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Barley Responses to Drought and Arbuscular Mycorrhizal Colonisation

Elizabeth Jessica Parker

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Abstract

Drought is a major agricultural challenge threatening production of crops. Barley is the fourth most important crop globally in terms of production quantity and yield is expected to suffer drought-related reductions of 17% by 2050 (FAO STAT 2020, Li *et al.* 2009). Arbuscular mycorrhizal (AM) fungi have been shown to alleviate drought-stress symptoms in many crop species but few studies have focussed on barley, particularly with regards to mycorrhiza-mediated drought alleviation (Jayne & Quigley 2014, Zhang *et al.* 2019).

This thesis characterises the effects of AM colonisation on the physiological and metabolomic responses of spring barley (*Hordeum vulgare* cv. Concerto) to drought under controlled glasshouse conditions.

In an initial experiment conducted in uncolonised plants, the imposed drought treatment reduced biomass, leaf relative water content and photosystem II efficiency of barley. Stomatal conductance (g_s) was reduced in droughted plants by the 5th day of drought, and photosynthetic rate (*A*) had declined by the 11th day of drought treatment. Metabolomic fingerprints of polar leaf extracts acquired by DI-ESI-MS showed clear distinction in response to drought at both the 11th and 18th day of drought, with flavonoids, flavonoid glycosides and cinnamate derivatives putatively identified as influential in the drought stress response of this cultivar.

In a separate fully factorial experiment, the only differences attributed to AM colonisation were a reduced root biomass in AM well-watered plants and a one day delay in the drought-induced decline of photosystem II efficiency. Metabolomic fingerprints of polar leaf extracts acquired by LC-ESI-MS suggested that phenylpropanoids and alkaloids were differentially expressed at tillering and stem elongation stages of development. Despite little AM-mediated drought alleviation in this cultivar, subtle metabolomic differences between AM and NM drought response suggested effects of the symbiosis on stress signalling (jasmonate biosynthesis) and protecting photosynthetic machinery (tetrapyrrole biosynthesis, electron transport).

Declaration

I, Elizabeth Parker, confirm that the work submitted in this thesis is my own except where work that has formed part of jointly authored publications has been included. My contribution and that of the other contributors to this work has been explicitly indicated below. I confirm that appropriate credit has been given within the thesis where reference has been made to the work of others.

Chapters 2 to 5 are presented as stand-alone research papers that reference each other and chapter 1 (methods). In collaboration, myself and my supervisors, Duncan Cameron, Susan Hartley and Julie Gray, conceived the ideas and designed the experiments for all data in this thesis (chapters 2, 3, 4 and 5). I conducted all experiments presented in this thesis with the following assistance:

- I received technical assistance with sample processing (harvesting plants and root washing) from Kirsty Elliott (chapters 2, 3, and 4) and with root staining from Nathan Howard (chapter 4).
- Heather Walker and Heather Grievson assisted with mass spectrometry (chapters 3 and 5).
- Anthony Turner assisted with C/N nutrient analysis and Sarah Thorne assisted with P and Si nutrient analysis (chapter 4 and appendix XII).

I analysed the data in this thesis and wrote the manuscripts, with critical contributions to the drafts by Duncan Cameron, Susan Hartley, Julie Gray, Alex Williams (chapter 5) and Joseph Llanos (General discussion) .

For development of a reproducible untargeted metabolomics workflow (chapter 6) my initial approach was guided by Duncan Cameron, Heather Walker and Alex Charles but was further developed with help from a University of Sheffield Library “Unleash your data and software” grant, on which I worked in collaboration with Kathryn C. Billane, Nichola Austen, Anne Cotton, Rachel M. George, David Hopkins, Janice A. Lake, James K. Pitman, James N. Prout, Heather J. Walker, Alex Williams and Duncan D. Cameron (specific contributions are detailed in chapter 6).

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The saying “it takes a village to raise a child” is certainly true when a thesis and a pandemic are also involved. So thanks to James & Zena, Josh & Jenny, Sara & Ewan, Tiphanie & Lewis, and Lynn for being our village. My parents and parents-in-law have listened to my PhD challenges for the last seven years with patience and compassion. Thank you Mum & Dad, Bob & Sue (and William, whose enthusiasm for biology has helped me remember why I got into this in the first place!).

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Abbreviations

% PC	Percentage of pot capacity
A	Photosynthetic rate
ABA	Abscisic acid
AM	Arbuscular mycorrhizal (treatment)
AMF	Arbuscular mycorrhizal fungi
ANOVA	Analysis of variance
BR	Brassinosteroids
C	Carbon
CI	Confidence interval
CK	Cytokinins
DI-ESI-MS	Direct injection electrospray ionisation mass spectrometry
DS	Drought stress treatment
DW	Dry weight
ET	Ethylene
Fv'/Fm'	Light-adapted chlorophyll fluorescence (photosystem II efficiency)
GA	Gibberellin
GLMM	Generalised linear mixed effects model
g_s	Stomatal conductance
IAA	Auxin
JA	Jasmonic acid
LC-ESI-MS	Liquid chromatography electrospray ionisation mass spectrometry
N	Nitrogen
NM	Non-mycorrhizal (treatment)
m/z	Mass to charge ratio
OPLS-DA	Directed analysis of orthogonal projections of latent structures
OPDA	Oxophytodienoic acid (12-OPDA)
P	Phosphorus
PC	Principal component
PCA	Principal component analysis
PERMANOVA	Permutational multivariate analysis of variance
PGPR	Plant growth promoting rhizobacteria
RT	Retention time
RWC	Relative water content
SA	Salicylic acid
Si	Silicon
SL	Strigolactones
WW	Well-watered treatment
ZR	Zeatin riboside

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General introduction

The challenges posed by drought

The challenge for society

Drought is a major abiotic stress causing crop losses across the globe; drought accounts for yield reductions of 20-40% in cereal crops (Daryanto *et al.* 2016) with extreme weather events that involve heat or drought reducing national cereal production by, on average, 9-10% (Lesk *et al.* 2016). Considering cereals provide 45% of global daily per capita calorie consumption (FAOstat 2017), drought-induced crop losses of cereals pose risks to food security at regional levels, as well as having impacts on global markets (Ding *et al.* 2010). As a growing population and competing anthropogenic demands on water sources collide with a warmer and more extreme climate, we face a global situation in which we need to produce more food, for more people, with less water (Gupta *et al.* 2020).

