 2. Briefly, we converted the raw output to mzXML format, and aligned them using the R package *erah* (Domingo-Almenara et al., 2016). We recovered missing compounds, carried out quality control corrections, and analysed using the in-house MATLAB script (Mathwork, MA; available at https://github.com/Biospec/cluster-toolbox-v2.0), before performing a PCA and followed this by adding ellipses to the treatments using confidence intervals from the centroid to inform the area (R version 3.6.0 "Planting of a Tree", The R Foundation for Statistical Computing, 2019). We then used MetaboAnalyst to look for treatment effects where there is a significant fold change in signal for a feature between control and drought on the GC-MS data. These were represented using volcano plots, which indicated significance of the fold change against the p-value of each metabolite, when comparing drought and control exudation fingerprints. We processed the transmission FTIR exudates data as above for the ATR-FTIR, and visualised the results of both analysis types using PCA. Because biological

samples produce a highly characteristic infra-red absorption spectrum with areas of absorbance peaks typical to common classes of biomolecules occurring in particular regions of the IR spectrum, it is also possible to categorise an FTIR spectrum into a number of regions: a fatty acid region, 3050-2800cm^{-1}; the amide region in which proteins and peptides absorb strongly, 1750-1500 cm^{-1}; the 'mixed' region in which carboxylic acid functional groups, unbonded amino acids, and polysaccharides absorb, 1500-1250 cm^{-1}; and the (poly)saccharide region, 1200-900 cm^{-1} (Schmidt and Flemming, 1998).

3.5 Results

3.5.1 Root traits

Root architectural traits were uniformly significantly different under drought conditions from the control: values for biomass, root length, root volume, and branching and number of root tips were all lower in droughted plants (Table 1). Surface area was also lower in droughted plants. However, morphological traits were not significantly different between control and drought. Using PCA to visualise the data a clear cluster of the control watering regime is visible, separating from the root traits of the droughted plants subjected to a reduced watering regime (Figure 2). On this PCA, the root traits of *Anthoxanthum odoratum* conformed to two primary axes which explained 85.6% of the variation. On axis 1, which explained 67.1% of the variation, there was a mixture of architectural and morphological traits, and the control pots were grouped around the end of the arrows that represented higher values of the traits. Axis 2, which explained 18.5% variation, was mainly explained by root diameter and specific root length, which are both morphological traits.

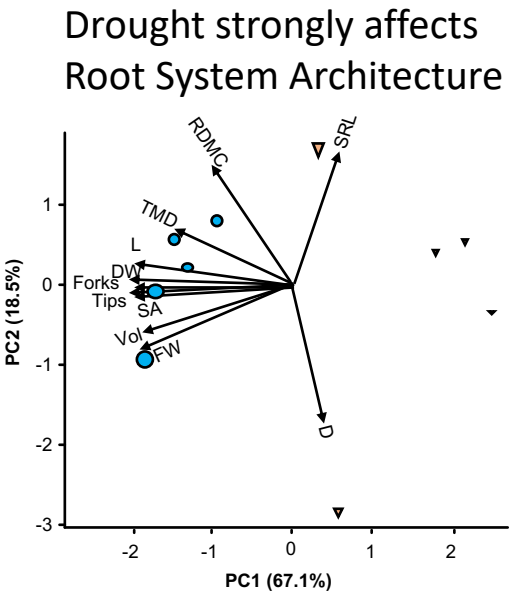

Figure 2 (left): Principal Components Analysis of root functional traits, where control plants are represented by blue circles, and drought plants are represented by orange triangles. The symbols are scaled in size based on the relative dry mass of the root system at harvest. The annotations used in this figure are defined in Table 1.

Table 1 (below): Root traits of *Anthoxanthum odoratum* subjected to control and drought conditions. Traits that showed a drought effect significant at the $p<0.05$ level, as determined by Welch's t-test, are shown in bold font.

Trait class	Trait & Annotation		Control mean (± 95% CI)	Drought mean (± 95% CI)	± 95% CI	t statistic	*P*-value
Architectural	Root biomass (g)	DW	**0.364**	**0.154**	**0.09**	**5.250**	**<0.001**
	Root length (cm)	L	**13642.071**	**8391.300**	**653.77**	**11.200**	**<0.001**
	Root volume (cm^3)	V	**5.364**	**2.948**	**0.99**	**5.690**	**<0.001**
	Number of root tips	Tips	**102657.400**	**48207.600**	**23023.70**	**5.711**	**0.001**
	Number of root forks	Forks	**203905.800**	**82988.800**	**25636.21**	**11.251**	**<0.001**
Morphological	Average root diameter (mm)	D	0.224	0.235	0.04	-0.803	0.455
	Root dry matter content (%)	RDMC	0.066	0.054	0.02	1.164	0.282
	Tissue mass density (mg cm^{-3})	TMD	0.068	0.053	0.03	1.175	0.292
	Specific root length (m g^{-1})	SRL	2512.010	2549.570	1023.08	-0.085	0.935
NA	Surface area (cm^2)	SA	**957.523**	**496.270**	**103.28**	**10.307**	**<0.001**

3.5.2 Root chemistry

The ATR-FTIR spectrum of the roots under control and drought conditions is extremely similar (Figure 3A). The metabolites detected in the whole root system by ATR-FTIR were optimally described by three PCA axes, with the first explaining 57.3% of the data, the second explaining 31.4%, and the third explaining 6.2% (94.9% in total). On the ordination plot there was a clear separation between the drought and control root chemistry on Principal Component 2 (Figure 3B). Plotting the loadings for PC2 against the original wavenumbers of the data yielded a number of peaks relatively enriched in drought at wavenumbers 1317cm^{-1}, 1287cm^{-1}, 1168cm^{-1}, 1058cm^{-1}, and 1032cm^{-1}.

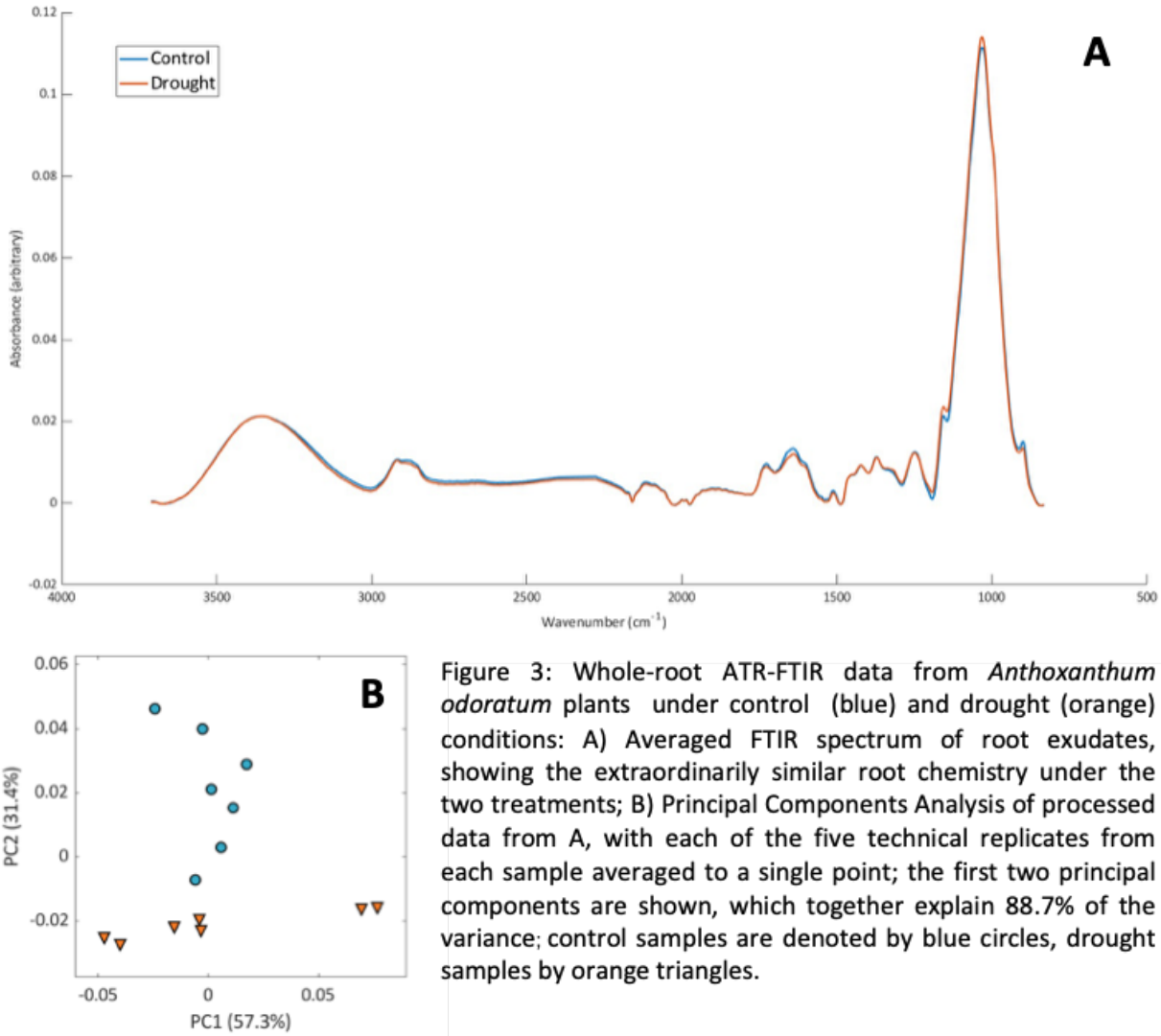

Figure 3: Whole-root ATR-FTIR data from *Anthoxanthum odoratum* plants under control (blue) and drought (orange) conditions: A) Averaged FTIR spectrum of root exudates, showing the extraordinarily similar root chemistry under the two treatments; B) Principal Components Analysis of processed data from A, with each of the five technical replicates from each sample averaged to a single point; the first two principal components are shown, which together explain 88.7% of the variance; control samples are denoted by blue circles, drought samples by orange triangles.

3.5.3 Root exudates

3.5.3.1 GC-MS

Principal Components Analysis of the GC-MS results showed no separation between the drought and control treatments when looking at the global dataset, with ellipses drawn around the treatments using a 95% confidence interval from the centroid to inform the area (Fig. 4A). Examining each feature individually by looking at its the relative fold-change against the p-value of each metabolite (Fig 4B), revealed 15 compounds enriched in drought (all three that could be annotated were amino acids, Fig. 4C:1,2,3) and 5 in the control (two were annotated, the sugars kestose and sorbose, Fig. 4C:4,5). In Figure 4C the mean values are represented by yellow dots, the median and upper and lower quartiles by the one thick and two thin black bars respectively, and the upper and lower quartiles as a function of 1.5 times the value of the interquartile range by the whiskers. The other metabolites were not identified or annotated.

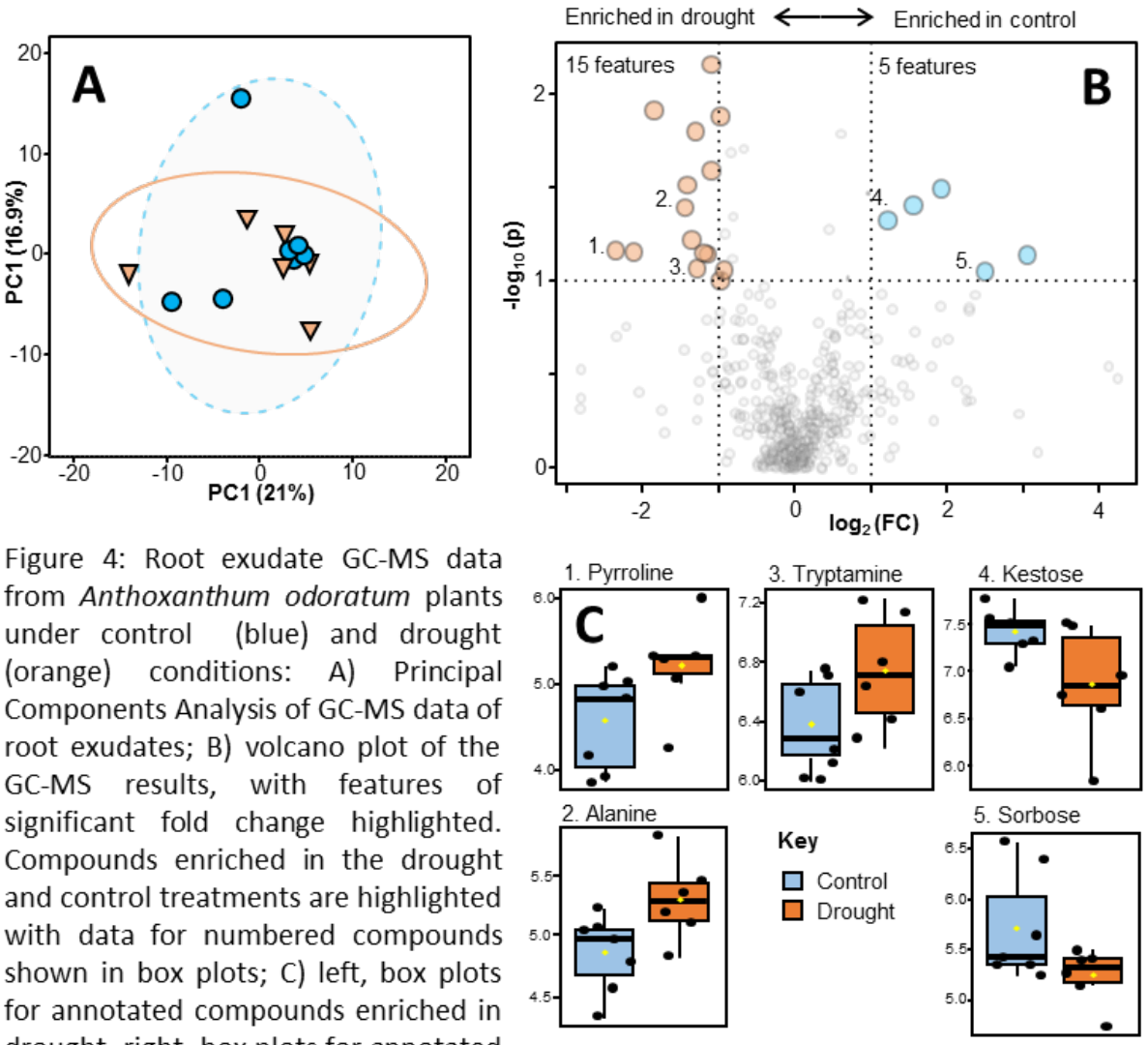

Figure 4: Root exudate GC-MS data from *Anthoxanthum odoratum* plants under control (blue) and drought (orange) conditions: A) Principal Components Analysis of GC-MS data of root exudates; B) volcano plot of the GC-MS results, with features of significant fold change highlighted. Compounds enriched in the drought and control treatments are highlighted with data for numbered compounds shown in box plots; C) left, box plots for annotated compounds enriched in drought, right, box plots for annotated compounds enriched in the control treatment.

3.5.3.2 FTIR

Transmission FTIR analysis of the root exudates found a strong drought signal (Fig, 5A), including when the data were visualized using PCA (Fig 5B). The drought treatment showed more tightly clustered points than the control treatment, so exudate chemistry became more uniform. There was a shift to increased relative abundance of chemical species in the amide region in the drought samples, and an attendant decrease in the saccharides present in drought samples (Fig 5A).

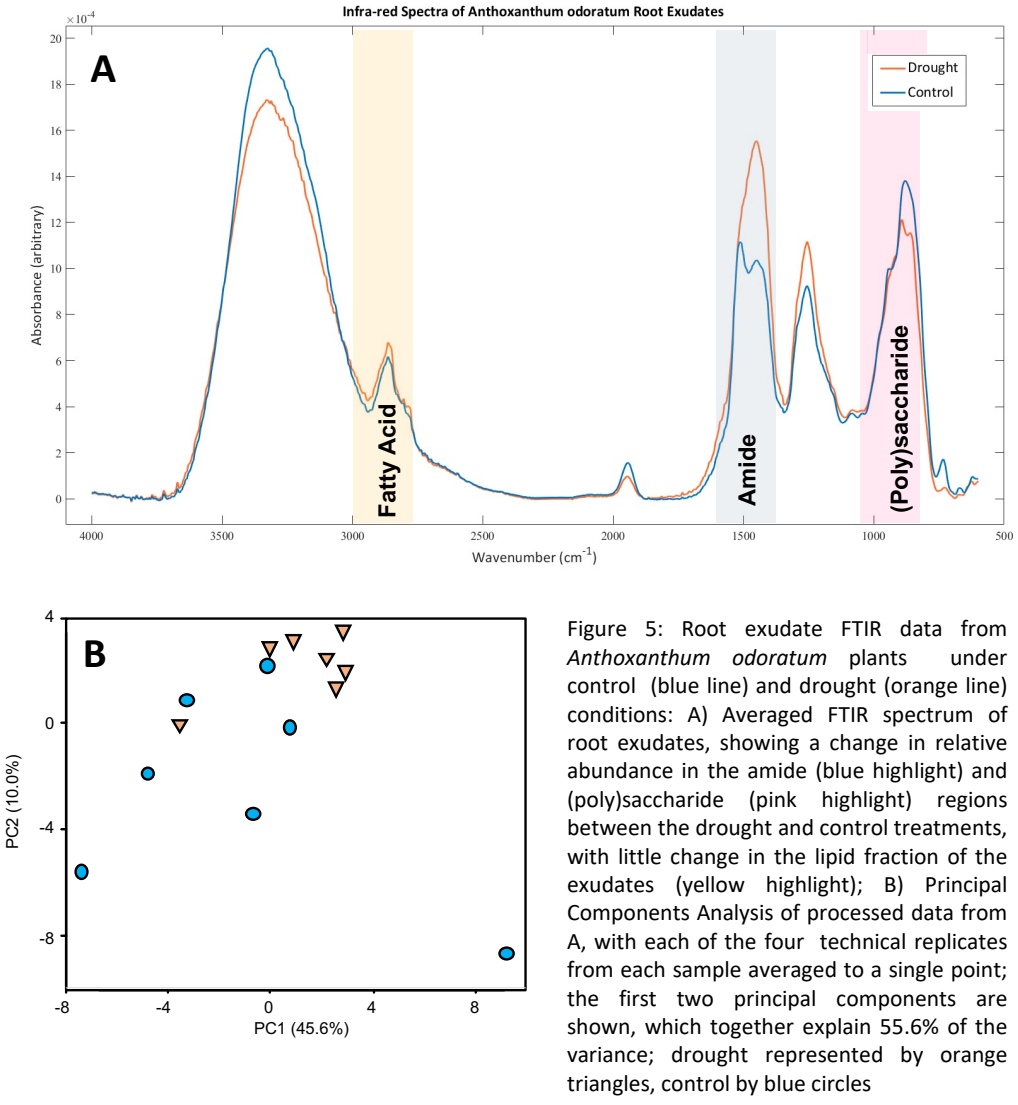

Figure 5: Root exudate FTIR data from *Anthoxanthum odoratum* plants under control (blue line) and drought (orange line) conditions: A) Averaged FTIR spectrum of root exudates, showing a change in relative abundance in the amide (blue highlight) and (poly)saccharide (pink highlight) regions between the drought and control treatments, with little change in the lipid fraction of the exudates (yellow highlight); B) Principal Components Analysis of processed data from A, with each of the four technical replicates from each sample averaged to a single point; the first two principal components are shown, which together explain 55.6% of the variance; drought represented by orange triangles, control by blue circles

3.6 Discussion

Here we aimed to provide a proof-of-concept study that showed that the soil-hydroponics hybrid method examined in Chapter 2 could detect drought effects, and that infra-red spectroscopy was appropriate for exudate analysis. It is the first time that anyone has used FTIR to identify stress effects in exudates, and such signal detection shows promise for future use of this analytical pipeline in analysis of real-world conditions. We hypothesised that plant response to drought would be reflected in root functional effects traits, whole root chemistry, and the chemistry of root exudates. We could detect these effects, and these differences were consistent throughout the root responses measured, indicating hybrid methods are appropriate for drought analyses.