The challenge for a plant

Drought represents a complex challenge for plants as sessile autotrophic organisms. Maintaining the transpiration stream is essential in order to take up water by capillary action and so keeping stomata open maintains the plant's water supply for processes including photosynthesis, maintaining turgor pressure and fruit or grain filling. However, this concurrently increases water loss from leaves. On the other hand, temporarily closing stomata can conserve water but limits gas exchange and, if drought persists, can reduce carbon intake and transpirational cooling, increase photorespiration, induce ROS production and oxidative damage, impeding photosynthesis and growth (Seleiman *et al.* 2021). Furthermore, reducing transpiration to slow water loss can slow the uptake, xylem transport, and energy-dependent unloading of inorganic nutrients such as nitrates (NO_3^-), phosphates (PO_4^{3-}) and potassium (K^+) leading to nutrient deficiency (Farooq *et al.* 2009). As such, drought poses a significant challenge to plants in terms of balancing water loss, photosynthesis and water and nutrient uptake.

The challenge for the plant's symbionts

For microorganisms living in the soil, soil drying can be extremely damaging. Arbuscular mycorrhizal (AM) fungi are symbionts that colonise the roots of plants and whose hyphae extend out into the surrounding soil to obtain nutrients such as phosphorus (P) and nitrogen (N) that are exchanged for carbon (C) from the host plant (Smith & Read 2008). For obligate biotrophic symbionts such as arbuscular mycorrhizal (AM) fungi that live within roots and rhizosphere simultaneously, drought threatens the plant-derived C supply, as well as altering

their intra-radical environment as the plant responds to oxidative damage (Forczek *et al.* 2022, Gong *et al.* 2015). At the same time an AM fungus must respond to soil drying and its direct effects on the extraradical mycelium of the fungus, as well as interactions with its own mycorrhizal-hyphae-associated microbiome (Emmet *et al.* 2021). So intimate, ancient, and complex is the AM symbiosis that it can be extremely challenging to separate the effects of drought on the plant, fungus or associated microorganisms and, due in part to a focus on agricultural yield and the complexity of understanding mycorrhiza as a network rather than an organism, a plant-centric approach to drought responses has dominated the research field (Silva & Lambers 2021, Chaudhary *et al.* 2022).

Plant responses to drought

For mesophytes (those plants adapted to living in semi-arid or sub-humid environments, including most crop plants), a lack of water for long enough to cause injury can be deemed a “drought” (Fang & Xiong 2015). Drought resistance is a complex trait encompassing the capability of a plant to survive and continue to function during drought and, according to Fang & Xiong (2015), comprises four major approaches:

- *Drought avoidance* - adjustment of growth and physiology to maintain functioning during mild to moderate drought by:
    - Reducing water loss (e.g. dynamic stomatal closure)
    - Increasing water uptake ability (e.g. increasing root depth)
    - Altering maturation to reproductive growth stages
- *Drought tolerance* - reducing and repairing damage caused by severe drought in order to maintain some level of functioning by:
    - Upregulating osmoprotective compounds
    - Increasing antioxidant accumulation and activity of antioxidant enzymes
- *Drought escape* - adjustment of life cycle to minimise the likelihood of encountering drought. For crops this can include altered planting times or choice of short growth season crops.
- *Drought recovery* - ability of a plant to return to growth (and achieve yield) despite experiencing severe drought, if water is subsequently made available.

Even within species, variation in drought avoidance and tolerance are observed, with some cultivars or genotypes able to maintain photosynthesis, growth and reach yield under more severe drought conditions than others. For example, drought tolerant cultivars of oat have been found to accumulate salicylic acid rapidly and sustain this accumulation in order to finely control stomatal closure and antioxidant response, whereas drought susceptible

cultivars shut stomata early and slowly accumulated antioxidants and were thus less able to continue normal physiological processes during drought (Sanchez-Martin *et al.* 2015).

Mycorrhiza-mediated drought alleviation

Arbuscular mycorrhizal (AM) fungi have been heralded as both a useful indicator of soil health in agro-ecosystems (Oehl *et al.* 2011) and a potentially useful biostimulant that can reduce the need for inorganic phosphate fertilisers, while also providing a range of benefits to plant health and crop value (Zhang *et al*. 2016, Thioub *et al.* 2019).

Associating with arbuscular mycorrhizal fungi can be beneficial for many crop plants, with the association having been shown to boost plant biomass production (Abdelmoneim *et al.* 2013, Zhou *et al.* 2015), crop yield (Bowles *et al.* 2017, Zhang *et al.* 2019) and crop quality (Al-Karaki *et al.* 2004, Subramanian *et al.* 2006, Gholamhoseini *et al.* 2013) for many species. Other benefits to a crop plant's general health have also been shown, for example, increased macro- and micro- nutrient accumulation (Rani *et al.* 2017, Symanczik *et al.* 2018), improved resistance to specific pathogens or herbivores (Kempel *et al*. 2010, Verosoglou & Rillig 2012), improved tolerance of heavy metal toxicity (Hristozkova *et al*. 2016, Chaturvedi *et al*. 2018), salinity (Chandrasekaran *et al*. 2014) and high temperatures (Mathur *et al.* 2018).

Evidence of the AM symbiosis alleviating symptoms of drought in plants subjected to water stress has been accumulating since the 1970s (Safir *et al.* 1971). Studies were initially concerned with whether AM effects on macronutrient nutrition and plant size were responsible for observed differences in host water relations but by the early 2000s a number of other mechanisms had been identified and were reviewed by Augé (2001). At that time, the consensus was that P nutrition was likely the strongest factor affecting the observed alleviation of drought symptoms in mycorrhizal plants. However, Augé also noted a number of other mechanisms by which AM fungi might improve host water relations, notably by influencing phytohormones; by changing the strength of the C sink to influence photosynthesis, gas exchange and osmotic adjustment; and by directly contributing to water absorption via the extraradical mycelium. The alteration of soil structure to improve water retention; the modulation of aquaporins (water channel proteins); and the altered activity of antioxidants were added to the list of proposed mechanisms for mycorrhiza-mediated drought alleviation by Ruiz-Lozano (2003). Meta-analysis by Jayne & Quigley (2014) corroborated the assumed trend that inoculation with AM fungi improves the growth and yield of crop plants in general under drought stress.

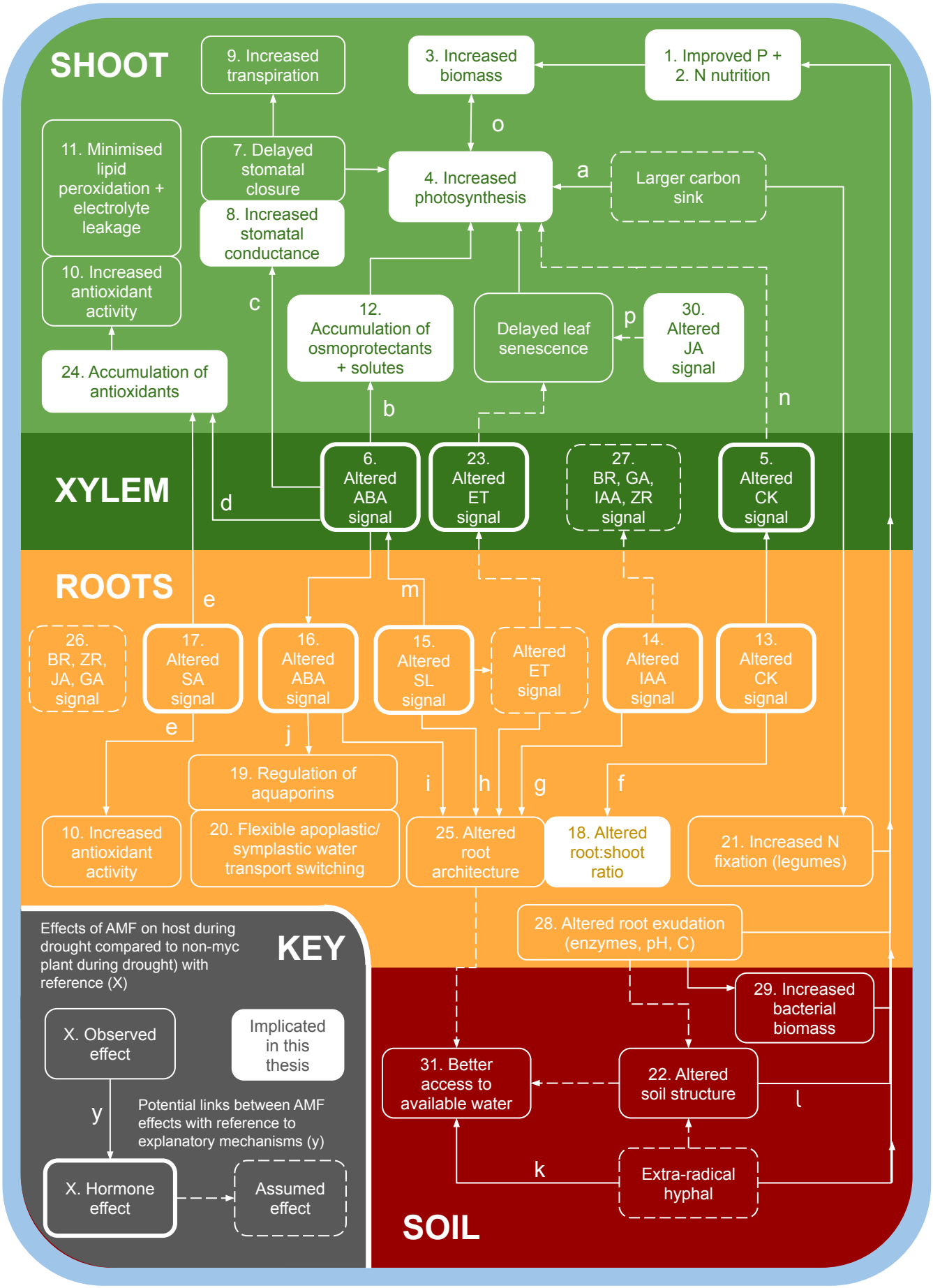

Fig. 1.1. (Panel I) The AM symbiosis has local and systemic effects on the host during exposure to drought. ABA = abscisic acid, BR = brassinosteroids, CK = cytokinins, ET = ethylene, GA = gibberellin, IAA = indole acetic acid (auxin), JA = jasmonic acid, SA = salicylic acid, SL = strigolactones, ZR = zeatin riboside. Potential links between mechanisms can be found on panel II overleaf along with references. Numbered references for effects of AM colonisation under drought can be found on panel III overleaf …

Possible explanations for observed effects of AMF during drought:

a. AMF increase the C sink strength and stimulate photosynthesis (Kaschuk et al. 2009, Gavito et al. 2019).