The first part of our hypothesis was that drought would have a large impact on plant root traits, either through developmental delay, or through a shift in root system architecture or changes in foraging patterns. We found that architectural traits were much more important than morphological traits. This indicates that although the plants are locked in to a particular morphological pattern at the species level, the different ways that this blueprint can be assembled shows a great deal of phenotypic plasticity resulting in a difference in architectural traits, something which has been shown before in response to water availability limited to certain locations in the soil column (Fry et al., 2018b); here we show this is also the case for whole-pot water availability. It is unclear from our results whether the differences seen are a) induced as a direct result of water availability, where these plants alter how to organise their RSA under reduced watering; or b) from developmental delay caused by drought stress, which has been theorised as a reason for drought effects on plants (Blum 1996; Salehi-Liser 2016). A further study where plants are harvested longitudinally could show whether the traits that have lower values due to drought are simply attributable to the smaller overall size - if droughted plants have a root system more similar to younger control plants, this would provide strong evidence for the effect being due to developmental delay. This may be likely; a study on drought and root traits in grasses by de Vries et al. (2016) found drought did not alter the relationship between most observed root traits, including those calculated independent of biomass, and root biomass, meaning droughted root systems were in terms of structure effectively smaller versions of control root systems.

Moreover, the two axes of trait values demonstrated by the PCA conform to the root economic space presented by Bergman et al. (2020), and in this study we have found support for the collaboration and the conservation gradients. SRL and root diameter are approximately negatively correlated in our study, indicating a collaboration gradient, but there was no significant difference in these values between groups. The conservation gradient is much more important under drought in our study, with a shift towards more conservative traits (fewer forks, root tips) indicating less foraging under drought (Lozano et al., 2020). Comas et al. (2013) suggest that plant root diameter decreases under drought, but our finding, that architectural traits are more responsive, reflects a shift towards conserving resources in line with Bergmann et al. (2020). Hernandez et al. (2009) have found that the SRL/Diameter axis exists across species and diameter alters with hydraulic water uptake ability, in Mediterranean species in a growth cabinet, but our results do not support this as an effect within *A. odoratum* exposed to drought. Zhou et al. (2018) similarly found, in a global synthesis of 128 field-based studies, that morphological traits are more important than architectural ones, but found that the effect on SRL for grasses is quite weak, which may indicate why it does not have a significant effect in our study. Taken together, our findings suggest that root traits of *A. odoratum* are highly responsive to drought, and tend towards delayed growth and conservation of resources, contrary to many other plant types.

The second part of our hypothesis suggested that ATR-FTIR would be appropriate to detect drought signals in the chemistry of whole root systems. ATR-FTIR has been used to analyse root chemistry in previous studies (e.g. Garrigues et al., 2000; Zhao et al., 2013), and has even been used to identify the proportion of plant species present from roots in soil cores from peatlands (Strakova et al., 2020). Here, we used this established technique to determine changes in metabolic fingerprint between the drought and control treatments. While we did observe separation of the treatments in ordination space, the chemistry of the roots under drought is extraordinarily similar to that under control conditions.

The third part of our hypothesis concerned root exudate chemistry, and whether the hybrid method discussed here would preserve a drought effect that is detectable with our analytical methods, despite a hydroponic root-repair stage. Gas-Chromatography Mass-Spectroscopy did not show differences in global exudate chemistry between the control and drought treatments, but did find a number of individual features that differed significantly between treatments, with several amino acids increasing under drought, and the sugars kestose and

sorbose decreasing. While previous studies (e.g. Privete et al., 2000; Swarcewicz et al., 2017) have found an increase in sugars such as kestose in response to drought, presumably up-regulated in order to facilitate a quick recovery, *A. odoratum* seems to be taking an approach similar to the drought-tolerant wild species *Solanum pernellii* in upregulating genes linked to amino acid synthesis - linked to longer-term drought tolerance rather than drought avoidance (Egea et al., 2018). Child et al. (2007) showed that various species of the microbial genus *Mycobacterium* use kestose as an interkingdom signal to associate with plant roots; this, together with the root trait data collected in this experiment which showed that a drought effect does not impact on the root economic spectrum collaboration gradient, indicates that the strategy of *A. odoratum* is to rather than increase in sugars in response to drought, undergo a complete architectural reorganisation. When considered alongside the trait data, it suggests that *A. odoratum* adopts a 'do-it-yourself' approach under drought, reducing root foraging and putting fewer resources into rhizosphere assembly. One caveat is that there are many molecules with a high degree of fold-change between treatments in the exudates that were not identified by the GC-MS, and these may have important roles in both plant drought tolerance and microbial-driven functions. There is therefore a need for a method to identify changes in exudation chemistry, even where the molecules are not known. Here we have used FTIR spectroscopy to support and extend the GC-MS results, and have shown a strong drought effect in exudate infra-red metabolic fingerprint for the first time. There has been a global shift from sugars to amide-containing compounds between the treatment groups. Importantly, we also found that the response in drought of the root system is not at the same wavenumbers as the response in exudates, indicating a different chemical response in the root system and in exudates to drought - possible evidence that root exudation is a highly controlled trait. We believe the use of infra-red spectroscopy has the potential to give important insight into the shifts in exudate chemistry in response to drought in future studies, as well as being an excellent first-use technique to inform further analytical choices in exudate studies. However, root exudation has been shown to differ between different growth stages of plants; Chaparro et al. (2013) found that exudates change over the whole lifetime of *Arabidopsis thaliana* plants (although this was collected on plants grown on agar). The metabolomic shifts identified in our study could therefore again be due to drought-induced developmental delay.

3.7 Conclusions

Here we have shown that drought effects on roots are evident at the architectural and chemical levels, although not at the morphological level. The chemistry of the whole root system does not exhibit extreme shifts under drought stress conditions. The change in exudates from sugars to amides under drought is consistent with the change in architectural traits, showing that *A. odoratum* completely reorganises its root form and function - although not its bulk chemistry - to withstand abiotic stress. This indicates that the plant is using the same developmental blueprint under drought, and it is possible that the developmental delay caused by drought is one cause of the large shifts in exudate chemistry. Future work should consider a detailed life stage exudation and root chemistry study to confirm this suggestion, as well as consider using the methods advanced here on more species exposed to multiple stresses.

3.8 Acknowledgements

R.M. was supported by a Biotechnology and Biological Sciences (BBSRC) Doctoral Training Partnership (reference 1792130), which also funded this work. F.T.d.V. was funded by a BBSRC David Phillips Fellowship (BB/L02456X/1). The suggestions of A.W. on the use of composite figures were much appreciated.

3.9 References

Balachowski, Jennifer A., and Florence A. Volaire. 2018. “Implications of Plant Functional Traits and Drought Survival Strategies for Ecological Restoration.” *Journal of Applied Ecology* 55 (2). John Wiley & Sons, Ltd:631–40. https://doi.org/10.1111/1365-2664.12979.

Begley, Paul, Sue Francis-McIntyre, Warwick B. Dunn, David I. Broadhurst, Antony Halsall, Andy Tseng, Joshua Knowles, HUSERMET Consortium, Royston Goodacre, and Douglas B. Kell. 2009. “Development and Performance of a Gas Chromatography−Time-of-Flight Mass Spectrometry Analysis for Large-Scale Nontargeted Metabolomic Studies of Human Serum.” *Analytical Chemistry* 81 (16). American Chemical Society:7038–46. https://doi.org/10.1021/AC9011599.

Bergmann, Joana, Alexandra Weigelt, Fons van der Plas, Daniel C. Laughlin, Thom W. Kuyper, Nathaly Guerrero-Ramirez, Oscar J. Valverde-Barrantes, et al. 2020. “The Fungal Collaboration Gradient Dominates the Root Economics Space in Plants.” *Science Advances* 6 (27). American Association for the Advancement of Science:eaba3756. https://doi.org/10.1126/SCIADV.ABA3756.

Blum, A. 1996. “Crop Responses to Drought and the Interpretation of Adaptation.” *Drought Tolerance in Higher Plants: Genetical, Physiological and Molecular Biological Analysis*. Springer, Dordrecht, 57–70. https://doi.org/10.1007/978-94-017-1299-6_8.

Briones, M. J.I., D. M.O. Elias, H. K. Grant, and N. P. McNamara. 2019. “Plant Identity Control on Soil Food Web Structure and C Transfers under Perennial Bioenergy Plantations.” *Soil Biology and Biochemistry* 138 (November). Pergamon:107603. https://doi.org/10.1016/J.SOILBIO.2019.107603.

Canarini, Alberto, Andrew Merchant, and Feike A. Dijkstra. 2016. “Drought Effects on Helianthus Annuus and Glycine Max Metabolites: From Phloem to Root Exudates.”

Rhizosphere 2 (December). Elsevier:85–97. https://doi.org/10.1016/J.RHISPH.2016.06.003.

Chaparro, Jacqueline M., Dayakar V. Badri, Matthew G. Bakker, Akifumi Sugiyama, Daniel K. Manter, and Jorge M. Vivanco. 2013. "Root Exudation of Phytochemicals in Arabidopsis Follows Specific Patterns That Are Developmentally Programmed and Correlate with Soil Microbial Functions." *PLoS ONE* 8 (2). Public Library of Science:e55731. https://doi.org/10.1371/journal.pone.0055731.

Child, R., C. D. Miller, Y. Liang, G. Narasimham, J. Chatterton, P. Harrison, R. C. Sims, D. Britt, and A. J. Anderson. 2007. "Polycyclic Aromatic Hydrocarbon-Degrading Mycobacterium Isolates: Their Association with Plant Roots." *Applied Microbiology and Biotechnology 2007 75:3* 75 (3). Springer:655–63. https://doi.org/10.1007/S00253-007-0840-0.

Comas, Louise, Steven Becker, Von Mark V. Cruz, Patrick F. Byrne, and David A. Dierig. 2013. "Root Traits Contributing to Plant Productivity under Drought." *Frontiers in Plant Science* 0 (NOV). Frontiers:442. https://doi.org/10.3389/FPLS.2013.00442.

De Vries, Franciska T., Alex Williams, Fiona Stringer, Robert Willcocks, Rosie McEwing, Holly Langridge, and Angela L. Straathof. 2019. "Changes in Root-Exudate-Induced Respiration Reveal a Novel Mechanism through Which Drought Affects Ecosystem Carbon Cycling." *New Phytologist* 224 (1). John Wiley & Sons, Ltd:132–45. https://doi.org/10.1111/nph.16001.

De Vries, Franciska T., and Tancredi Caruso. 2016. "Eating from the Same Plate? Revisiting the Role of Labile Carbon Inputs in the Soil Food Web." *Soil Biology and Biochemistry* 102 (November). Pergamon:4–9. https://doi.org/10.1016/J.SOILBIO.2016.06.023.

De Vries, Franciska T., Caley Brown, and Carly J. Stevens. 2016. "Grassland Species Root Response to Drought: Consequences for Soil Carbon and Nitrogen Availability." *Plant and Soil 2016 409:1* 409 (1). Springer:297–312. https://doi.org/10.1007/S11104-016-2964-4.

De Vries, Franciska T., Rob I. Griffiths, Mark Bailey, Hayley Craig, Mariangela Girlanda, Hyun Soon Gweon, Sara Hallin, et al. 2018. “Soil Bacterial Networks Are Less Stable under Drought than Fungal Networks.” *Nature Communications 2018 9:1* 9 (1). Nature Publishing Group:1–12. https://doi.org/10.1038/s41467-018-05516-7.

Delhaize, E., P. R. Ryan, and P. J. Randall. 1993. “Aluminum Tolerance in Wheat (Triticum Aestivum L.) (II. Aluminum-Stimulated Excretion of Malic Acid from Root Apices).” *Plant Physiology* 103 (3). Oxford Academic:695–702. https://doi.org/10.1104/PP.103.3.695.

Domingo-Almenara, Xavier, Jesus Brezmes, Maria Vinaixa, Sara Samino, Noelia Ramirez, Marta Ramon-Krauel, Carles Lerin, et al. 2016. “ERah: A Computational Tool Integrating Spectral Deconvolution and Alignment with Quantification and Identification of Metabolites in GC/MS-Based Metabolomics.” *Analytical Chemistry* 88 (19). American Chemical Society:9821–29. https://doi.org/10.1021/ACS.ANALCHEM.6B02927.

Egea, Isabel, Irene Albaladejo, Victoriano Meco, Belén Morales, Angel Sevilla, Maria C. Bolarin, and Francisco B. Flores. 2018. “The Drought-Tolerant Solanum Pennellii Regulates Leaf Water Loss and Induces Genes Involved in Amino Acid and Ethylene/Jasmonate Metabolism under Dehydration.” *Scientific Reports 2018 8:1* 8 (1). Nature Publishing Group:1–14. https://doi.org/10.1038/s41598-018-21187-2.

Fry, Ellen L., Joanna Savage, Amy L. Hall, Simon Oakley, W. J. Pritchard, Nicholas J. Ostle, Richard F. Pywell, James M. Bullock, and Richard D. Bardgett. 2018a. “Soil Multifunctionality and Drought Resistance Are Determined by Plant Structural Traits in Restoring Grassland.” *Ecology* 99 (10). John Wiley & Sons, Ltd:2260–71. https://doi.org/10.1002/ECY.2437.

Fry Ellen L.**,** Amy L. Evans, Craig J. Sturrock and Richard D. Bardgett. 2018b. “Root architecture governs plasticity in response to drought.” *Plant and Soil* 433: 189-200. 10.1007/s11104-018-3824-1.

Gargallo-Garriga, Albert, Catherine Preece, Jordi Sardans, Michal Oravec, Otmar Urban, and Josep Peñuelas. 2018. “Root Exudate Metabolomes Change under Drought and

Show Limited Capacity for Recovery." *Scientific Reports 2018 8:1* 8 (1). Nature Publishing Group:1–15. https://doi.org/10.1038/s41598-018-30150-0.

Gaume, Alain, Felix Mächler, Carlos De León, Luis Narro, and Emmanuel Frossard. 2001. "Low-P Tolerance by Maize (Zea Mays L.) Genotypes: Significance of Root Growth, and Organic Acids and Acid Phosphatase Root Exudation." *Plant and Soil 2001 228:2* 228 (2). Springer:253–64. https://doi.org/10.1023/A:1004824019289.

Hernández, E. I., A. Vilagrosa, J. G. Pausas, and J. Bellot. 2009. "Morphological Traits and Water Use Strategies in Seedlings of Mediterranean Coexisting Species." *Plant Ecology 2009 207:2* 207 (2). Springer:233–44. https://doi.org/10.1007/S11258-009-9668-2.

JM, Garrigues, Akssira M, Rambla FJ, Garrigues S, and de la Guardia M. 2000. "Direct ATR-FTIR Determination of Sucrose in Beet Root." *Talanta* 51 (2). Talanta:247–55. https://doi.org/10.1016/S0039-9140(99)00258-1.

Lozano, Yudi M., Carlos A. Aguilar-Trigueros, Isabel C. Flaig, and Matthias C. Rillig. 2020. "Root Trait Responses to Drought Are More Heterogeneous than Leaf Trait Responses." *Functional Ecology* 34 (11). John Wiley & Sons, Ltd:2224–35. https://doi.org/10.1111/1365-2435.13656.

Naveed, M., M. A. Ahmed, P. Benard, L. K. Brown, T. S. George, A. G. Bengough, T. Roose, N. Koebernick, and P. D. Hallett. 2019. "Surface Tension, Rheology and Hydrophobicity of Rhizodeposits and Seed Mucilage Influence Soil Water Retention and Hysteresis." *Plant and Soil 2019 437:1* 437 (1). Springer:65–81. https://doi.org/10.1007/S11104-019-03939-9.

Pinheiro, C., and M. M. Chaves. 2011. "Photosynthesis and Drought: Can We Make Metabolic Connections from Available Data?" *Journal of Experimental Botany* 62 (3). Oxford Academic:869–82. https://doi.org/10.1093/JXB/ERQ340.