b. ABA upregulates the accumulation of osmoprotectants that help confer partial tolerance to heat stress (Kumar et al. 2012) and a similar mechanism could be at play during drought stress.

c. Drought-induced ABA signals are possibly modulated by xylem pH, JA, CK, peptides, microRNAs, and malate in their effects on stomata (Schachtman & Goodger 2008).

d. ABA biosynthesis in leaves is induced by mycorrhization (Adolfsson *et al.* 2017). ABA has a concentration dependent effect on superoxide dismutase, catalase, and ascorbate peroxidase in wheat (Agarwal et al. 2005).

e. SA has been implicated in the accumulation of antioxidant compounds and the protection of plants against oxidative stress (Agarwal et al. 2005, Rivas-San Vicente & Plasencia 2011).

f. CKs are thought to regulate root:shoot ratio (Fusconi 2014).

g. IAA has been implicated in AM-mediated changes in root architecture (Sukumar et al. 2013, Fusconi 2014) particularly in promoting lateral root formation (Fusconi 2014).

h. SLs block lateral root formation in the absence of AM colonisation (probably via an interaction with ET) (Fusconi 2014).

i. ABA maintains root growth under drought conditions possibly by suppressing ET accumulation (Liu et al. 2005).

j. Studies show varying effects of ABA on aquaporins (Groppa et al. 2012), such as upregulation of some PIP genes concurrent with downregulation of others (Aroca et al. 2006).

k. Transport of water along hydrophobic hyphal surface, within hyphae, and through channels formed by “wrapped” hyphae (Allen 2007). Also hyphae able to access water in micropores >2µm (Allen 2007).

l. Improved soil structure improves availability of nutrients including N (Veresoglou et al. 2012).

m. SL signals from roots have been found to increase shoot sensitivity to ABA (Vinsentin *et al.* 2016). SLs may also act as an endogenous drought signal inducing stomatal regulation via a pathway independent of ABA (Lv *et al.* 2018).

n. In *Arabidopsis* mutant studies, timing and method of induced CK up-regulation allows modulation of water loss to cope with different drought scenarios (Prerostova *et al.* 2018). Exogenous foliar application of CK in pomegranate produces similar amelioration of drought stress as AM inoculation (Bompadre *et al.* 2015).

o. AM colonisation increases photosynthetic rate via an increase in total leaf surface area (Adolfsson *et al.* 2015).

p. JA biosynthesis is initiated in chloroplasts and induces leaf senescence (Ullah *et al.* 2019).

Fig. 1.1. (Panel 2) The AM symbiosis has local and systemic effects on the host during exposure to drought. The diagram aims to link the diverse effects of AM colonisation during exposure to drought that have been observed in the literature (numbered references). The lettered references provide potential explanations based on studies which have **not** been conducted under drought stress and/or with AM fungi. (continued overleaf)...

References:

1. (Al-Karaki & Al-Raddad 1997, Davies et al. 2002, Al-Karaki et al. 2004, Asrar & Elhindi 2011, Asrar et al. 2012, Abdelmoneim et al. 2014, Ganjeali et al. 2018, Mirshad & Purthur 2016 & 2017, Symanczik et al. 2018, Badr et al. 2020, Langeroodi et al. 2020, Hu et al. 2020)
2. (Subramanian et al. 2006, Asrar et al. 2012, Grümberg et al. 2015, Mirshad & Purthur 2016 & 2017, Metwaly & El-Khateeb 2019, Symanczik et al. 2018, Badr et al. 2020, Langeroodi et al. 2020, Hu et al. 2020)
3. (Khalvati et al. 2005, Subramanian et al. 2006, Wu & Xia 2006, Ruiz-Sánchez et al. 2010, Asrar et al. 2012, Abdelmoneim et al. 2014, Liu et al. 2016, Wang et al. 2017, Ganjeali et al. 2018, Quiroga et al. 2017, Liu et al. 2018, Bakr et al. 2018, Metwaly & El-Khateeb 2019, Ren et al. 2019, Li et al. 2019, Langeroodi et al. 2020, Olalde-Portugal et al. 2020, Hu et al. 2020)
4. (Sánchez-Blanco et al. 2004, Khalvati et al. 2005, Wu et al. 2008, Ruiz-Sánchez et al. 2010, Bárzana et al. 2012, Ruiz-Lozano et al. 2015, Wang et al. 2017, Bakr et al. 2018, Moradtalab et al. 2018, Li et al. 2019, Mathur et al. 2019, Ren et al. 2019, Fracasso et al. 2020, Olalde-Portugal et al. 2020)
5. (Goicoechea et al. 1995)
6. (Duan et al.1996, Wang et al. 2017, Ren et al. 2019, Ouledali et al. 2019, Langeroodi et al. 2020)
7. (Allen & Boosalis 1983)
8. (Sánchez-Blanco et al. 2004, Khalvati et al. 2005, Wu & Xia 2006, Bárzana et al. 2015, Wang et al. 2017, Ganjeali et al. 2018, Bakr et al. 2018, Li et al. 2019, Ouledali et al. 2019, Ren et al. 2019, Symanczik et al. 2018, Badr et al. 2020, Olalde-Portugal et al. 2020, Hu et al. 2020)
9. (Davies Jr et al. 1996, Duan et al. 1996, Bolandnazar et al. 2007, Wang et al. 2017, Ganjeali et al. 2018, Moradtalab et al. 2018, Ouledali et al. 2019)
10. (Porcel & Ruiz-Lozano 2004, Ruiz-Sánchez et al. 2010, Zhu et al. 2011, Baslam & Goicoechea 2012, Sohrabi et al. 2012, Yaghoubian et al. 2014, Bárzana et al. 2015, Gong et al. 2015, Grümberg et al. 2015, Ganjeali et al. 2018, Mirshad & Purthur 2016 & 2017, Moradtalab et al. 2018, Langeroodi et al. 2020)
11. (Porcel & Ruiz-Lozano 2004, Ruiz-Sánchez et al. 2010, Zhu et al. 2011, Baslam & Goicoechea 2012, Sohrabi et al. 2012, Yaghoubian et al. 2014, Bárzana et al. 2015, Gong et al. 2015, Grümberg et al. 2015, Mirshad & Purthur 2016 & 2017, Quiroga et al. 2017, Li et al. 2019, Ren et al. 2019)
12. (Wu & Xia 2006, Baslam & Goicoechea 2012, Sohrabi et al. 2012, Abdelmoneim et al. 2014, Bárzana et al. 2015, Grümberg et al. 2015, Mirshad & Purthur 2016 & 2017, Quiroga et al. 2017, Moradtalab et al. 2018, Metwaly & El-Khateeb 2019, Hu et al. 2020) vs (Porcel & Ruiz-Lozano 2004, Ruiz-Sánchez et al. 2010)
13. (Goicoechea et al. 1996)
14. (Sánchez-Romera et al. 2015, Liu et al. 2018, Quiroga *et al.* 2018, Zhang et al. 2019, Quiroga *et al.* 2020)
15. (Ruiz-Lozano et al. 2015)
16. (Ruiz-Lozano et al. 2015, Liu et al. 2016, Quiroga *et al.* 2018, Zhang et al. 2019, Quiroga *et al.* 2020)
17. (Sánchez-Romera et al. 2015, Quiroga *et al.* 2018, Quiroga *et al.* 2020)
18. (Davies Jr et al. 1996, Davies et al. 2002, Boyer et al. 2015, Grümberg et al. 2015)
19. (Porcel et al. 2006, Bárzana et al. 2015, Quiroga et al. 2017)
20. (Bárzana et al. 2012)
21. (Saia et al. 2014)
22. (Wu et al. 2008, Ji et al. 2019)
23. (Langeroodi et al. 2020)
24. (Liu et al. 2016, Ren et al. 2019, Langeroodi et al. 2020)
25. (Liu et al. 2016, Liu et al. 2018, Ji et al. 2019, Zhang et al. 2019, Liu *et al.* 2020)
26. (Liu et al. 2016, Zhang et al. 2019)
27. (Wang et al. 2017)
28. (Cheng et al. 2022, Hoang et al. 2022)
29. (Hoang et al. 2022)
30. (Pedranzani et al. 2016)
31. (Saharan et al. 2018, Singh et al. 2019)

Fig. 1.1. (Panel 3) The AM symbiosis has local and systemic effects on the host during exposure to drought. Studies referred to in the diagram (numbered references) in which the effects of AM colonisation on drought response of host plants have been investigated. This is by no means an exhaustive collection but instead highlight the focus of research to date and potential future avenues for research.

Suggested mechanisms of mycorrhiza-mediated drought alleviation

As drought response is such a complex process, affecting many diverse plant traits, understanding how AM colonisation might mitigate drought symptoms is challenging. The following summary of the literature highlights those mechanisms of mycorrhiza-mediated drought alleviation that have received the most attention to date, many of which are interconnected and may be more, or less, important in certain species or cultivars.

Figure 1.1. connects the effects of AM colonisation observed under drought (in crop plants). Numbered references in panel 1 refer to panel 3 where a list of studies that have observed a given effect can be found. Letters in panel 1 relate to arrows that link effects observed under drought with potential explanatory mechanisms that have not, as yet, been demonstrated in studies including both an AM inoculation and drought treatment, but where other literature suggests they may be relevant (panel 2 gives more detail of these potential links with references).

Increased photosynthetic rate, nutrition, biomass and size effects

Macronutrient nutrition and plant biomass are straight-forward to quantify and are regularly reported in both drought and AM colonisation studies as key markers of the drought- or AM-response. Under drought conditions, positive effects of AM colonisation on total plant or above-ground biomass have been widely reported (Jayne & Quigley 2014, see 3. in fig.1.1), and this is regularly associated with an improvement in plant P nutrition, plant N nutrition and legume nodule N fixation compared to uncolonised (NM) plants under the same drought treatment (see 1, 2 and 21 in fig.1.1). It should be noted that some studies do observe increased P or N nutrition of host plants without an associated increase in biomass (e.g. Davies *et al*. 2002, Grumberg *et al.* 2015).

Meta-analysis by Augé *et al.* (2015) found that in studies where AM plants were larger or had higher P content than NM controls, the effects of colonisation on stomatal conductance under drought were more marked. Plant size and nutrition are good indicators of the drought alleviation of AM colonisation and in this respect are symptomatic, rather than mechanistic, explanations of the drought-response. Improved photosynthetic rate or photosystem II efficiency of AM plants under drought are regularly reported (see 4 in fig. 1.1). An active AM symbiosis may protect photosynthetic function under drought by improving nutrition and increasing biomass and leaf area (Asrar *et al.* 2012, Adolfsson *et al.* 2015); increasing stomatal conductance to maintain gas exchange (Wu & Xia 2006); or by influencing antioxidant activity and osmolyte accumulation to protect photosynthetic pigments and

structures (Barzana *et al*. 2015). Maintaining photosynthetic function is advantageous for the fungal symbiont, which relies on the host plant for its C supply (Smith & Read 2008).

The increase in C sink strength represented by the AM fungus may itself help to maintain the rate of photosynthesis during drought episodes. A review of the effects of AM fungi and rhizobia contribution to sink strength on photosynthetic rate in legumes calculated that AM significantly increased host photosynthetic rate by 14%; that this was substantially more than the C demand of the fungus; and that the improved photosynthetic rate could not be fully explained by improved nutrition (Kaschuk *et al*. 2009). Gavito *et al.* (2019) have since shown that removing part of the extraradical mycelium of AM fungi colonising cucumber plants as a way of suddenly reducing the size of the C sink temporarily reduced photosynthetic rate by 10-40%.