Preece, Catherine, and Josep Peñuelas. 2016. "Rhizodeposition under Drought and Consequences for Soil Communities and Ecosystem Resilience." *Plant and Soil 2016 409:1* 409 (1). Springer:1–17. https://doi.org/10.1007/S11104-016-3090-Z.

Puntase, Janjira, Chuckree Senthong, Sawit Meechoui, and Keith T Ingram. 2004. “Effect of Root Exudates on Drought and Aflatoxin Resistance of Peanut Genotypes.” *Journal of Chemical Information and Modeling* 53 (9):1689–99.

Schmitt, Jürgen, and Hans Curt Flemming. 1998. “FTIR-Spectroscopy in Microbial and Material Analysis.” *International Biodeterioration & Biodegradation* 41 (1). Elsevier:1–11. https://doi.org/10.1016/S0964-8305(98)80002-4.

Salehi-Lisar, Seyed Yahya, and Hamideh Bakhshayeshan-Agdam. 2016. “Drought Stress in Plants: Causes, Consequences, and Tolerance.” *Drought Stress Tolerance in Plants, Vol 1: Physiology and Biochemistry* 1 (January). Springer, Cham:1–16. https://doi.org/10.1007/978-3-319-28899-4_1.

Shi, Shengjing, Alan E. Richardson, Maureen O’Callaghan, Kristen M. Deangelis, Eirian E. Jones, Alison Stewart, Mary K. Firestone, and Leo M. Condron. 2011. “Effects of Selected Root Exudate Components on Soil Bacterial Communities.” *FEMS Microbiology Ecology* 77 (3):600–610. https://doi.org/10.1111/j.1574-6941.2011.01150.x.

Swarcewicz, Barbara, Aneta Sawikowska, Łukasz Marczak, Magdalena Łuczak, Danuta Ciesiołka, Karolina Krystkowiak, Anetta Kuczyńska, Mariola Piślewska-Bednarek, Paweł Krajewski, and Maciej Stobiecki. 2017. “Effect of Drought Stress on Metabolite Contents in Barley Recombinant Inbred Line Population Revealed by Untargeted GC–MS Profiling.” *Acta Physiologiae Plantarum* 39 (8). Springer:1–16. https://doi.org/10.1007/s11738-017-2449-y.

Williams, Alex, and Franciska T. de Vries. 2020. “Plant Root Exudation under Drought: Implications for Ecosystem Functioning.” *New Phytologist* 225 (5). John Wiley & Sons, Ltd:1899–1905. https://doi.org/10.1111/NPH.16223.

Williams, Alex, Holly Langridge, Angela L. Straathof, Graeme Fox, Howbeer Muhammadali, Katherine A. Hollywood, Yun Xu, Royston Goodacre, and Franciska T. de Vries. 2021. “Comparing Root Exudate Collection Techniques: An Improved Hybrid Method.” *Soil Biology and Biochemistry* 161 (October). Pergamon:108391. https://doi.org/10.1016/J.SOILBIO.2021.108391.

Williams, Alex, Holly Langridge, Angela L. Straathof, Howbeer Muhamadali, Katherine A. Hollywood, Royston Goodacre, and Franciska T. de Vries. 2021. “Root Functional Traits Explain Root Exudation Rate and Composition across a Range of Grassland Species.” *Journal of Ecology* 00. John Wiley & Sons, Ltd:1–13. https://doi.org/10.1111/1365-2745.13630.

Winder, C L, E Carr, R Goodacre, R Seviour, E L Carr, P Kämpfer, B K C Patel, and V Gürtler. 2003. “The Rapid Identification of Acinetobacter Species Using Fourier Transform Infrared Spectroscopy.” *International Journal of Systematic and Evolutionary Microbiology* 53:953–63. https://doi.org/10.1046/j.1365-2672.2003.02154.x.

Zhao, Xin, Xiaoju Yang, Yong Shi, Guoxiong Chen, Xinrong Li, Xin Zhao, Xiaoju Yang, Yong Shi, Guoxiong Chen, and Xinrong Li. 2013. “Protein and Lipid Characterization of Wheat Roots Plasma Membrane Damaged by Fe and H2O2 Using ATR-FTIR Method.” *Journal of Biophysical Chemistry* 4 (1). Scientific Research Publishing:28–35. https://doi.org/10.4236/JBPC.2013.41004.

Zhou, Guiyao, Xuhui Zhou, Yuanyuan Nie, Shahla Hosseini Bai, Lingyan Zhou, Junjiong Shao, Weisong Cheng, Jiawei Wang, Fengqin Hu, and Yuling Fu. 2018. “Drought-Induced Changes in Root Biomass Largely Result from Altered Root Morphological Traits: Evidence from a Synthesis of Global Field Trials.” *Plant, Cell & Environment* 41 (11). John Wiley & Sons, Ltd:2589–99. https://doi.org/10.1111/PCE.13356.

4. Interactive effects of drought and warming on root exudates and soil chemistry in two contrasting grass species

Reuben Margerison[1,2], Alex Williams[1], Alistair Hanak[1], Cassio Lima[2], Jurre Bleeker[5], Harro Bouwmeester[5], Ellen L. Fry[6], Giles Johnson[1], Patrick Gallois[3], Royston Goodacre[2,4] and Franciska T. de Vries[1,5]

[1]School of Natural Sciences, The University of Manchester, Oxford Road, M13 9PT, Manchester, UK

[2]Department of Biochemistry and Systems Biology, Institute of Systems, Molecular and Integrative Biology (ISMIB), University of Liverpool, Biosciences Building, Crown Street, L69 16 7ZB, Liverpool, UK

[3]School Biological Sciences, The University of Manchester, Oxford Road, M13 9PT, Manchester, UK

[4]Manchester Institute of Biotechnology, The University of Manchester, Princess Road, 18 Manchester M1 7DN, UK

[5]Institute of Biodiversity and Ecosystem Dynamics, University of Amsterdam, PO Box 94240, 20 1090 GE Amsterdam, The Netherlands

[6]Department of Biology, Edge Hill University, St Helens Road, Ormskirk, L39 4AP, Lancashire UK

4.1 Key words

Drought; warming; climate change; root exudates; leachates; ATR-FTIR; root traits; metabolomics; plant-soil interactions

4.2 Abstract

Plant-derived carbon compounds in the form of root exudates are key drivers of nutrient cycling and ecosystem function, but it is unclear how they are affected under climate change. Exudation is observed to be higher in volume in species with a more acquisitive resource economics strategy, thought to be due to the need to rapidly recruit a specific growth-promoting microbial community. However, acquisitive plant species are also known to be less resilient to climate change. Accordingly, the precise changes in exudate chemistry, and the downstream effects on soil chemistry, caused by change in temperature and water availability, under species with contrasting resource economics strategies are not well understood. Using Attenuated Total Reflectance - Fourier Transform Infra-Red spectroscopy to analyse soil leachates and root exudates collected using a recently developed soil-hydroponic hybrid method, we found in a controlled environment experiment that the conservative grass *Anthoxanthum odoratum* and the acquisitive grass *Dactylis glomerata* respond similarly to environmental stresses despite their different life strategies. Here we show that the primary driver of changes in root exudation under simulated climate change in these species is increased temperature, and that these inputs have downstream effects on soil chemistry. Our results also indicate that resource strategy is not an important indicator of climate-driven changes in plant inputs into the soil.

4.3 Introduction

Climate change will result in multiple simultaneous stresses on plants. These will cause complex plant responses which are aimed at tolerating or avoiding the stress. Stress tolerance describes a capability to endure stressful conditions; stress tolerance responses to drought can include osmotic adjustments to alter water potential gradients, a target for crop improvement (Cattavelli et al., 2008). Stress avoidance responses attempt to minimize the effect (Puijalon et al., 2011); in a drought this could include shedding older roots as these take up water less effectively (Eissenstat et al., 2000). These tolerance and avoidance responses may have direct effects on photosynthetic rate and availability of sugars for exudation, as well as root system architectures (RSA). Consequently drought and warming are associated with shifts in plant exudate quality and quantity, leading to further changes in rhizosphere community composition and ecosystem respiration (de Vries et al., 2019), carbon priming effects and nutrient cycling. Increasingly there are calls to investigate interactive effects of multiple stresses in plants because of non-linear and unpredictable responses, wherein small changes can push an ecosystem past a breakpoint beyond which regression to the previous stable state is not possible (Folke et al., 2004). Such non-linear responses to environmental stresses have been previously uncovered in root traits and tissue chemistry (Gargallo-Garriga et al., 2015; Rillig et al., 2021). Understanding of plant responses to multiple stresses in terms of exudation, and how this impacts the chemistry of the soil, is currently limited, although some *in situ* studies have shown that both drought and warming interact to alter plant exudate volume (Jakoby et al., 2020; Gargallo-Garriga et al., 2021).

However, there is little information on these interactive effects on exudate chemistry, and their consequences for microbial community structure and function. Some theorise that any effect of warming will arise from exacerbating drought effects, although increases in metabolic activity through increased kinetic energy are also possible (Gargallo-Garriga et al., 2021). In a warming study in the Arctic tundra, Gargallo-Garriga and colleagues hypothesised that the changes they observed in exudate chemistry were due to a shift in plant metabolism from growth under ambient conditions, to anti-stress and protection under warming.

However, small-scale mechanistic studies that seek to uncover changes in exudates under controlled conditions with modern collection methods are few. Difficulties with the methodology in creating a realistic drought and warming treatment in hydroponic plants, and the questions about the value of the exudates collected, has led to the development and investigation of soil-hydroponic hybrid exudate collection methods. This work has recently shown that exudates collected from roots left to recover from washing damage before collection have a metabolome less similar to that of damaged root tissue, exudate composition that is distinct from pure hydroponic growth, and that historic drought effects can be detected in exudates using these methods even after a period of recovery (Williams et al., 2021a; Chapter 2; Chapter 3), but the suitability of these methods for studying interactive climate shifts has yet to be determined.

Effects of individual climate changes such as increased temperature or reduced water availability (drought) on plant-microbial interactions are well studied but often confounding; under drought, root exudation has been shown to increase (Dyer et al., 2008), remain constant (Karlowsky et al., 2018) or decrease (de Vries et al., 2019), and where changes are seen, microbial composition and function are observed to respond (Dyer et al., 2008). A study by de Vries et al. (2019) showed that the exudates from droughted grassland plants increased microbial respiration more than those from control plants, despite lower overall exudate volume. They surmised that this indicated that there was a shift in metabolites towards molecular forms more palatable to microbes, in order to facilitate rapid recovery when drought was alleviated. A shift in composition of exudates under drought has since been shown to occur in further studies (Chapter 3). Warming has consistently been shown to increase the rate of root exudation in a wide variety of plants (Uselman et al., 2000; Yin et al., 2013; Wang et al., 2020), and it has also recently been shown to alter exudate chemistry in a species-dependent way (Wu and Yu, 2019). Studies on the effects of multiple climate stressors on exudates using modern collection methods are currently lacking.

Plant functional traits are well characterised in response to individual stresses, less so for multiple stresses. There is strong support for application of the 'resource economics spectrum' as a useful tool when seeking to explain differences in stress response (Wright et al., 2004). In the resource economics spectrum, ranging from conservative to acquisitive strategies, plants tend to align with either conservative resource use strategies, which are often associated with nutrient-poor, stable environments, or acquisitive strategies, which are observed in high

nutrient or disturbed environments (de la Riva et al., 2016). These relationships are observed in both above and belowground plant parts, meaning that the paradigm is consistent (Perez-Ramos et al., 2012). Accordingly, inferences can be made as to the speed of tissue turnover, the speed of nutrient cycling and the characteristics of the soil food web that associates with the plants (de Vries et al., 2012). Studies have shown that often, plants on the conservative end of the resource economics spectrum tend to be more resilient to drought (Fry et al., 2018). This is thought to be because investment is made into more complex structural carbohydrates, potentially with deeper roots for accessing water at deep soil layers, with slower uptake rates. Recent evidence suggests that the resource economics strategy of herbaceous species can be an important variable in root exudation chemistry (Williams et al., 2021), with investment into exudation increased in more competitive, resource acquisitive species driving increased soil nitrogen (N) cycling (Kastovska et al., 2015). The increase in exuded metabolites, and changes in their chemistry, was inferred to be the cause of increased denitrification and respiration in soils under grasses with acquisitive resource strategies compared with conservative (Guyonnet et al., 2018), although it has been theorised that the N poor root systems of conservative species can minimise N losses in the case of disturbances (Kastovska et al., 2015), which could include root shedding under drought conditions. Given the contrast in resource use and exudation by conservative and acquisitive plant species, it is important to address the knowledge gap concerning their responses to abiotic stress to better understand ecosystem responses and ensure correct inputs to climate models.

Here we aim to begin to address the knowledge gaps outlined above, and to quantify the effects of abiotic stresses that would be consistent with those likely to seen more frequently under a warmer, drier climate on the plant inputs to, and chemical responses of, soil. We hypothesise that drought and warming will affect root exudate composition, and that drought and warming will interact to perturb the composition of plant inputs to soil more than each variable individually. We also hypothesise that leachate composition, here used as a proxy for soil chemistry, will also shift under drought and warming, and that the leachates from unplanted soil will differ fundamentally in their responses to stressors to leachates from planted soil, due to root exudates driving the soil response in planted pots. Finally, we hypothesise that exudates of plants and soil leachates with a different resource economics strategy will have differing responses to the same abiotic stresses, due to their different carbon economy, root system architecture, and microbial communities. To address these

hypotheses we ran a multifactorial controlled-environment experiment with the grasses *Anthoxanthum odoratum* (sweet vernal grass), and *Dactylis glomerata* (cock's foot), and pots of unplanted soil, subjecting them to drought, warming, or both, before collecting leachates and exudates. These two species of grass were selected because of their high incidence in mesotrophic grasslands and contrasting life history strategies, with one (*D. glomerata*) being acquisitive, with a rapid growth rate and high specific leaf area (SLA), and the other (*A. odoratum*) being conservative, with a slow growth rate and low SLA (Baxendale et al., 2014).

4.5 Materials and Methods

4.5.1 Experimental design

We germinated seed of *Anthoxanthum odoratum* and *Dactylis glomerata* (Emorsgate Seeds, Somerset UK) on potting soil for one week in a growth chamber set to 20℃, 18/6h photoperiod until cotyledon leaves appeared, before seedlings were transferred to transferred to an individual pot. We filled ninety-six pots (round, 10cm diameter, 10cm deep) with 400g of a field soil mixed 50/50 v/v with sand. The soil was collected from Colt Park in the north of England (54°11' 37.1''N 2°20' 54.9''W, altitude 348m), a lightly-grazed mesotrophic grassland with a history of limited addition of fertiliser. The soil was collected in May 2019, sieved at 4mm, and stored at 4℃ until use. We grew them for 6 weeks before subjecting them to two weeks of the treatment effect followed by two weeks of recovery. Twenty-four mesocosms were maintained at 60% water holding capacity (WHC) throughout the experiment, and twenty-four plants were for two weeks subjected to a drought treatment, to be dried to 30% water holding capacity. Twelve of these from each watering regime were put into a 25℃ cabinet concurrently with the drought stage, whilst the others remained at 20℃, to give four treatments (Fig. 1). Due to limited plant growth space, only two cabinets were available. As well as these planted mesocosms, four pots of unplanted soil were subjected to the same four treatment effects.

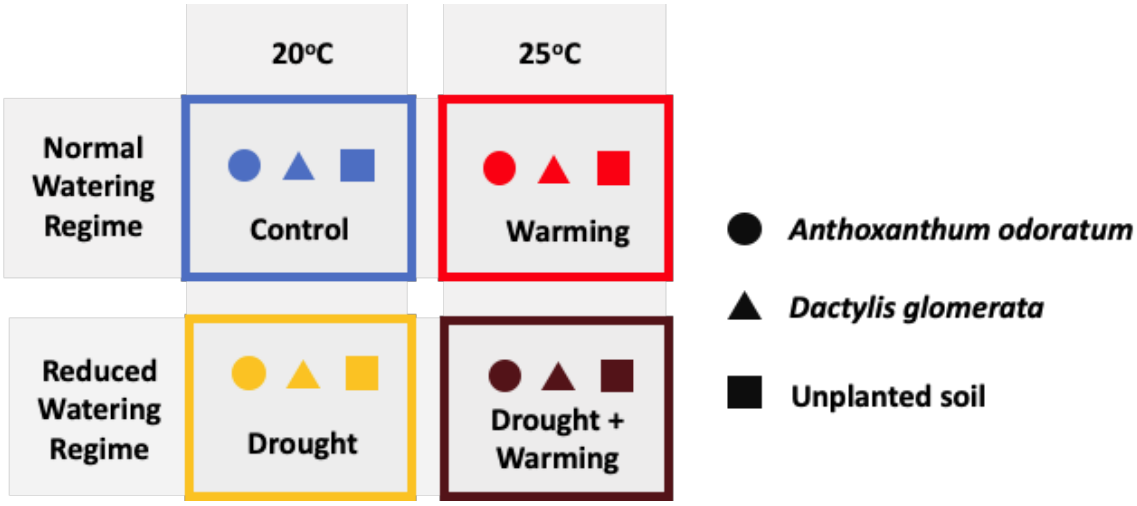

Figure 1: A summary of the conditions used in the treatment stage of this experiment, with the colours, symbols, and the four names used to refer to each treatment group: Control, 20°C and normal watering, in blue; Warming, 25°C and normal watering, in red; Drought, 20°C and reduced watering, in yellow; and Drought + Warming, 25°C and reduced watering, in burgundy. *Anthoxanthum odoratum* is shown with a circle, *Dactylis glomerata* with a triangle, and unplanted soil with a square.

4.5.2 Leachate Collection

After a total of ten weeks growth in the mesocosms, the soluble fraction of the soil column - leachates - were collected by dripping MilliQ water through the intact root-soil system into a 100ml container until filled. The container was placed on ice during collection to reduce biochemical turnover. This leachate sample was then filtered using a 0.22µm filter (Merck Millipore (U.K.) Limited, Watford, SLGP033RS) that had been previously washed with MilliQ water, and transferred to three 50 ml Falcon Tubes (Greiner Bio-one CellStar 227261), and immediately frozen at -80°C. The frozen exudate solution was freeze-dried using a Scavac CoolSafe 55-9 Pro (LaboGene, Lynge, Denmark) for 48 hours (Williams et al., 2021b).