Under a mild drought scenario, photosynthesis is not necessarily limited in the same way that growth is, so AM hyphae, by providing a sink for surplus C, could prevent subsequent inhibition of photosynthesis, as argued by Prescott (2022). Being able to maintain photosynthesis during a short mild or moderate drought may be advantageous upon rewetting (Hossain *et al.* 2014). Furthermore, the host plant may be able to preferentially supply C to “better” symbionts, depending on the environmental conditions, and thus protect itself from drought stress via its “choice” of AM partner. Early evidence for this has been demonstrated in *Medicago,* though the fungal traits that cause host preference remain unclear (Forczek *et al.* 2022).

Altered root architecture and host resource partitioning

The AM symbiosis has long been observed to alter host root architecture (Hetrick *et al.* 1988, Hooker *et al*. 1992). Under optimum conditions, AM colonisation is reported to increase root length, lateral branching and root: shoot ratio (Hetrick 1991). Under drought stress, increases in root length as a result of AM colonisation have been observed, as well as increases in root density, root volume, root hair length, and lateral root formation (see 25 in fig. 1.1). Having a longer, more branched root system could theoretically improve access to water during periods of low precipitation, by increasing the surface area for absorption of available water and reaching a deeper water table. Better plant performance under drought via AM alteration of root architecture has been primarily demonstrated in citrus species (Liu *et al.* 2018, Liu *et al*. 2020).

In contrast, allocation of resources to root growth is reduced by AM colonisation in some crop plants under drought conditions. For example in soybean, three strains of AM fungi and a mixed inoculum containing all three strains, all reduced root: shoot ratio under drought

stress but not under well-watered conditions compared to uninoculated controls (Grümberg *et al.* 2015). Since hyphae can provide some of the plant's requirements of macronutrients and water, AM colonisation may reduce the need for the host to invest in a larger or more complex root system (de Vries *et al.* 2021).

Compatible solute accumulation

In response to drought stress, plants can alter osmotic potential to maintain turgor pressure and protect photosynthetic structures by accumulating osmolytes or compatible solutes such as sugars, amino acids, proline and glycine betaine (Downton 1983, Martinez *et al.* 2004). AM colonisation has been observed to improve osmotic adjustment of the host plant during period of drying (Kubikova *et al.* 2001) and has simultaneously been shown in many cases to increase the accumulation of soluble sugars, non-structural carbohydrates, soluble starch, inorganic solutes such as K^+, Ca^{2+} and Mg^{2+}, and soluble protein in host plant tissues that can all act as osmotic regulators (see 12 in fig. 1.1).

Proline is a non-protein amino acid accumulated by many plants in response to osmotic stress and is thought to act as an osmoprotectant (Ruiz-Lozano *et al.* 2003). Studies have found evidence of proline both accumulating (Abdelmoneim *et al.* 2013, Porcel & Ruiz-Lozano 2004, Ruiz-Sanchez *et al.* 2010, Asrar *et al.* 2012, Wu & Xia 2006, Zhu *et al.* 2011, Doubkova *et al.* 2013) and being downregulated (Benhiba *et al.* 2015, Grumberg *et al.* 2015, Porcel & Ruiz-Lozano 2004, Barzana *et al.* 2015) in response to AM colonisation under drought stress. This inconsistency is perhaps reflective of the complexity of interpreting accumulation of osmoprotective compounds such as proline. AM plants may increase the accumulation of osmoprotective compounds as a mechanism to reduce oxidative damage but in other cases, a reduced levels of osmoprotectants in AM plants may simply indicate that the plant is experiencing lower stress levels thanks to other mechanisms of mycorrhizal drought alleviation (Doubkova *et al.* 2013, Sharma *et al.* 2015).

In field trials with flax, AM colonisation has been found to reduce drought-induced glycine betaine accumulation (Rahimzadeh & Prizad 2017). However in buckwheat, a drought-induced accumulation of glycine betaine was observed but was not significantly altered by AM colonisation while soluble sugar accumulation was (Mohammadi *et al.* 2022). Thus the specific osmolytes affected by AM symbiosis are likely to vary from species to species.

Antioxidants and antioxidant enzyme activity

While the production of damaging reactive oxygen species (ROS) occurs under non-stressed conditions as a result of reactions including photosynthesis and respiratory electron transport, their production is greatly increased as a result of drought stress (Noctor & Foyer 1998). This induces oxidative stress causing damage to DNA, enzymes and cellular structures and ultimately impeding processes essential to growth and survival (Ruiz-Sanchez *et al.* 2010). Where they have been assessed, ROS concentrations during drought stress have generally been found to be lower in AM plants compared to uninoculated controls (see 10 and 24 in fig. 1.1). As a result, lipid peroxidation causing damage to membranes, DNA, proteins and enzymes, is also observed to be lower in AM inoculated plants under drought stress (see 11 in fig. 1.1).

Plants produce a range of antioxidant compounds and enzymes capable of inhibiting, processing or scavenging ROS without becoming destructive radicals themselves (Noctor & Foyer 1998). Alteration of antioxidant enzyme activity has been proposed as an important mechanism in mycorrhiza-mediated drought-alleviation, and meta-analysis has recently corroborated the general trend of drought-stress alleviation in AM treated plants concurrent with increases in antioxidant enzyme activity (Chandrasekaran & Paramasivan 2022).

Other non-enzymatic antioxidant compounds have also been linked to mycorrhiza-mediated drought-alleviation. These include components of the phenylpropanoid biosynthesis pathway (particularly flavonoids), carotenoids, α-tocopherol and other secondary metabolites (Zou *et al.* 2021). In the roots, there is some evidence to suggest that transcription of AM fungal genes contribute directly to the increased antioxidant activity locally (Zou *et al.* 2021).

Altered hormonal signalling

Phytohormones play an important but complex role in the establishment of the arbuscular mycorrhizal symbiosis as well as in coordinating drought stress signalling and response in plants (Pozo *et al.* 2015). As such, alteration of phytohormone balance has been proposed as a mechanism by which AM colonisation may help to alleviate the effects of drought in host plants (Pozo *et al.* 2015).

The AM symbiosis is understood to allow the host to regulate abscisic acid (ABA) – often dubbed “the stress hormone” – levels better and faster than NM plants (see 6 and 16 in fig. 1.1). However, an increasing number of other phytohormones have been implicated in AM-mediated drought alleviation as the endogenous roles of these hormones are investigated further (see 5, 6, 13, 14, 15, 16, 17, 23 in fig. 1.1). Studies that have been able

to quantify a diverse range of phytohormone concentrations in the same plants suggest complex effects of AM colonisation on the host's phytohormone balance (see 26 and 27 in fig. 1.1).

Understanding the subtleties of phytohormone balance and its role in AM-mediated drought alleviation is particularly challenging since many of the intermediates in phytohormone biosynthesis can share properties and roles. For example, some jasmonate (JA) precursors are bioactive and can be considered to fulfil similar endogenous roles to JA, such as 12-oxophytodienoic acid (OPDA) acting as an antitranspirant (Savchenko *et al.* 2014). In digitgrass, compounds in the JA biosynthesis pathway including OPDA, 11-OH-JA and 12-OH-JA showed higher accumulation in AM plants than in NM plants under drought conditions while JA did not differ significantly between AM and NM droughted plants (Pendranzani *et al.* 2016).

Altered maturation and phenological development

Under drought conditions, some authors have noted a marked acceleration of phenological development in AM colonised plants in comparison to NM plants. For example, Fracasso *et al.* 2020 found that AM inoculation reduced the length of the vegetative stage of tomato development under drought-stress conditions, resulting in shorter plants bearing more flower branches and larger individual fruits than NM plants, and ultimately producing a greater fresh weight of fruit. In non-stress conditions, other authors have remarked on the altered biochemical maturation of both pea and *Medicago truncatula* (Schtark *et al.* 2019, Yurkov *et al*. 2021).

Maintaining a transpiration stream

In response to drought, plants close their stomata in order to conserve water but AM colonisation has been found to delay stomatal closure (Allen & Boosalis 1983) allowing AM plants to maintain a higher stomatal conductance than NM plants under drought conditions (see 8 in fig. 1.1). Meta-analysis estimated AM stomatal conductance to be 24% higher than in NM plants during drought stress (Augé *et al.* 2015). In itself, delayed stomatal closure and maintaining stomatal conductance are a mechanism by which AM symbiosis can alleviate drought as maintaining a transpiration stream maintains water and inorganic nutrient uptake.

Alternatively, maintaining stomatal conductance may result from other mechanisms of mitigating drought (such as hyphal water uptake, altered root architecture) delaying the need for the plant to close its stomata. Either way, studies generally report that AM plants maintain a higher transpiration rate than NM plants during drought conditions (see 9 in fig. 1.1).

Barley: a model for understanding the effects of drought in cereals

Typically, mycorrhiza-mediated drought alleviation is investigated using plant hosts such as strawberry, tomato, lettuce or citrus, that are strongly responsive to mycorrhizal colonisation, have high water demand, high value fruit, or a perennial growth habit, thus making the cost of AM inoculation more economically viable (Jayne & Quigley 2014). Mechanisms relevant in these fruit crops are likely to differ in importance compared to cereal crops that have a lower water demand and are grown under very different conditions. Studies using cereals are less represented in the literature, and AM effects on barley under drought are under-reported, despite its use as a model cereal crop (Harwood 2019), and indeed no consensus on the ability of AM inoculation to alleviate drought in barley has been reached due to the low number of studies conducted to date (Jayne & Quigley 2014, Zhang *et al.* 2019).

Barley is a self-pollinating cereal in the Poaceae family and is used as a model for the Triticeae (including wheat) due to its diploid genome and wide range of genetic and genomic resources (Harwood 2019). Globally, barley is the fourth most important cereal crop in terms of production quantity after maize, rice, and wheat (FAO STAT 2020). Though the majority of barley is used for animal fodder and in brewing, an increasing interest in barley for human consumption has arisen from an improved understanding of the health benefits of its leaves and grains, which have high antioxidant and beta-glucan contents respectively (Kowalczewski *et al.* 2020, Harwood 2019).

Seeing as the effects of a changing climate include a global increase in extreme drought and heat events, barley yields are expected to suffer, with global average yield losses of between 3 and 17% due to extreme heat and drought events by the end of the century (Xie *et al.* 2018), though some predictions suggest even higher impacts such as drought-related yield reductions of 17% by 2050 (Li *et al.* 2009).

In temperate regions, spring barley has traditionally been a reliable crop and a useful alternative to wheat in years when late winter is wet, or where blackgrass is a particular problem for wheat production (Farmers' Guide 2021). Whilst we associate a need for drought tolerant crops with arid climates, it is becoming increasingly apparent that adaptation to more frequent, more extreme droughts is necessary for temperate agriculture (Holman *et al.* 2021). If carbon emissions continue to rise as per the IPCC's highest emissions scenario (RCP8.5), parts of the UK are expected to experience an increase in soil moisture deficit of over 250mm, and a 15-35% increase in the lengths of severe agricultural drought over the next 50-70 years (Arnell & Freeman 2021). Knowledge of drought-tolerance mechanisms in crops such as barley are important for maximising reliable production in the face of a climate of increasing extremes.