4.5.3 Exudate Collection

To prepare the plants for the hydroponic root repair stage, we carefully washed the roots clean of all soil particles and placed the plants into 100ml Schott Duran® bottles (SCHOTT UK Ltd., Stafford, UK) with a soil solution. The plants were suspended with their roots submerged in the soil solution, and the aboveground fraction held clear of the fluid using Azpak non-absorbent cotton wool. The soil solution was created by mixing the same soil as for the mesocosm work in water at a 1:1 v/v soil/water mix and leaving to settle at 5℃ for three days, before removing the settled soil. The hydroponics were aerated using a HiDOM HD-603 aquarium pump (Shenzhen Hidom Electric Co., Ltd, Shenzhen, China) set to a flow rate of approximately 1ml s^{-1}. The hydroponic treated plants were returned to the growth chamber under the same conditions for three days before harvest (Williams et al., 2021a and Williams et al., 2021b).

All plants were removed from the hydroponic recovery system, and had their root systems carefully rinsed in two 1L beakers of water and one 1L beaker of milliQ water to wash off any remaining soil solution. Plants were suspended in a milliQ-rinsed 100mL glass Schott bottle with Parafilm M (Bemis Company, Inc. Neenah, WI, USA), with their roots immersed in 100mL of milliQ water. These bottles were placed on ice at 60rpm on a rotary shaker for 120 minutes. This exudate sample was filtered, frozen and freeze-dried as above.

4.5.4 ATR-FTIR

We chose ATR-FTIR as it is a method that produces high-quality infra-red spectroscopic data even at low volumes. In order to avoid any potential issues with signal-to-noise ratio with the low absolute volume of chemical species in exudates, and to avoid signal saturation on transmission FTIR with the high absolute volume of chemicals in the leachate samples, ATR-FTIR was therefore chosen for analysis of both sample types, enabling the results to be directly comparable. The data produced will be infra-red spectra of the leachate and exudate samples, and will allow us to address our hypotheses by showing differences in metabolic fingerprint between treatments. The collected exudates and leachates were resuspended in two microlitres of HP-LC-grade water, and transferred onto the diamond of an Attenuated Total

Reflectance (ATR) accessory for the Bruker Invenio S FTIR (Bruker, Billerica, MA, USA) (Fig. 2), and dried there using a commercially available hairdryer on the cold setting. Data were collected in the 4000–400 cm^{-1} range at a resolution of 4 cm^{-1}. 70% ethanol was used to clean the diamond between samples, and a new background measurement was taken before every sample.

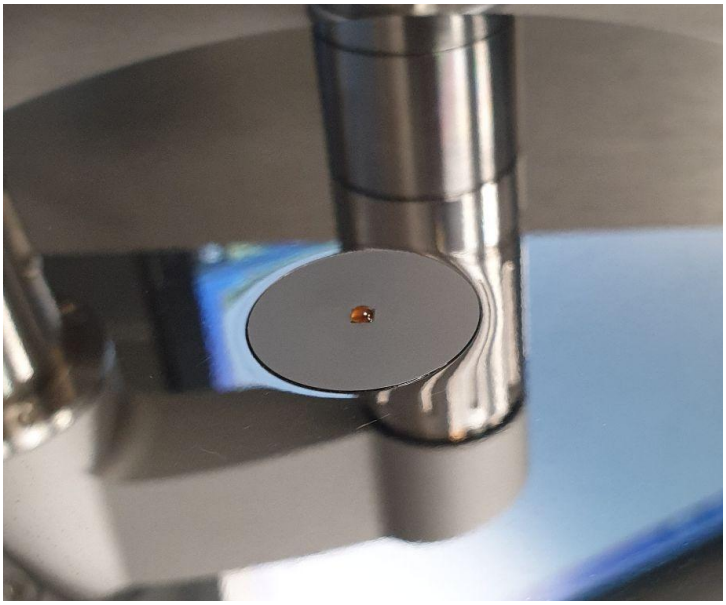

Figure 2: Two microlitres of soil leachate on the diamond of an ATR-FTIR, before the drying stage

4.5.5 Statistical analysis

Spectral data were processed to remove interference from CO_2 in the 2400-2275 wavenumber region and trimmed to the 4000-800 wavenumber range before undergoing baseline correction and normalisation using the cluster toolbox in MATLAB (Mathwork, MA; Cluster Toolbox available at https://github.com/Biospec/cluster-toolbox-v2.0). The exudates and leachates data were analysed separately, with the exudates data having samples from two species, *A. odoratum* and *D. glomerata,* and the leachate dataset having these two species and additionally having samples from unplanted soil. The processed FTIR data of the exudates and leachates were analysed using a Principal Coordinate Analysis (PCoA) using the metaMDS function of the *vegan* package (Oksanen et al., 2020) in R4.0.3 (Bunny-Wunnies Freak Out). This creates a Bray-Curtis dissimilarity matrix of the data and iteratively performs non-metric

multi-dimensional scaling (NMDS) to find an optimal stress solution, before scaling and rotating it. The data were plotted to display significant treatment effects. A permutational Analysis of Variance (PERMANOVA) was conducted on the NMDS scores using the envfit command, which tested for significant treatment effects through 1000 permutations.

4.6 Results

4.6.1 Exudates

Principal Coordinate Analysis in non-metric multidimensional scaling was run on the exudates FTIR data. The first two axes explain 65.7% of the variation, with almost all of this explained by the first axis (Figure 3). The ordination stress was 0.152, which is below the 0.2 level at which caution must be used in interpretation, and we did not see unexpected sub-populations in the data.

When we interrogated the data using PERMANOVA we found the overall ordination space only showed a significant effect of species (r^2=0.098, p<0.001). However, examination of the individual PCoA axes found that on axis one there was a significant effect of warming ($F_{1,88}$=5.59, p=0.020). There was a highly significant effect of species on both axes (axis 1 $F_{1,88}$=17.39, p<0.001; axis 2 $F_{1,88}$=45.61, p<0.001). There was no interaction observed, so both species responded to warming in a similar manner. The effect is highlighted in the PCoA in Figure 3. The loadings from the first PCoA axis were then plotted against the wavenumbers from the original data to reveal what spectral features were driving the differences behind the significant effect of warming on this axis, which can be seen in Figure 4 along with overlays highlighting regions containing bond types in the fatty acid, (poly)saccharide, amide, and mixed regions as defined by Schmidt and Flemming (1998). The amide and mixed regions were relatively enriched in the warmed samples, with strong differentiating wavenumbers around 1604 cm^{-1} and 1401 cm^{-1}, with the type of poly(sacharride) linkages changing between 20°C and 25°C treatments, with the 20°C treatments being relatively enriched around wavenumbers 1015 cm^{-1} and 987 cm^{-1}, with 1156 cm^{-1} being relatively enriched in the 25°C treatments.

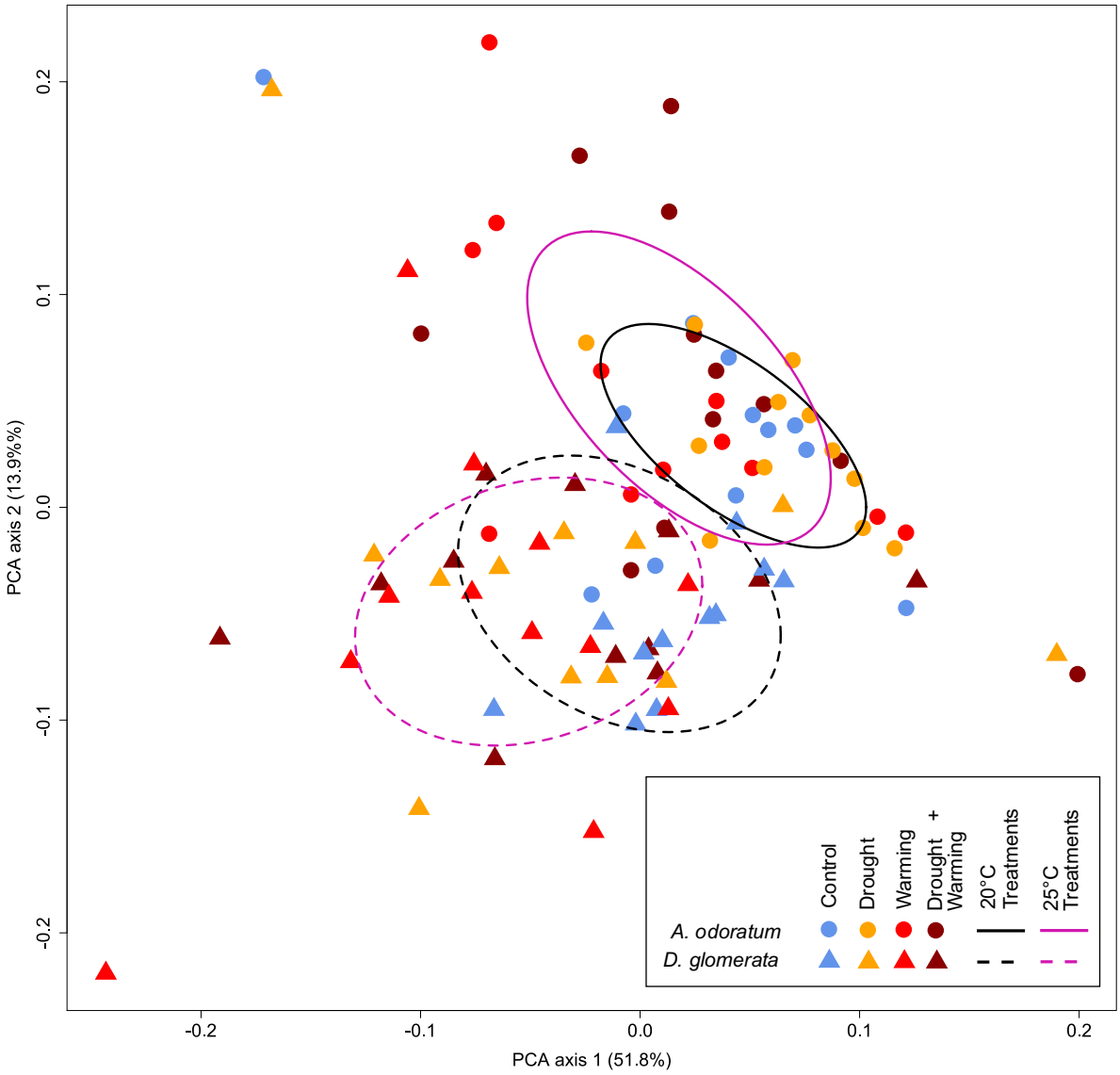

Figure 3: Principal Coordinate Analysis plot of exudate FTIR data. *Anthoxanthum odoratum* are shown as circles, with solid ellipses, and *Dactylis glomerata* as triangles, with dashed ellipses. Treatments are shown by point colour, with Control shown in blue, Drought shown in orange, Warming in red, and Drought + Warming in burgundy. 95% confidence ellipses are shown for the four groups highlighted as significantly different by PERMANOVA, with the groups subjected to 25°C during the treatment phase (Warming and Drought + Warming) drawn with a purple line, and the ellipses of the groups not subjected to warming (kept at 20°C during treatment phase; Drought and Control) drawn with black lines.

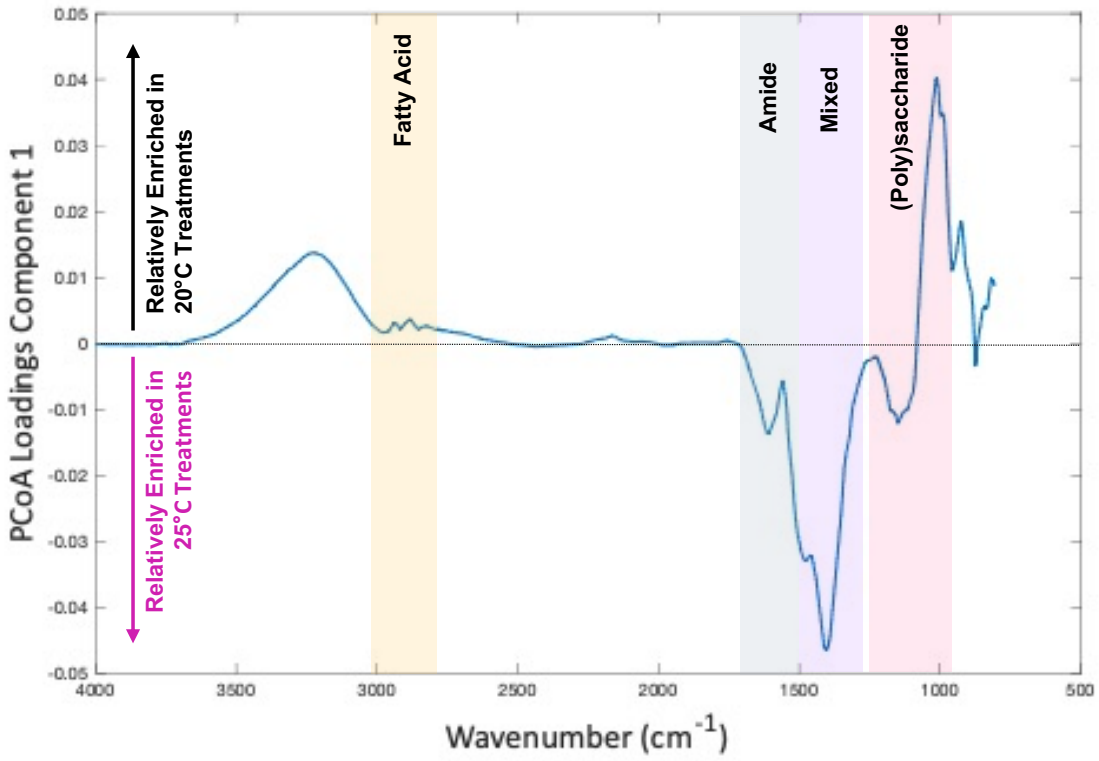

Figure 4: The most discriminating wavenumbers between the 20°C and 25°C treatments groups for exudates collected from both *Anthoxanthum odoratum* and *Dactylis glomerata* (blue line). Overlays are given for regions containing bond types in the fatty acid, (poly)saccharide, amide, and mixed regions as defined by Schmidt and Flemming (1998).

4.6.2 Leachates

Non-metric multidimensional scaling through PCoA was performed on the leachate FTIR data. The first axis explains the overwhelming majority of the variation in the data, at 94.9%. This is largely due to the huge difference between planted and unplanted soil that drove almost all of this variation (the split between planted and unplanted soil can be seen in Figure 5A). Together, the first and second axes explain 98.4% of the variation. The ordination stress was 9.21×10^{-5}. When the two species and the soil are contrasted in ordination space, there is a perfect fit ($r^2=1$, $p<0.001$). On closer inspection of the individual axes, axis 1 showed a highly significant three-way interaction between the species/soil treatment and the drought and the warming treatments ($F_{2,35}=5.91$, $p=0.006$). This three-way interaction takes the form of

opposing responses between soil and species (Fig 5A). Axis 2 showed a highly significant interaction between drought and warming, but no effect of species ($F_{1,35}$=7.62, p=0.009). Figure 4B shows this effect, with the Warming treatments of both species moving down the plot further from Controls than the Drought + Warming treatment. The loadings from the first and second PCoA axis were then plotted against the wavenumbers from the original data to show which spectral features were driving the differences behind the significant effects of planting on axis 1 and of warming on axis 2, which can be seen in Figure 6A and 6B respectively, along with overlays highlighting regions containing bond types in the fatty acid, (poly)saccharide, amide, and mixed regions as defined by Schmidt and Flemming (1998). The planted soils differed from the unplanted soils in terms of the types of proteins and peptides present with a shift from 1587cm^{-1} to 1644 cm^{-1}, and the mixed region was relatively enriched in unplanted soil, the differences driven by a strong peak at 1323cm^{-1}. The differences were also driven by a relative abundance in the planted samples of the bonds in the poly(saccharide) region, with peaks at 1107 cm^{-1}, 1054 cm^{-1}, 1031 cm^{-1}, and 989 cm^{-1}. The warmed treatments, the differentiator on axis 2, differed in amine content, with a peak at 1650 cm^{-1} showing relative enrichment in 25°C treatments. The mixed region shows a number of peaks driving the differences between warmed and unwarmed groups, with peaks at 1419 cm^{-1} and 1310 cm^{-1} showing enrichment in the 20°C treatments. The type of poly(saccharide) linkages also changed between 20°C and 25°C treatments, with the controls showing comparative enrichment at wavenumbers 1126 cm^{-1}, 1044 cm^{-1}, and 987 cm^{-1}.

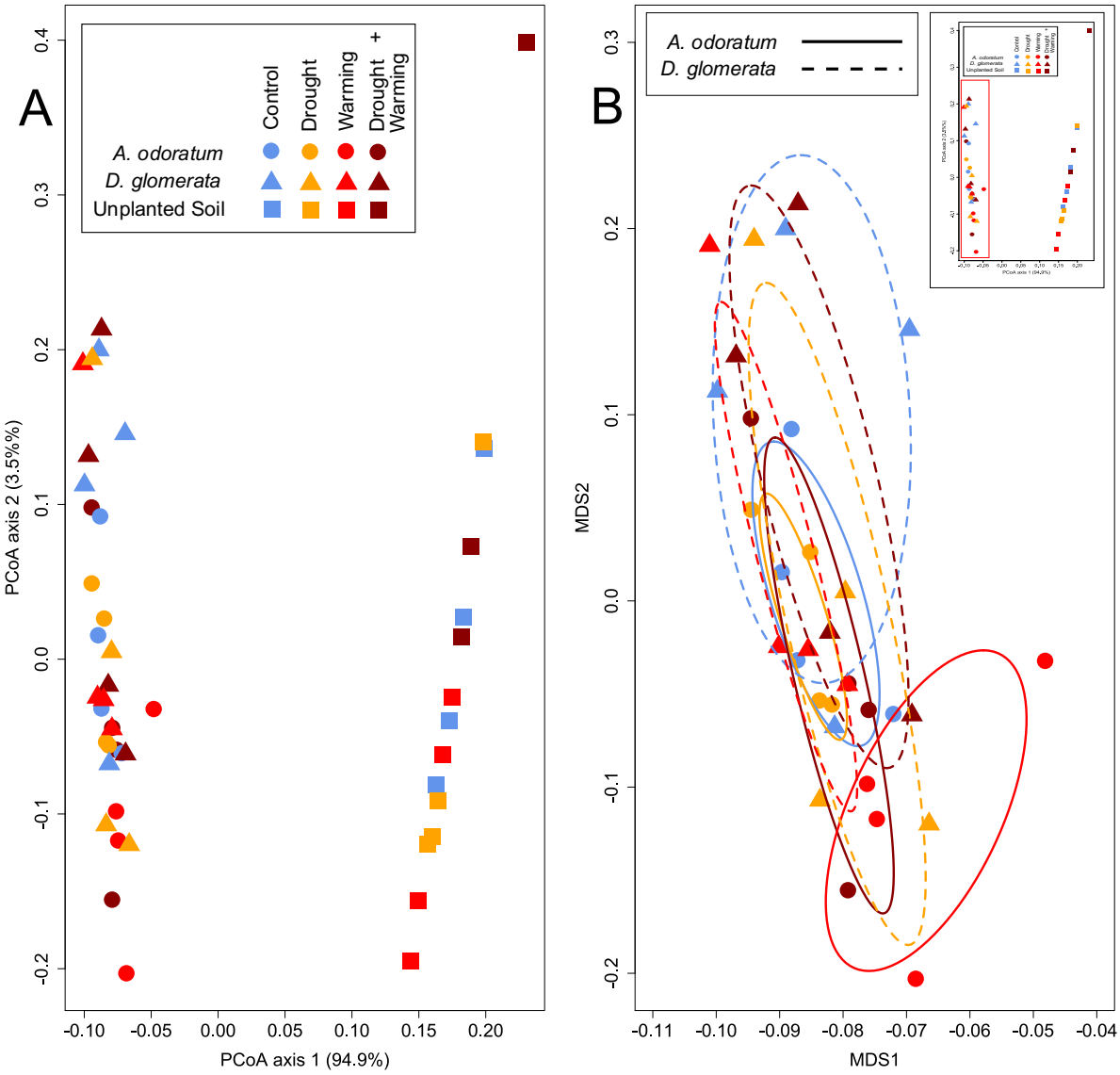

Figure 5: Principal Coordinate Analysis plot of leachate FTIR data. A) *Anthoxanthum odoratum* are shown as circles, and *Dactylis glomerata* as triangles, with bare soil shown as squares. Treatments are shown by point colour, with control shown in blue, drought shown in orange, warming in red, and drought and warming in burgundy. B) Inset of the Principal Coordinate Analysis plot of leachate FTIR data shown in (A), *Anthoxanthum odoratum* are shown as circles, with solid 95% confidence ellipses, and *Dactylis glomerata* as triangles, with dashed 95% confidence ellipses. Treatments are shown by point and ellipse colour, with Control shown in blue, Drought shown in orange, Warming in red, and Drought + Warming in burgundy.

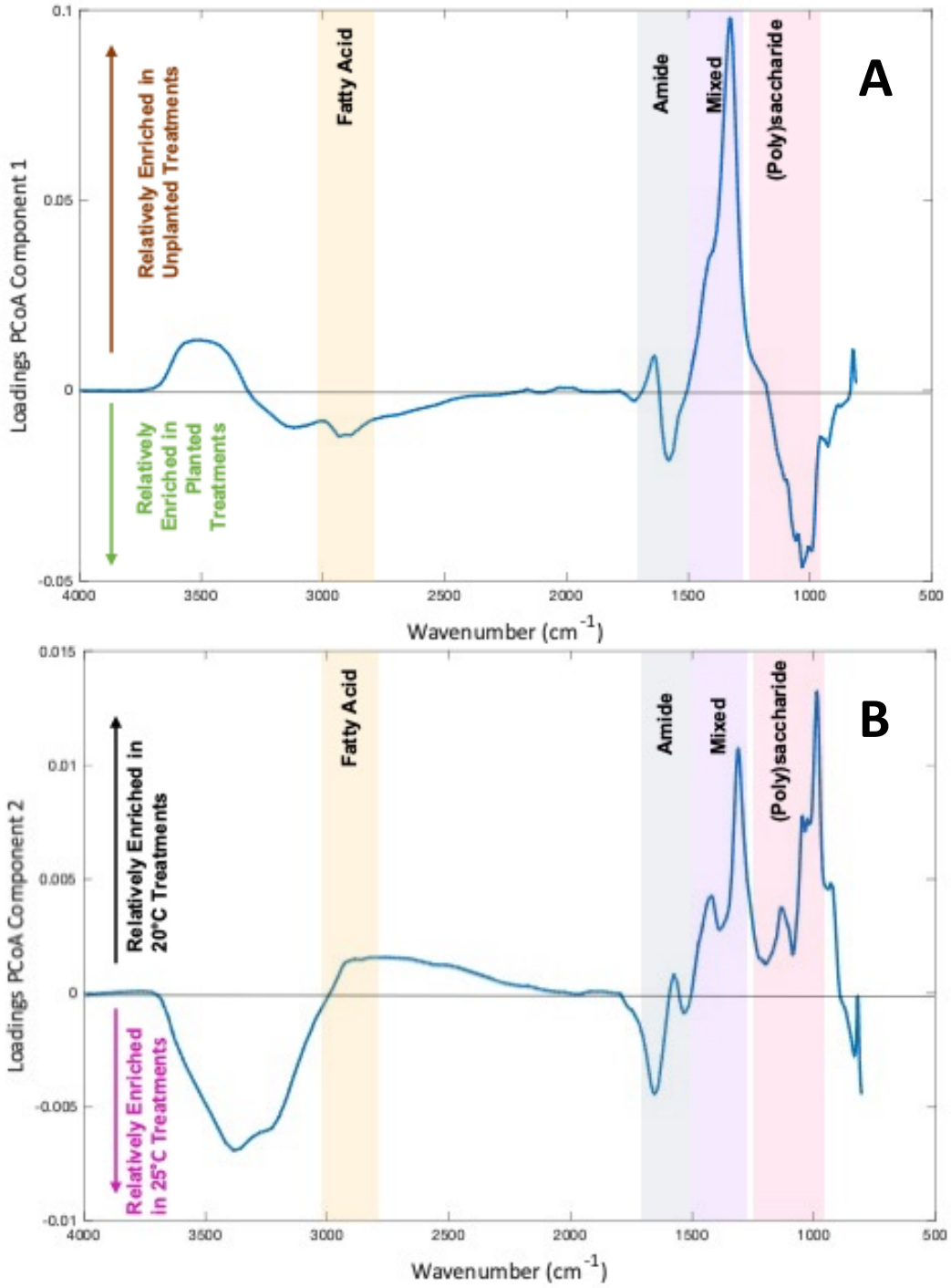

Figure 6: The most discriminating wavenumbers driving the analysis shown in Figure 5. Overlays are given for regions containing bond types in the fatty acid, (poly)saccharide, amide, and mixed regions as defined by Schmidt and Flemming (1998). A) The most discriminating wavenumbers (blue line) driving the first component of the analysis shown in Figure 5, which separates planted and unplanted soil. Wavenumbers where the loadings trace below y=0 (dashed black line) are relatively enriched in the planted treatments; B) The most discriminating wavenumbers (blue line) between 20°C and 25°C treatments for exudates collected from both *Anthoxanthum odoratum* and *Dactylis glomerata*, Wavenumbers where the loadings trace below y=0 (dashed black line) are relatively enriched in 25°C.

4.7 Discussion

The aim of this experiment was to quantify the effects of abiotic stresses that would be consistent with those likely to be frequently seen under a warming, drier climate on the plant inputs to, and chemical responses of, soil. We hypothesised that drought and warming would have strong and synergistic effects on both exudates and leachates, and that the two grasses, selected because their resource use strategy puts them at different ends of the fast-slow spectrum, would show different responses to the climate treatments. Our study found that in both species warming was a much more important driver of exudation changes than drought, and that while the plant species showed different exudation patterns, these both responded to warming in a similar manner. Our findings indicate that resource strategy is not an important indicator of climate-driven changes in plant inputs into the soil.

Our first hypothesis was concerned with the impact of drought and warming on root exudation of *A. odoratum* and *D. glomerata*. We anticipated that both treatments would have a strong effect, and that the interaction would have a synergistic effect, amplifying shifts in exudation chemistry. Surprisingly, we only found an effect of warming. The warming effected a change in the proportion of biomolecules collected in exudates from poly(saccharides) to amide-containing compounds such as proteins, peptides, and possibly free amino acids (using the labels for wavenumber as defined by Schmidt and Flemming (1998)). This could imply that there has been a stress-induced drop in exudate absolute volume or carbon investment, or a change in chemical makeup of exudates. This could corroborate the results of De Vries et al. (2019), who found that exudates collected from plants that had been exposed to drought induced the same amount of microbial respiration as exudates from plants that had not been subjected to drought stress, even though there was a lower amount of total carbon in exudates of droughted plants. Taken together, this is evidence of plants changing exudate chemical composition in response to a stress, and effecting a change in microbial responses. In previous work strong effects of drought have been shown in exudates of *A. odoratum* (e.g. Chapter 3), but these were not apparent in this study. However, it is possible that the strength of the warming effect was such that drought was non-significant by comparison. Previous *in situ* studies have found effects of interaction of drought and warming on exudation, including a study on plant responses to the natural warming and drying seen in the Mediterranean ecosystem over summer (Jakoby et al., 2020). Here tree roots were uncovered from the soil,

and exudates were collected along with a tissue sample to identify the species by DNA. For all species studied, drought and warming had the most profound effect on the chemical makeup of the root exudates. The different result compared with our study may be due to the focus on trees, or due to the species being adapted to drought and warming, which may not be the case for the grasses studied here. Other studies have shown that warming often triggers increased exudation in order to galvanise microbial enzyme activity, which will produce plant-available nutrients and thus keep up with the increased metabolic requirements of the plant (Zhang et al., 2020). There is also evidence that warming increases membrane permeability of root cells, which increases exudation (Allison et al., 2010). This raises the question of how much control plants have over the exact composition of exudation, and whether some exudates are merely the removal of waste or potentially harmful molecules. If the membrane becomes more permeable under warming, this could indicate that plants are exuding molecules more indiscriminately, which could have effects on microbial recruitment and function. Our use of a recovery period back at standard temperature should reduce the effect of warming on such factors, and likewise the drought recovery period in our study removes a possible confounding factor where increased exudation under drought conditions may be due to root mortality (Henry et al., 2007). There is also the possibility that a small fraction of the collected exudate is microbial in origin, even though this collection method is set up to minimise this; thus the differences may be partially due to differential microbial responses to the treatments.

Our second hypothesis was that we expected a larger change in leachate chemistry in soils that had been subjected to both drought and warming than the treatment factors individually, and that there would be a large contrast between soils that hosted a plant, and bare soils. We accepted part of this hypothesis: the difference between planted and unplanted soils resulted in almost total explained variance. Given the role of ecosystem engineers that plants fulfil, this is unsurprising in itself, but the strong relative abundance of bonds in the poly(saccharide) region in planted samples driving the differences between these groups is of note, as clearly this is a plant-derived component of the soil chemistry. Therefore plants alter soil chemistry in this system in a profound way, and any changes to the plant will likely have strong consequences for exudation and soil chemistry. However, we cannot accept our hypothesis that warming and drought together are more disruptive to soil chemistry than warming and drought individually. Whilst there is an interaction between drought and warming in both axes

of ordination space in our results, we see the reverse of the hypothesized effect - the most disruptive effect here to soil chemistry is warming alone. This may imply that plant-soil systems have inbuilt resilience to a combined effect of drought and warming, although many temperate areas of the globe are predicted to become warmer and *wetter*, rather than warmer and drier (IPCC, 2021), so the systems plants have evolved to cope with effects of high temperature may not be sufficient under an altered climate. The warming effect on the leachates was strong, and led to a difference in amine content, which showed relative enrichment in 25°C treatments, but the type of poly(saccharide) linkages also changed, with the control temperature of 20°C showing comparative enrichment. The strength of the response to warming seen in the exudates of this experiment may be driving the more extreme shifts in soil chemistry with this factor.

Our third hypothesis suggested that the two grass species would respond differently to drought and warming due to their contrasting resource economics strategy. While the species did have different metabolic fingerprints, they both changed in the same direction after warming, both changing the proportion of poly(saccharide) to amide, and did not strongly respond to drought. This indicates that there is a general response to warming that is independent of resource use, and could add support to the theory that membrane permeability increases under warming (Allison et al., 2010). We chose *A. odoratum* and *D. glomerata* because they contrast on the resource economics spectrum: *D. glomerata* is more acquisitive, faster growing and forages more rapidly in the soil (Baxendale et al., 2014). It has previously been inferred that root exudation differs dramatically between acquisitive and conservative species by measuring proxies such as soil ecosystem nitrogen cycling, with the soil microbial biomass also differing, being lower and less variable over seasonal changes for conservative plant species (Kaštovská et al., 2015). Consequently, we expected higher volumes of exudation, but for it to be potentially more impacted by the climate changes with our *D. glomerata* samples. The separation seen between the the two species indicated that there was a strong difference in exudate composition, but that warming had a broadly similar effect. The lack of a significant interaction between species identity and the climate treatments indicates that, contrary to our expectation, more acquisitive species did not seem to be more strongly affected than conservative. Of further interest is the lack of effect of drought. This is unexpected because in other studies both species are highly responsive to

climate changes in terms of root and shoot traits (Baxendale et al., 2014; de Vries et al., 2016) and, in the case of *A. odoratum*, exudation (Chapter 3).

4.8 Conclusions

Plants are known to engineer rhizosphere conditions in order to drive carbon cycling and nutrient acquisition, and that warming increases activity (Kuzyakov & Razavi, 2019). Here we have shown in two grass species of contrasting life strategies that exudation chemistry changes, mainly in response to warming, and less to drought, and that these changes have downstream effects on the composition of soil chemistry, the first time that warming effects have been explored using these methods of exudate collection or spectroscopy. The successful use of infra-red spectroscopy to illuminate these effects in this study opens the possibility for an inexpensive, high-throughput method of tracking changes in soil chemistry, and together with the non-destructive nature of leachate sampling enables the design of longitudinal studies of soil chemistry on low budgets. Further work could involve such studies, and also examine whether these changes result in an alteration of microbial community structure.

4.9 Acknowledgements

R.M. was supported by a Biotechnology and Biological Sciences (BBSRC) Doctoral Training Partnership (reference 1792130), which also funded this work. F.T.d.V. was funded by a BBSRC David Phillips Fellowship (BB/L02456X/1).

4.10 References

Allison, Steven D., Matthew D. Wallenstein, and Mark A. Bradford. 2010. “Soil-Carbon Response to Warming Dependent on Microbial Physiology.” *Nature Geoscience 2010 3:5* 3 (5). Nature Publishing Group:336–40. https://doi.org/10.1038/ngeo846.

Baxendale, Catherine, Kate H. Orwin, Franck Poly, Thomas Pommier, and Richard D. Bardgett. 2014. “Are Plant-Soil Feedback Responses Explained by Plant Traits?” *Journal of Physiology*. https://doi.org/10.1111/nph.12915.

Cattivelli, Luigi, Fulvia Rizza, Franz-W Badeck, Elisabetta Mazzucotelli, Anna M Mastrangelo, Enrico Francia, Caterina LastNameLastNameMarè, Alessandro Tondelli, and A Michele Stanca. 2008. “Drought Tolerance Improvement in Crop Plants: An Integrated View from Breeding to Genomics.” *Field Crops Research*. https://doi.org/10.1016/j.fcr.2007.07.004.

Chapin, F S, Autumn K Iii, and F Pugnaire. 2000. “Root Structure and Function in an Ecological Context.” *New Phytologist* 148 (3). John Wiley & Sons, Ltd:353–54. https://doi.org/10.1046/J.1469-8137.2000.00781.X.

De Vries, Franciska T., Alex Williams, Fiona Stringer, Robert Willcocks, Rosie McEwing, Holly Langridge, and Angela L. Straathof. 2019. “Changes in Root-Exudate-Induced Respiration Reveal a Novel Mechanism through Which Drought Affects Ecosystem Carbon Cycling.” *New Phytologist* 224 (1). John Wiley & Sons, Ltd:132–45. https://doi.org/10.1111/nph.16001.

De Vries, Franciska T., Caley Brown, and Carly J. Stevens. 2016. “Grassland Species Root Response to Drought: Consequences for Soil Carbon and Nitrogen Availability.” *Plant and Soil 2016 409:1* 409 (1). Springer:297–312. https://doi.org/10.1007/S11104-016-2964-4.

De Vries, Franciska T., Mira E. Liiri, Lisa Bjørnlund, Matthew A. Bowker, Søren Christensen, Heikki M. Setälä, and Richard D. Bardgett. 2012. “Land Use Alters the Resistance and Resilience of Soil Food Webs to Drought.” *Nature Climate Change 2012 2:4* 2 (4). Nature Publishing Group:276–80. https://doi.org/10.1038/nclimate1368.

Dyer, Carmen L., Peter M. Kopittke, Anna R. Sheldon, and Neal W. Menzies. 2008. “Influence of Soil Moisture Content on Soil Solution Composition.” *Soil Science Society of America Journal* 72 (2). John Wiley & Sons, Ltd:355–61. https://doi.org/10.2136/SSSAJ2007.0124.

Folke, Carl, Steve Carpenter, Brian Walker, Marten Scheffer, Thomas Elmqvist, Lance Gunderson, and C. S. Holling. 2004. “Regime Shifts, Resilience, and Biodiversity in Ecosystem Management.” *Http://Dx.Doi.Org/10.1146/Annurev.Ecolsys.35.021103.105711* 35 (November). Annual Reviews:557–81. https://doi.org/10.1146/ANNUREV.ECOLSYS.35.021103.105711.

Fry, Ellen L., Joanna Savage, Amy L. Hall, Simon Oakley, W. J. Pritchard, Nicholas J. Ostle, Richard F. Pywell, James M. Bullock, and Richard D. Bardgett. 2018. “Soil Multifunctionality and Drought Resistance Are Determined by Plant Structural Traits in Restoring Grassland.” *Ecology* 99 (10). John Wiley & Sons, Ltd:2260–71. https://doi.org/10.1002/ECY.2437.

Gargallo-Garriga, Albert, Jordi Sardans, Marta Ayala-Roque, Bjarni D. Sigurdsson, Niki I.W. Leblans, Michal Oravec, Karel Klem, Ivan A. Janssens, Otmar Urban, and Josep Peñuelas. 2021. “Warming Affects Soil Metabolome: The Case Study of Icelandic Grasslands.” *European Journal of Soil Biology* 105 (July). Elsevier Masson:103317. https://doi.org/10.1016/j.ejsobi.2021.103317.

Gargallo-Garriga, Albert, Jordi Sardans, Míriam Pérez-Trujillo, Michal Oravec, Otmar Urban, Anke Jentsch, Juergen Kreyling, Carl Beierkuhnlein, Teodor Parella, and Josep Peñuelas. 2015. "Warming Differentially Influences the Effects of Drought on Stoichiometry and Metabolomics in Shoots and Roots." *New Phytologist* 207 (3). John Wiley & Sons, Ltd:591–603. https://doi.org/10.1111/NPH.13377.

Guyonnet, Julien P., Amélie A. M. Cantarel, Laurent Simon, and Feth el Zahar Haichar. 2018. "Root Exudation Rate as Functional Trait Involved in Plant Nutrient-Use Strategy Classification." *Ecology and Evolution* 8 (16). John Wiley & Sons, Ltd:8573–81. https://doi.org/10.1002/ECE3.4383.

Guyonnet, Julien P., Amélie A.M. Cantarel, Laurent Simon, and Fethel Zahar Haichar. 2018. "Root Exudation Rate as Functional Trait Involved in Plant Nutrient-Use Strategy Classification." *Ecology and Evolution* 8 (16). John Wiley & Sons, Ltd:8573–81. https://doi.org/10.1002/ECE3.4383.

Henry, Amelia, William Doucette, Jeanette Norton, and Bruce Bugbee. 2007. "Changes in Crested Wheatgrass Root Exudation Caused by Flood, Drought, and Nutrient Stress." *Journal of Environmental Quality* 36 (3). John Wiley & Sons, Ltd:904–12. https://doi.org/10.2134/JEQ2006.0425SC.

IPCC, 2021: Climate Change 2021: The Physical Science Basis. Contribution of Working Group I to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change [Masson-Delmotte, V., P. Zhai, A. Pirani, S.L. Connors, C. Péan, S. Berger, N. Caud, Y. Chen, L. Goldfarb, M.I. Gomis, M. Huang, K. Leitzell, E. Lonnoy, J.B.R. Matthews, T.K. Maycock, T. Waterfield, O. Yelekçi, R. Yu, and B. Zhou (eds.)]. Cambridge University Press. In Press.

J Oksanen, F Guillaume Blanchet, Michael Friendly, Roeland Kindt, Pierre Legendre, Dan McGlinn, Peter R Minchin, RB O'Hara, GL Simpson, P Solymos, MHH Stevens, E Szoecs, H Wagner. 2020 "vegan: Community Ecology Package. R package version 2.5-6

Jakoby, Gilad, Ido Rog, Shacham Megidish, and Tamir Klein. 2020. "Enhanced Root Exudation of Mature Broadleaf and Conifer Trees in a Mediterranean Forest during

the Dry Season." *Tree Physiology* 40 (11). Oxford Academic:1595–1605. https://doi.org/10.1093/TREEPHYS/TPAA092.

Karlowsky, Stefan, Angela Augusti, Johannes Ingrisch, Roland Hasibeder, Markus Lange, Sandra Lavorel, Michael Bahn, and Gerd Gleixner. 2018. "Land Use in Mountain Grasslands Alters Drought Response and Recovery of Carbon Allocation and Plant-Microbial Interactions." *Journal of Ecology* 106 (3). John Wiley & Sons, Ltd:1230–43. https://doi.org/10.1111/1365-2745.12910.

Kaštovská, Eva, Keith Edwards, Tomáš Picek, and Hana Šantrůčková. 2015. "A Larger Investment into Exudation by Competitive versus Conservative Plants Is Connected to More Coupled Plant–Microbe N Cycling." *Biogeochemistry* 122 (1). Kluwer Academic Publishers:47–59. https://doi.org/10.1007/S10533-014-0028-5.

Kuzyakov, Yakov, and Bahar S. Razavi. 2019. "Rhizosphere Size and Shape: Temporal Dynamics and Spatial Stationarity." *Soil Biology and Biochemistry* 135 (August). Pergamon:343–60. https://doi.org/10.1016/J.SOILBIO.2019.05.011.

Pérez-Ramos, Ignacio M., Catherine Roumet, Pablo Cruz, Alain Blanchard, Paul Autran, and Eric Garnier. 2012. "Evidence for a 'Plant Community Economics Spectrum' Driven by Nutrient and Water Limitations in a Mediterranean Rangeland of Southern France." *Journal of Ecology* 100 (6). John Wiley & Sons, Ltd:1315–27. https://doi.org/10.1111/1365-2745.12000.

Puijalon, Sara, Tjeerd J. Bouma, Christophe J. Douady, Jan van Groenendael, Niels P.R. Anten, Evelyne Martel, and Gudrun Bornette. 2011. "Plant Resistance to Mechanical Stress: Evidence of an Avoidance–Tolerance Trade-Off." *New Phytologist* 191 (4). John Wiley & Sons, Ltd:1141–49. https://doi.org/10.1111/J.1469-8137.2011.03763.X.

Rillig, Matthias C., Anika Lehmann, James A. Orr, and Walter R. Waldman. 2021. "Mechanisms Underpinning Nonadditivity of Global Change Factor Effects in the Plant–Soil System." *New Phytologist* 232 (4):1535–39. https://doi.org/10.1111/nph.17714.

Riva, Enrique G. de la, Ambra Tosto, Ignacio M. Pérez-Ramos, Carmen M. Navarro-Fernández, Manuel Olmo, Niels P.R. Anten, Teodoro Marañón, and Rafael Villar. 2016. "A Plant Economics Spectrum in Mediterranean Forests along Environmental Gradients: Is There Coordination among Leaf, Stem and Root Traits?" *Journal of Vegetation Science* 27 (1). John Wiley & Sons, Ltd:187–99. https://doi.org/10.1111/JVS.12341.

Schmitt, Jürgen, and Hans Curt Flemming. 1998. "FTIR-Spectroscopy in Microbial and Material Analysis." *International Biodeterioration & Biodegradation* 41 (1). Elsevier:1–11. https://doi.org/10.1016/S0964-8305(98)80002-4.

Uselman, Shauna M, Robert G Qualls, and Richard B Thomas. 2000. "Effects of Increased Atmospheric CO 2 , Temperature, and Soil N Availability on Root Exudation of Dissolved Organic Carbon by a N-Fixing Tree (Robinia Pseudoacacia L.)." *Plant and Soil* 222:191–202.

Wang, Qitong, Lanying Chen, Hang Xu, Kexin Ren, Zhenggang Xu, Ying Tang, and Juan Xiao. 2021. "The Effects of Warming on Root Exudation and Associated Soil N Transformation Depend on Soil Nutrient Availability." *Rhizosphere* 17 (March). Elsevier:100263. https://doi.org/10.1016/J.RHISPH.2020.100263.

Williams, Alex, Holly Langridge, Angela L. Straathof, Graeme Fox, Howbeer Muhammadali, Katherine A. Hollywood, Yun Xu, Royston Goodacre, and Franciska T. de Vries. 2021. "Comparing Root Exudate Collection Techniques: An Improved Hybrid Method." *Soil Biology and Biochemistry* 161 (October). Pergamon:108391. https://doi.org/10.1016/J.SOILBIO.2021.108391.

Williams, Alex, Holly Langridge, Angela L. Straathof, Howbeer Muhamadali, Katherine A. Hollywood, Royston Goodacre, and Franciska T. de Vries. 2021. "Root Functional Traits Explain Root Exudation Rate and Composition across a Range of Grassland Species." *Journal of Ecology* 00. John Wiley & Sons, Ltd:1–13. https://doi.org/10.1111/1365-2745.13630.

Wright, Ian J., Peter B. Reich, Mark Westoby, David D. Ackerly, Zdravko Baruch, Frans Bongers, Jeannine Cavender-Bares, et al. 2004. "The Worldwide Leaf Economics

Spectrum." *Nature 2004 428:6985* 428 (6985). Nature Publishing Group:821–27. https://doi.org/10.1038/nature02403.

Wright, Ian J., Peter B. Reich, Mark Westoby, David D. Ackerly, Zdravko Baruch, Frans Bongers, Jeannine Cavender-Bares, et al. 2004. "The Worldwide Leaf Economics Spectrum." *Nature 2004 428:6985* 428 (6985). Nature Publishing Group:821–27. https://doi.org/10.1038/nature02403.

Wu, Jiahui, and Shixiao Yu. 2019. "Effect of Root Exudates of Eucalyptus Urophylla and Acacia Mearnsii on Soil Microbes under Simulated Warming Climate Conditions." *BMC Microbiology 2019 19:1* 19 (1). BioMed Central:1–12. https://doi.org/10.1186/S12866-019-1604-6.

Yin, Huajun, Yufei Li, Juan Xiao, Zhenfeng Xu, Xinyin Cheng, and Qing Liu. 2013. "Enhanced Root Exudation Stimulates Soil Nitrogen Transformations in a Subalpine Coniferous Forest under Experimental Warming." *Global Change Biology* 19 (7). John Wiley & Sons, Ltd:2158–67. https://doi.org/10.1111/GCB.12161.

Zhang, Xuechen, Yakov Kuzyakov, Huadong Zang, Michaela A. Dippold, Lingling Shi, Sandra Spielvogel, and Bahar S. Razavi. 2020. "Rhizosphere Hotspots: Root Hairs and Warming Control Microbial Efficiency, Carbon Utilization and Energy Production." *Soil Biology and Biochemistry* 148 (September). Pergamon:107872. https://doi.org/10.1016/J.SOILBIO.2020.107872.

5. Discussion

Root exudation is a notoriously difficult process to measure accurately: when collected from soil, there is likely to be interference from biological and chemical agents of the soil, when collected from hydroponics, the lack of mechanical resistance on the roots could result in a totally different morphology that has consequences for exudate volume and composition. These, and many other problems including accurate characterisation of exudate chemistry and imposition of treatments such as abiotic stress, have meant that the field has advanced slowly, with much criticism and painstaking evaluation. At the time of writing there is little synthesis: as so much argument is still occurring regarding appropriate protocols, the search for general principles is in its infancy. In recent years, members of the De Vries lab have begun experimenting with a soil-hydroponic hybrid method, and received some success in showing robust, species-specific patterns in exudation (Williams et al. 2021). This thesis aimed to test this soil-hydroponic hybrid method to explore the root exudation patterns of model grass species under abiotic stress. In order to explore this I first presented a rigorous proof-of-concept study using barley as a model species, to demonstrate that hydroponic treatments produced a different root blueprint at the morphological, tissue chemical, and exudation levels, compared with soil-hydroponic hybrids (Chapter 2). From here, I was able to use the soil-hydroponic hybrid method to test root exudation as a plant functional trait in response to climate change, which, to my knowledge, has not been carried out before. Further, I have demonstrated the use of a range of analyses that complement one another in order to describe the changes occurring in root and exudate chemistry. The overarching hypothesis for the thesis was that multiple climate change factors will interact to influence root exudation. I have shown that not only are drought and warming effects detectable both alone and in combination, but that there is also a plant species-specific response. These responses are strongly influenced by the plant, as shown by the control treatment in Chapter 4 that used a no-plant control. In the following chapter, I will consider the three main themes of the thesis and describe how they advance the field, before discussing limitations and future directions.

5.1 Infra-red methods

Screening complex chemical solutions in order to characterise their composition is an important objective in many fields. Root exudates are one such example of a complex chemical solution, and were usually characterised using GC-MS or LC-MS, both techniques that can be unable to identify exact chemical constituents in a mixture (Herz et al. 2018). If identification or annotation is not possible with mass spectral methods, information on identity can be unable to be inferred. In IR spectroscopy, an IR-active molecule will always make a contribution to the collected spectrum, even if it is unknown. There is a long history of the use of IR spectroscopy in examining materials, including plant extracts and plant tissue, which enables the collection of metabolic fingerprints from samples, and can illuminate broad shifts in molecular makeup between treatments and sample types. This makes IR spectroscopy particularly well-suited to sample types that are as-yet poorly understood or under-characterised - such as root exudates. It is also unnecessary to use complex chemical processes to prepare a sample, unlike the process of derivatization that is needed with GC-MS, which can save on costs, effort, and time.

Mesocosm experiments using plants tend to be large, highly replicated, full factorial studies. This can mean that costs for experiments using mass spectral methods can increase, which can in turn make the endeavour, particularly for time series experiments acting as a cost multiplier, unfeasible. By using FTIR coupled with a GC-MS approach, I have improved the tractability of root exudate research. From a root tissue perspective, Chapter 2 is the first time to my knowledge that optical photothermal microscopy (O-PTIR, mIRage) has been used on root tissues, and the extremely small size of required sample could enable multiple samplings from a plant across the lifetime of a study. By coupling mIRage and FTIR, I was able to demonstrate the significant shift in exudate composition and plant metabolome between soil-hydroponic hybrids and solely hydroponic growth systems. There is also evidence for a general "stress" phenotype in root tissue, shown in a shift in poly(saccharides) present between treatment groups in whole-root ATR-FTIR in Chapters 2 and 3, and this should be explored further. This could be due to an effect similar to that seen in a study on oats exposed to salinity stress by Xu and colleagues (2021), who showed using transcriptomics coupled with metabolic

techniques that when stressed, the proportion of saccharides changed in the plant tissues as genes for glycolysis were upregulated to provide energy to deal with the stress. This has the potential to be used as a simple method to detect a signal of root stress, and could potentially even be combined with soil core sampling to determine root stress responses in mature field experiments.

One important finding from this work is that FTIR is an appropriate analysis for identifying the effects of drought on the root exudation pattern of a slow-growing grass, having replicated the findings of the traditionally used GC-MS (Chapter 3). FTIR in Chapter 3 also offered more information than GC-MS on a chemical level, validating its utility in exudate studies, by showing a shift in the proportion of poly(saccharide) to amide bonds in exudates. IR in Chapter 4 extended this, finding that plant species with contrasting life strategies both respond in a similar way to the effect of warming, both changing the proportion of poly(saccharide) to amide under stress. Chapter 4 additionally validated the use of IR spectroscopy for leachates as a sample type, and its use offered evidence that changes in plant inputs to soil altered the chemistry of the soil column, but not in an identical fashion - there is clearly a mediator of this change, most likely the soil microbiome.

Taking small root samples for O-PTIR (mIRage, Chapter 2) shows a potential role of IR methods to carry out repeated root metabolic measurements through the lifetime of the plant system, and could be complementary to taking leachate samples (Chapter 4), as this would also allow an indication of the in-plant and soil metabolome during the experiment, and can be taken in a non-destructive fashion. A time series of leachate samples would be particularly illuminating when used with FTIR, as it would show how the chemistry of the soil column responds to various treatments, and could be analysed in a high-throughput setting.

In this thesis I sought to improve the current methodology of experiments using microcosms for exudate studies by testing a range of methods, and have shown that broad scale metabolic fingerprinting can offer insight into belowground chemistry. This thesis pioneers the use of FTIR for the analysis of root exudates, and shows its potential both in isolation and in combination with mass spectrometry. There is a need for tools to give a window into the biochemistry of plant tissues and their interactions belowground during long-term experiments, and O-PTIR investigation of root tissue samples and FTIR analysis of soil leachates offer two such tools.

5.2 Exudation as a plant functional trait

Plant functional traits have received enormous research efforts over the past two decades, and their utility in explaining and predicting plant strategies in response to abiotic stress has been well characterised (Violle et al., 2007; Bjorkman et al., 2018; Kattga et al., 2019; Thomas et al., 2020). While architectural, morphological and basic chemical (C:N ratio) traits are straightforward to measure, metabolic processes and complex chemical interactions are much more challenging, leading to the distinction between 'hard' and 'soft' traits (Hodgson et al. 1999; Weiher et al. 1999). Bellau and Shipley (2018) showed in an elegant study that hard traits in herbaceous dicots have superior predictive power to soft traits when measured in a drought study, which clearly shows that hard traits are desirable - if possible. Their example used hard traits based on water economy (stomatal conductance at wilt point and so on), and did not include exudation. Nevertheless, root exudation should be considered a hard trait, particularly in light of the evidence presented in this thesis, which shows a predictable species-specific signature with significant plasticity in response to stress (Chapter 4), which supports other recent work showing similar results (Williams et al. 2021). Traits are useful as they tend to be more variable between species rather than within, while also exhibiting measurable and informative intra-specific plasticity (Kattge et al. 2013). Root exudation chemistry when measured using the methods described in this thesis appears to fulfil these criteria (although exudates collected using purely hydroponic methods may not, see Monchgesang et al., 2016), and as methods become more time- and cost-effective, we believe exudation will be recognised as an important predictive trait.

Another facet of functional trait studies is the classification of 'response' and 'effect' traits (Diaz & Cabido 2001; Lavorel & Garnier 2001; Klumpp & Soussana 2009). According to the framework, response traits change in *response* to some kind of perturbation or change, while effects traits have a direct effect on ecosystem functions and processing. Most studies are aimed at effect traits, although it can be argued, given the plasticity many traits exhibit, that there is much overlap. Exudates have the potential to be both a response and an effect trait.

In this thesis, I have purely considered their role in responding to changes in environment, as this is where the state of the art is currently. In Chapter 2 I showed that root traits, tissue chemistry and exudate chemistry all shift in tandem in response to growth medium. There have been some forays into using exudates as effectors, for example collecting exudates and applying them to other plants or soils and measuring consequent plant and soil effects (e.g. De Vries et al., 2019). However, this work is still in its infancy and beyond the scope of this thesis.

In further support for the exudate-as-trait theory, studies show that exudation is directly linked with other root traits, which could be added to the two-dimensional root trait spectrum presented by Bergmann et al. (2021). In an elegant study using ecotypes of *Arabidopsis thaliana*, Caffaro and colleagues (2011) found that using active carbon in soil removed 90% of root exudates, thus allowing the plant to grow in an almost exudate free, sterile culture. They observed radical shifts in plant rooting architecture as a result, with reduced lateral rooting numbers and increased lateral root length, which is strikingly similar to the effect observed in this thesis in Chapter 2, where I showed that hydroponics resulted in lower lateral rooting, longer root lengths, and an overall shift to a less acquisitive resource use strategy in barley. An implication therefore is that hydroponics dilute the concentration of exudates around the roots to such an extent as to become similar to removing them altogether. Strikingly, reapplication of exudates to the active-charcoal treated plants resulted in a reversion of architectural traits back to those where lateral rooting became almost indistinguishable from the control. This association between architecture and exudates allows us to make further associations between traits and exudates under other scenarios. In Chapter 3, I showed that *Anthoxanthum odoratum* was highly responsive to drought in terms of rooting architecture, and exudation. Interestingly there was no significant effect of drought on root morphology and very little on tissue chemistry, and I ascribed this effect to a drought-induced change in the organisation of tissue structure, while maintaining the fixed developmental blueprint. In reorganising tissue structure, there has been a concomitant shift in exudate chemistry, which indicates that under drought these two are strong response traits. Tissue morphology and chemistry may therefore be an effect trait, as they will naturally have implications for litter quality, decomposition and other carbon-based functions.

In Chapter 4, I concentrated on the effect of drought and warming on leachate and exudate chemistry. While leachates cannot be considered a trait, as they comprise a mix of

rhizodeposits, microbial nutrient release, and molecules washed from the soil surface, we noted a strong similarity in the response of leachates to the treatments in terms of chemical composition. In warmed soils, both leachates and exudates comprised of an increased proportion of amide-based molecules, while the control had far higher saccharides (although slightly different composition). This leaves an interesting question: could leachates be a useful proxy for exudates that is experimentally robust? It would certainly mean avenues of experimentation would open that had been formerly closed, such as community-level leachate profiles, and a huge conservation of time from root washing and so on. It would also allow a time series to be implemented, something I have called for as desirable in other areas of the thesis. This last point is highly speculative, but if further work does seem to indicate that leaching is highly correlated with exudation as a trait, this could offer huge opportunities to address some of the gaps that are currently present.

5.3 Climate change effects on exudation

One of the overarching themes of this thesis is to advance climate change research by considering the impact of drought and warming on exudation chemistry, and linking it to root traits and whole root chemistry. Most current research focuses on plant morphological or architectural trait expression, and links to ecosystem functions. Exudate studies are fraught with issues (Oburger & Jones 2018), and as the most common methods previously involved hydroponics, this naturally led to issues implementing drought in any realistic way. Here I not only considered the missing link between physical root traits and exudate chemistry, but I also considered interactive climate change effects.

The methods shown in Chapter 2 enabled a proof-of-concept of use of soil-hydroponic hybrid growth systems for use with drought treatments in Chapter 3. Important insights from Chapter 3 include a stress-induced change in exudate composition from sugars to amides under drought, and root architectural traits change significantly. However, unlike root architectural traits, in *Anthoxanthum odoratum* tissue chemistry is not enormously shifted to withstand a single abiotic climate stress. This indicates the drought response may actually be

developmental delay caused by reduced water availability, something which has been previously suggested (e.g. Blum, 1996; Salehi-Liser, 2016). This could be confirmed through longitudinal studies that measure changes in exudation and root system architecture over time, examining the hypothesis that the exudate profile of older plants that have survived a drought is more similar to that of young, undroughted plants than older undroughted plants. Such a study has not been performed with exudates, but has been with other root traits; de Vries and colleagues (2016) found that drought did not affect the relationships between root traits, indicating plants subjected to drought were structurally merely smaller versions of plants not subjected to drought. Should root exudation follow the relationship of other root traits, then this may also be true for them.

Chapter 3 laid the groundwork for the multi-species, multifactorial study of Chapter 4. There have been recent calls to design experiments with multiple climate change effects, because variables such as drought and warming have different effects on plants and their rhizosphere community (Rillig et al. 2021). In Chapter 4, I combined drought and warming in a full factorial study which aimed to test whether traits and exudates respond in additive or synergistic ways to multiple climate change factors. Here, the effect of warming was even more disruptive to the content of exudates than drought, or drought combined with warming. A periodic increase of just five degrees from 20°C to 25°C caused large changes to the content of exudates, but intriguingly both Chapter 3 and Chapter 4 found a shift from relative enrichment of poly(saccharides) to relative enrichment of amides in the unstressed to stressed conditions. If this holds in other species, this could mean a conserved environmental stress exudate phenotype exists. Further studies should consider verifying these findings, and expanding the use of the soil-hydroponic hybrid methods discussed in this thesis outside of the grasses. It is also important to test that the conclusions hold in a variety of soil types and biomes; for example, is there a similar, or opposite exudate effect if tropical soils experience low temperature stresses? Do plants that make interesting symbioses, such as legumes with nitrogen fixers, have similar patterns in exudation? There is a wealth of opportunity for future studies, and an almost unlimited number of research questions that could be asked of root exudates, making this a very exciting time to be at the cutting edge of the research.

5.4 Future of exudation science

There are clear objectives that need to be met in order to advance the field. Firstly, the growth methodology must reach some form of consensus. The review of Oburger and Jones (2018), presents a long list of possible growth formats, including hydroponics, soil-hydroponics hybrid methods, and rhizoboxes, and discusses pros and cons for each. In this thesis I have rigorously tested the efficacy of the soil-hybrid method against hydroponics, and shown a difference in traits, tissue and exudate chemistry. However, as it is still a proxy for undisturbed field conditions, I believe that there are still steps to be taken to increase realism in these studies. The second objective is simple, rapid, cheap quantification of exudate chemistry, ideally cross-referenced by soil type to see if these are linked. The FTIR work I have pioneered here is instrumental to showing changes in groups of molecules in response to stress. It is possible that further characterisation of individual molecules would be an exercise in diminishing returns, as the thousands of possibilities may be unlikely to increase mechanistic understanding, although it may enable elucidation of regulatory pathways. However, one key factor that should receive consideration is the volume of exudates and the exudate chemistry as a function of this. Going further, how would the exudation volume and chemistry vary between high and low root orders? The volume question is one that arises as a consequence of the methodology, and will not be easily resolved, although proxies such as total organic carbon exist.

Moving on to wider questions and research areas, next steps involve adding complexity and realism to experimental designs. Because plants rarely grow in isolation, the hybrid method of exudate collection and analysis also must be validated in multiple-species systems, so experiments in mesocosms should be considered - though separating out intact root systems from a mesocosm would present significant practical challenges, not least due to the difficulty in untangling and washing enmeshed root systems for the collection of exudates. If this is possible to overcome, future experiments could include growing species mixtures both together and separately in order to examine how plant-plant and plant-microbe-plant interactions impact root exudation. The relatively low noise in the exudates collected with the hybrid method also opens the possibility of identifying the genes guiding root exudation in plants. *Arabidopsis thaliana*, a model plant, has many sequenced ecotypes that could be used in this endeavour, and coupling exudate metabolomics studies with transcriptomics could

unveil the genetic components of exudation. Barley also has multiple, sequenced landraces – genetic time capsules throughout its 12,000-year cultivation – that show adaptations to climates from the arid Mediterranean to wetter northern Europe, and therefore has enormous potential for use as a model of climate-driven effects on root exudation.

Due to the role of root exudates in shaping plant growth, stress tolerance, and rhizosphere community function, in recent years there has been increased interest in manipulating root exudation to promote crop resilience to abiotic stress (Preece and Penuelas, 2020; De Vries et al, 2020). Improved mechanistic understanding of the underlying interactions between exudation and microbe-induced growth promotion and stress tolerance could facilitate these developments. A further step therefore could be to integrate the changes in root exudation in time, which would show highly detailed responses to shifts in external conditions. Repeated measures experiments on the same plants would also indicate how dynamic the plants are in modulating their root exudates, and could even lead to surveillance for soil health in agro-ecosystems.

The response of the microbial community to specific changes in exudation is also unclear, and the combination of these methods with microbial community sequencing could be highly informative. We know that exudation is one method plants use to recruit a desirable microbial community, and the consistency of unique exudation fingerprints in this thesis adds support to this. While characterising microbial responses to exudates and the feedback to plant performance is beyond the scope of this thesis, it is a natural next step and absolutely crucial to explore experimentally. The research field is gradually addressing these gaps, especially with regard to the plant response to pathogens, on which some work has been done (Dutta et al. 2013). There is also an accruing body of work on mycorrhizal fungi, which are known to receive a significant amount of plant-derived carbon (Kaiser et al. 2015; Meier et al. 2017; Bell et al. 2021). The question of whether the mycorrhizae will select molecular types from the array on offer from the plant is an interesting one, and warrants further study.

5.5 Limitations

While the soil-hydroponic hybrid system appears to be a marked improvement on purely hydroponic systems, not least because of the change in architectural traits, it is not without its problems. Trauma to the roots during the root washing stage is still possible, and moving the plant system to a hydroponic system, which will naturally have a microbial community that is different to the soil, is likely to cause some artificiality. Even though the hydroponic solutions used in the soil-hydroponic hybrid method were created from the same soil as used to grow the plants, there is an enormous stress caused by transition to liquid phase, and those microbes that are adapted to survive in solution, without osmotic problems, are likely to be a very small subset of the original soil community. Another key drawback of this method is that due to the toilsome and time-consuming nature of the work involved in any root washing, high throughputs might be difficult to achieve with this method. Additionally, O-PTIR, here explored using mIRage, has incredible potential for analysing structural features and plant microbe interactions, but further work is needed on sample mounting and preparation. The cylindrical samples used in this work resulted in challenges in data acquisition and high noise.

5.6 Conclusion

Root exudation research is at an exciting stage: while the current limitations of analysis and methodology mean that exudates are still measured as response traits, the research presented in this thesis, as well as of the wider De Vries lab group, mean that shortly we can move into exploring exudates as an effect trait. This will mean that we can gain a true handle on the role of exudates in ecosystem functions such as carbon sequestration, nutrient cycling and plant-soil feedbacks. Overall I have demonstrated a way forward in the problem of assessing exudate patterns in response to stress, while reducing the artificial elements used previously. Using these methods, complex changes in metabolite chemistry are detectable, and this has opened up opportunities for a wide range of other study designs, thus closing the gap between plant and soil functions. Here I have offered an advance in the use of infra-red spectroscopy to illuminate this topic, which has now been shown to be a powerful method to interrogate belowground ecosystem interactions. One of the key points that has arisen from the work in this thesis, is that both species-specific exudate fingerprints and stress response fingerprints are reproducible and consistent. This is an extremely important point going forward, as it means that exudates are a valid and useful trait, and that the exudation of low molecular weight compounds is likely to be tightly controlled and responsive to environmental changes. This is a critical stepping stone as we move towards a greater understanding of aboveground-belowground ecosystem interactions, which becomes ever more important in our changing climate. I would like to emphasise that the soil-hybrid method used in this thesis is still a work in progress on the road to truly characterising exudates from plants. It demonstrates an important step forward, but can only ever be a proxy for natural field conditions. It remains to be seen whether future research can bridge this gap, however, doing so would represent a revolution in our understanding of plant and soil interactions.

5.6 References

Bell, Christopher A., Emily Magkourilou, Peter E. Urwin, and Katie J. Field. 2021. “The Influence of Competing Root Symbionts on Below-Ground Plant Resource Allocation.” *Ecology and Evolution* 11 (7). John Wiley & Sons, Ltd:2997–3003. https://doi.org/10.1002/ECE3.7292.

Belluau, Michaël, and Bill Shipley. 2018. “Linking Hard and Soft Traits: Physiology, Morphology and Anatomy Interact to Determine Habitat Affinities to Soil Water Availability in Herbaceous Dicots.” *PLOS ONE* 13 (3). Public Library of Science:e0193130. https://doi.org/10.1371/JOURNAL.PONE.0193130.

Bjorkman, Anne D., Isla H. Myers-Smith, Sarah C. Elmendorf, Signe Normand, Nadja Rüger, Pieter S.A. Beck, Anne Blach-Overgaard, et al. 2018. “Plant Functional Trait Change across a Warming Tundra Biome.” *Nature 2018 562:7725* 562 (7725). Nature Publishing Group:57–62. https://doi.org/10.1038/s41586-018-0563-7.

Caffaro, María M., Jorge M. Vivanco, Flavio H.Gutierrez Boem, and Gerardo Rubio. 2011. “The Effect of Root Exudates on Root Architecture in Arabidopsis Thaliana.” *Plant Growth Regulation 2011 64:3* 64 (3). Springer:241–49. https://doi.org/10.1007/S10725-011-9564-3.

De Vries, Franciska T. , Rob I. Griffiths, Mark Bailey, Hayley Craig, Mariangela Girlanda, Hyun Soon Gweon, Sara Hallin, et al. 2018. “Soil Bacterial Networks Are Less Stable under Drought than Fungal Networks.” *Nature Communications 2018 9:1* 9 (1). Nature Publishing Group:1–12. https://doi.org/10.1038/s41467-018-05516-7.

De Vries, Franciska T. , Caley Brown, and Carly J. Stevens. 2016. “Grassland Species Root Response to Drought: Consequences for Soil Carbon and Nitrogen Availability.” *Plant and Soil 2016 409:1* 409 (1). Springer:297–312. https://doi.org/10.1007/S11104-016-2964-4.

De Vries, Franciska T. , Alex Williams, Fiona Stringer, Robert Willcocks, Rosie McEwing, Holly Langridge, and Angela L. Straathof. 2019. “Changes in Root-Exudate-Induced Respiration Reveal a Novel Mechanism through Which Drought Affects Ecosystem Carbon Cycling.”

New Phytologist 224 (1). John Wiley & Sons, Ltd:132–45. https://doi.org/10.1111/nph.16001.

De Vries, Franciska T., Rob I. Griffiths, Christopher G. Knight, Oceane Nicolitch, and Alex Williams. 2020. "Harnessing Rhizosphere Microbiomes for Drought-Resilient Crop Production." *Science*. American Association for the Advancement of Science. https://doi.org/10.1126/science.aaz5192.

Díaz, Sandra, and Marcelo Cabido. 2001. "Vive La Différence: Plant Functional Diversity Matters to Ecosystem Processes." *Trends in Ecology & Evolution* 16 (11). Elsevier Current Trends:646–55. https://doi.org/10.1016/S0169-5347(01)02283-2.

Dutta, Swarnalee, T. Swaroopa Rani, and Appa Rao Podile. 2013. "Root Exudate-Induced Alterations in Bacillus Cereus Cell Wall Contribute to Root Colonization and Plant Growth Promotion." *PLOS ONE* 8 (10). Public Library of Science:e78369. https://doi.org/10.1371/JOURNAL.PONE.0078369.

Herz, Katharina, Sophie Dietz, Karin Gorzolka, Sylvia Haider, Ute Jandt, Dierk Scheel, and Helge Bruelheide. 2018. "Linking Root Exudates to Functional Plant Traits." *PLOS ONE* 13 (10). Public Library of Science:e0204128. https://doi.org/10.1371/JOURNAL.PONE.0204128.

Hodgson, J. G., P. J. Wilson, R. Hunt, J. P. Grime, and K. Thompson. 1999. "Allocating C-S-R Plant Functional Types: A Soft Approach to a Hard Problem." *Oikos* 85 (2). JSTOR:282. https://doi.org/10.2307/3546494.

Kaiser, Christina, Matt R. Kilburn, Peta L. Clode, Lucia Fuchslueger, Marianne Koranda, John B. Cliff, Zakaria M. Solaiman, and Daniel V. Murphy. 2015. "Exploring the Transfer of Recent Plant Photosynthates to Soil Microbes: Mycorrhizal Pathway vs Direct Root Exudation." *New Phytologist* 205 (4). John Wiley & Sons, Ltd:1537–51. https://doi.org/10.1111/NPH.13138.

Kattge, J., S. Díaz, S. Lavorel, I. C. Prentice, P. Leadley, G. Bönisch, E. Garnier, et al. 2011. "TRY – a Global Database of Plant Traits." *Global Change Biology* 17 (9). John Wiley & Sons, Ltd:2905–35. https://doi.org/10.1111/J.1365-2486.2011.02451.X.

Kattge, Jens, Gerhard Bönisch, Sandra Díaz, Sandra Lavorel, Iain Colin Prentice, Paul Leadley, Susanne Tautenhahn, et al. 2020. “TRY Plant Trait Database – Enhanced Coverage and Open Access.” *Global Change Biology* 26 (1). John Wiley & Sons, Ltd:119–88. https://doi.org/10.1111/GCB.14904.

Klumpp, Katja, and Jean François Soussana. 2009. “Using Functional Traits to Predict Grassland Ecosystem Change: A Mathematical Test of the Response-and-Effect Trait Approach.” *Global Change Biology* 15 (12). John Wiley & Sons, Ltd:2921–34. https://doi.org/10.1111/J.1365-2486.2009.01905.X.

Lavorel, S., and E. Garnier. 2002. “Predicting Changes in Community Composition and Ecosystem Functioning from Plant Traits: Revisiting the Holy Grail.” *Functional Ecology* 16 (5). John Wiley & Sons, Ltd:545–56. https://doi.org/10.1046/J.1365-2435.2002.00664.X.

Meier, Ina C., Adrien C. Finzi, and Richard P. Phillips. 2017. “Root Exudates Increase N Availability by Stimulating Microbial Turnover of Fast-Cycling N Pools.” *Soil Biology and Biochemistry* 106 (March). Pergamon:119–28. https://doi.org/10.1016/J.SOILBIO.2016.12.004.

Oburger, Eva, and David L. Jones. 2018. “Sampling Root Exudates – Mission Impossible?” *Rhizosphere* 6 (June). Elsevier:116–33. https://doi.org/10.1016/J.RHISPH.2018.06.004.

Preece, Catherine, and Josep Peñuelas. 2016. “Rhizodeposition under Drought and Consequences for Soil Communities and Ecosystem Resilience.” *Plant and Soil 2016 409:1* 409 (1). Springer:1–17. https://doi.org/10.1007/S11104-016-3090-Z.

Rillig, Matthias C., Anika Lehmann, James A. Orr, and Walter R. Waldman. 2021. “Mechanisms Underpinning Nonadditivity of Global Change Factor Effects in the Plant–Soil System.” *New Phytologist* 232 (4). John Wiley & Sons, Ltd:1535–39. https://doi.org/10.1111/NPH.17714.

Thomas, H. J.D., A. D. Bjorkman, I. H. Myers-Smith, S. C. Elmendorf, J. Kattge, S. Diaz, M. Vellend, et al. 2020. “Global Plant Trait Relationships Extend to the Climatic Extremes of the Tundra Biome.” *Nature Communications 2020 11:1* 11 (1). Nature Publishing Group:1–12. https://doi.org/10.1038/s41467-020-15014-4.

Violle, Cyrille, Marie Laure Navas, Denis Vile, Elena Kazakou, Claire Fortunel, Irène Hummel, and Eric Garnier. 2007. “Let the Concept of Trait Be Functional!” *Oikos* 116 (5). John Wiley & Sons, Ltd:882–92. https://doi.org/10.1111/J.0030-1299.2007.15559.X.

Williams, Alex, Holly Langridge, Angela L. Straathof, Graeme Fox, Howbeer Muhammadali, Katherine A. Hollywood, Yun Xu, Royston Goodacre, and Franciska T. de Vries. 2021. “Comparing Root Exudate Collection Techniques: An Improved Hybrid Method.” *Soil Biology and Biochemistry* 161 (October). Pergamon:108391. https://doi.org/10.1016/J.SOILBIO.2021.108391.

Weiher, Evan, Adrie Werf, Ken Thompson, Michael Roderick, Eric Garnier, and Ove Eriksson. 1999. “Challenging Theophrastus: A Common Core List of Plant Traits for Functional Ecology.” *Journal of Vegetation Science* 10 (5). Wiley:609–20. https://doi.org/10.2307/3237076.

Xu, Zhongshan, Xiaojing Chen, Xiaoping Lu, Baoping Zhao, Yanming Yang, and Jinghui Liu. 2021. “Integrative Analysis of Transcriptome and Metabolome Reveal Mechanism of Tolerance to Salt Stress in Oat (Avena Sativa L.).” *Plant Physiology and Biochemistry* 160 (March). Elsevier Masson:315–28. https://doi.org/10.1016/J.PLAPHY.2021.01.027.

Appendix

S.2 Supplementary Material Chapter 2

A
Belowground Biomass mg
0.05
0.10
0.15
c
a
b
B
Root Length Density m m⁻³
4.0e+06
8.0e+06
1.2e+07
b
a
b
C
Root Volume cm³
0.5
1.0
1.5
2.0
2.5
c
a
b
D
Specific Root Length m g⁻¹
50000
100000
200000
a
b
b
E
Number of root tips
5000
10000
20000
b
a
b
F
Number of root forks
10000
30000
50000
b
a
b
G
Root Tissue Density mg mm⁻³
0.02
0.06
0.10
H
Root Surface Area by Mass m² kg⁻¹
2000
6000
10000
a
ab
b
I
Root Dry Matter Content mg g⁻¹
0.02
0.04
0.06
0.08
J
Aboveground Biomass mg
0.2
0.4
0.6
0.8
1.0
c
a
b
K
Aboveground Dry Matter Content mg g⁻¹
4
6
8
10
b
a
b
Hydroponics Air
Soil
Hydroponics No Air

Figure S2.1 (previous page): Box plots of individual root traits shown in the biplot in Figure 4. Statistically significant differences at the $p<0.05$ level, as determined by Tukey's Honest Significant Difference test, are represented by letters above the boxes.

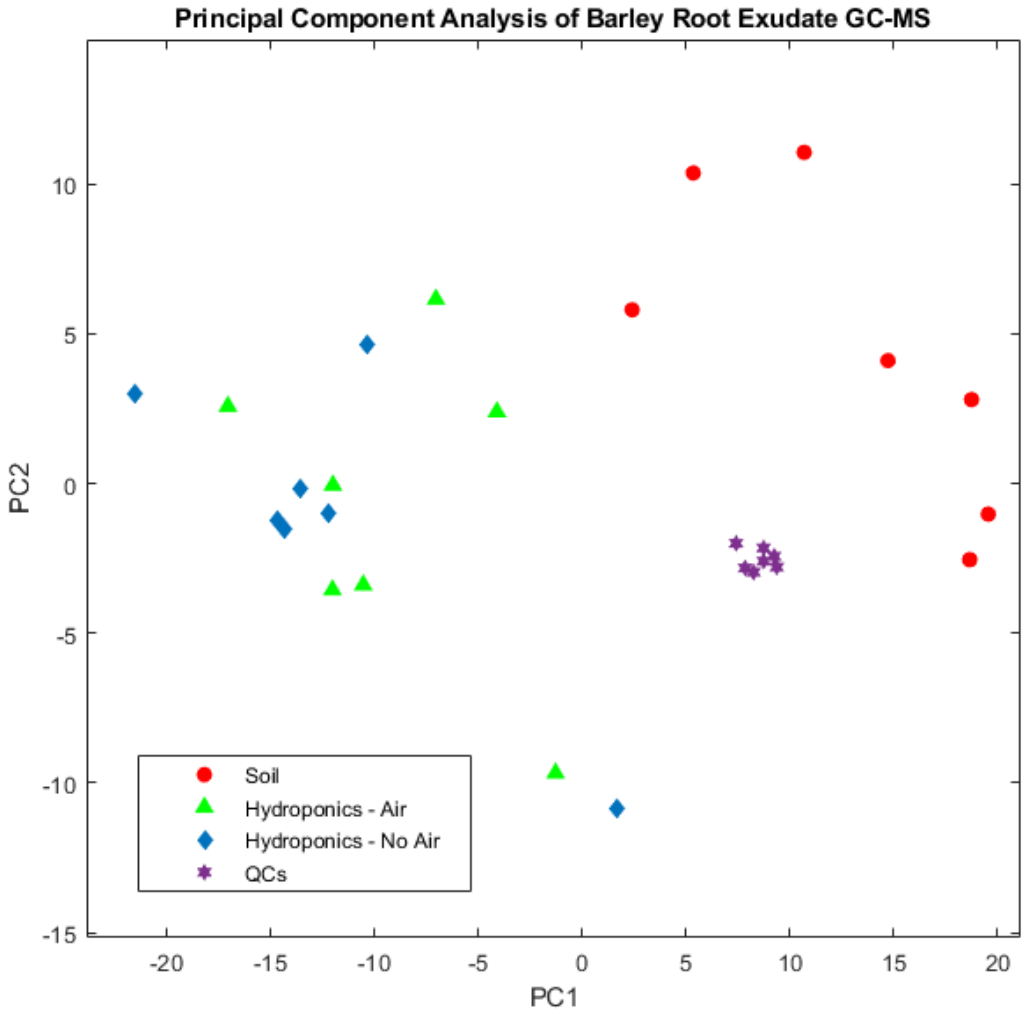

Figure S2.2: Principal Component Analysis of barley root exudates shown in Figure 2.8, showing clustering of Quality Control Samples (QCs)

Table S2.1: Experimental structure showing replicates and distribution between mIRage and root scanning for trait analysis as the analysis stream of the root system of each plant

Plant ID	Pooled Exudate Sample ID	Treatment	O-PTIR mIRage & Whole-root ATR-FTIR	Root Trait Analysis
S15	1	Soil	No	Yes
S16	1	Soil	No	Yes
S3	1	Soil	No	Yes
S4	1	Soil	Yes	No
A25	2	Hydroponics - Air	No	Yes
A27	2	Hydroponics - Air	No	Yes
A28	2	Hydroponics - Air	No	Yes
A26	2	Hydroponics - Air	Yes	No
W18	3	Hydroponics - No Air	No	Yes
W5	3	Hydroponics - No Air	No	Yes
W6	3	Hydroponics - No Air	No	Yes
W17	3	Hydroponics - No Air	Yes	No
W21	4	Hydroponics - No Air	No	Yes
W22	4	Hydroponics - No Air	No	Yes
W9	4	Hydroponics - No Air	No	Yes
W10	4	Hydroponics - No Air	Yes	No
A16	5	Hydroponics - Air	No	Yes
A3	5	Hydroponics - Air	No	Yes
A4	5	Hydroponics - Air	No	Yes
A15	5	Hydroponics - Air	Yes	No
W15	6	Hydroponics - No Air	No	Yes
W16	6	Hydroponics - No Air	No	Yes
W4	6	Hydroponics - No Air	No	Yes
W3	6	Hydroponics - No Air	Yes	No
W1	7	Hydroponics - No Air	No	Yes
W13	7	Hydroponics - No Air	No	Yes

W2	7	Hydroponics - No Air	No	Yes
W14	7	Hydroponics - No Air	Yes	No
A17	8	Hydroponics - Air	No	Yes
A18	8	Hydroponics - Air	No	Yes
A5	8	Hydroponics - Air	No	Yes
A6	8	Hydroponics - Air	Yes	No
A19	9	Hydroponics - Air	No	Yes
A7	9	Hydroponics - Air	No	Yes
A8	9	Hydroponics - Air	No	Yes
A20	9	Hydroponics - Air	Yes	No
W11	10	Hydroponics - No Air	No	Yes
W12	10	Hydroponics - No Air	No	Yes
W24	10	Hydroponics - No Air	No	Yes
W23	10	Hydroponics - No Air	Yes	No
S25	11	Soil	No	Yes
S27	11	Soil	No	Yes
S28	11	Soil	No	Yes
S26	11	Soil	Yes	No
S10	12	Soil	No	Yes
S21	12	Soil	No	Yes
S9	12	Soil	No	Yes
S22	12	Soil	Yes	No
S17	13	Soil	No	Yes
S18	13	Soil	No	Yes
S6	13	Soil	No	Yes
S5	13	Soil	Yes	No
S20	14	Soil	No	Yes
S7	14	Soil	No	Yes
S8	14	Soil	No	Yes
S19	14	Soil	Yes	No
A1	15	Hydroponics - Air	No	Yes
A13	15	Hydroponics - Air	No	Yes

A2	15	Hydroponics - Air	No	Yes
A14	15	Hydroponics - Air	Yes	No
W19	16	Hydroponics - No Air	No	Yes
W20	16	Hydroponics - No Air	No	Yes
W8	16	Hydroponics - No Air	No	Yes
W7	16	Hydroponics - No Air	Yes	No
A11	17	Hydroponics - Air	No	Yes
A12	17	Hydroponics - Air	No	Yes
A24	17	Hydroponics - Air	No	Yes
A23	17	Hydroponics - Air	Yes	No
W25	18	Hydroponics - No Air	No	Yes
W26	18	Hydroponics - No Air	No	Yes
W27	18	Hydroponics - No Air	No	Yes
W28	18	Hydroponics - No Air	Yes	No
S1	19	Soil	No	Yes
S14	19	Soil	No	Yes
S2	19	Soil	No	Yes
S13	19	Soil	Yes	No
A21	20	Hydroponics - Air	No	Yes
A22	20	Hydroponics - Air	No	Yes
A9	20	Hydroponics - Air	No	Yes
A10	20	Hydroponics - Air	Yes	No
S12	21	Soil	No	Yes
S23	21	Soil	No	Yes
S24	21	Soil	No	Yes
S11	21	Soil	Yes	No

S.3 Supplementary Material Chapter 3

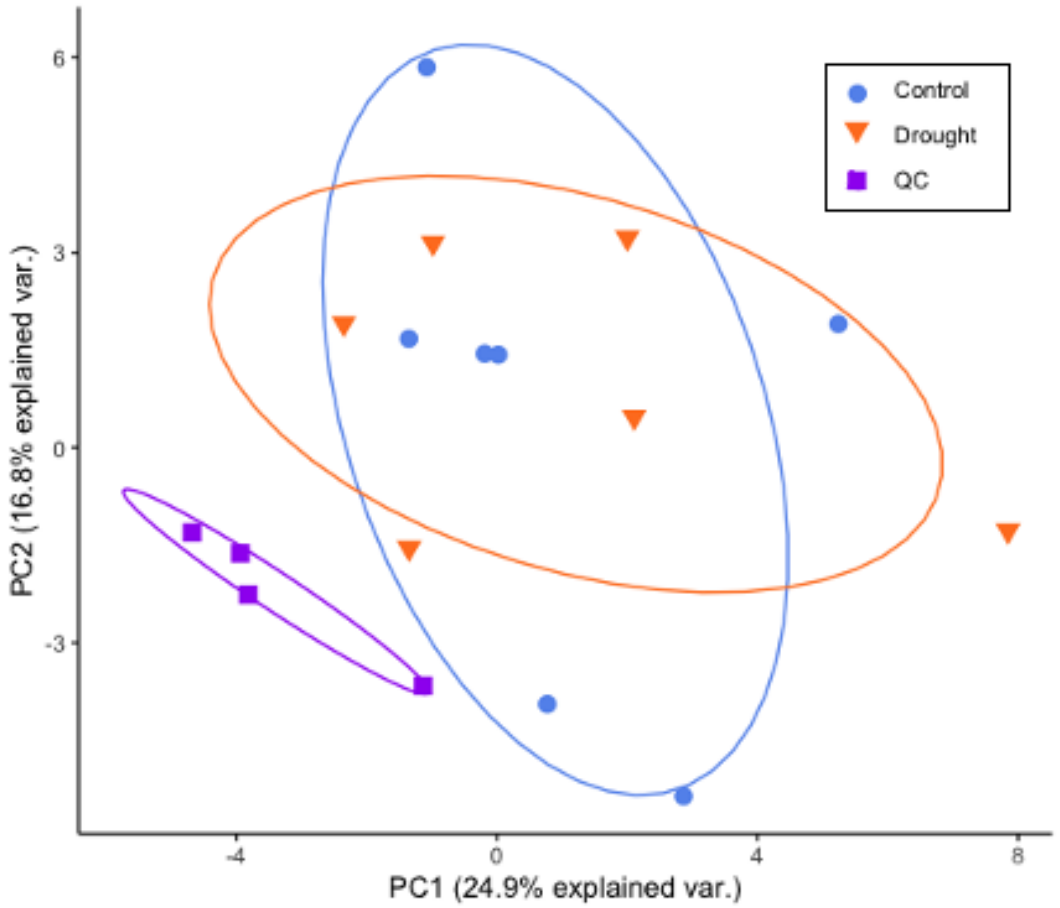

Figure S3.1: GC-MS results presented in figure 4A including the quality control samples (QC).

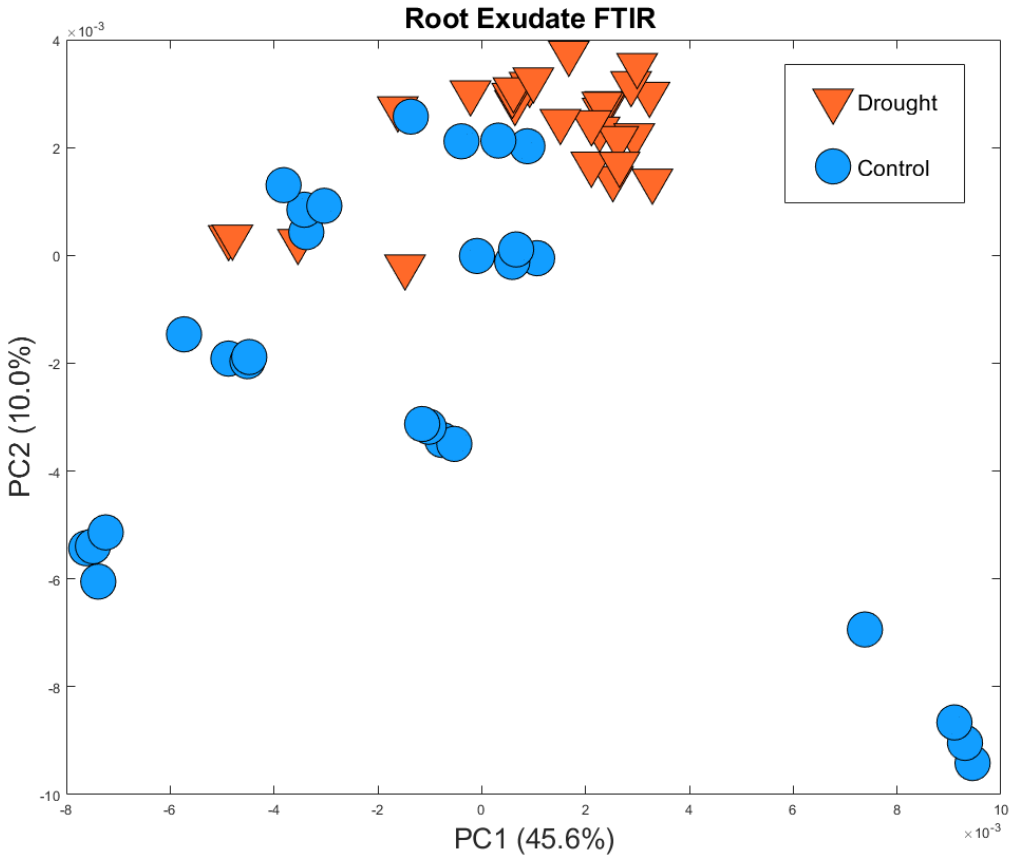

Figure S3.2: Root exudate FTIR results presented in figure 5B before averaging of technical replicates.