According to a review by Sallam *et al.* 2019, wheat and barley cultivars achieve better yields during drought stress conditions via drought tolerance mechanisms including:

- An increased accumulation of antioxidants and compatible solutes (specifically reduced-glutathione, ascorbate, polyamines and glycine betaine);
- Maintained levels of primary metabolites (sugars, amino acids, proline);
- Increased antioxidant enzyme activity.

Drought avoidance mechanisms of wheat and barley have been identified including:

- A longer root system;
- Increased leaf waxiness and trichome density.

Drought sensitivity in wheat and barley is associated with:

- Membrane deterioration due to lower antioxidant activity;
- High levels of reactive oxygen species (ROS) and products of oxidative damage;
- Reduced leaf area;
- Reduction in chlorophylls and carotenoids.

Comparison of the transcriptomic responses to drought in four key cereal species (rice, maize, barley and *Brachypodium*) has suggested that the balance of maintaining photosynthesis and delaying initiation of leaf senescence is important in differentiating between drought tolerant and drought sensitive cereal cultivars and that fine-tuning the balance of these processes may permit improvements in drought tolerance (Baldoni *et al.* 2021). The same study found a number of “conserved drought tolerance genes” (those that were consistently up- or down- regulated between drought tolerant and drought susceptible cultivars in each of the four grass species). As well as those involved directly in photosynthesis or leaf senescence, and known stress response genes, genes involved in chlorophyll biosynthesis, jasmonic acid signalling, auxin signalling, cellular transport and secondary metabolism were all found to be conserved between the transcriptomic drought responses of the four grass species (Baldoni *et al.* 2021).

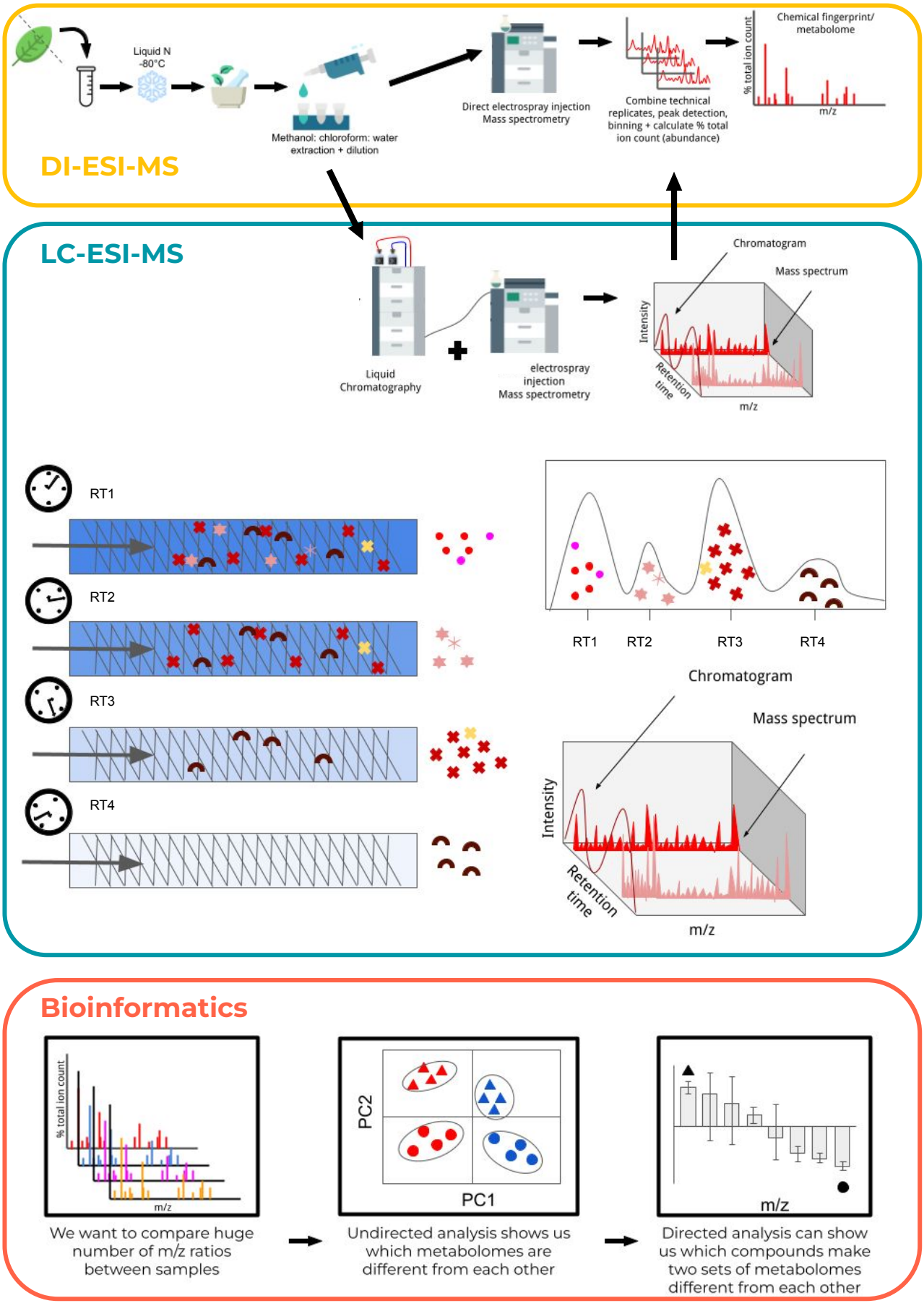

Fig. 1.2. Untargeted metabolomics workflow using direct injection mass spectrometry (DI-ESI-MS) or liquid chromatography coupled to MS (LC-ESI-MS). Sequential elution of compounds from the liquid chromatography column filters entry of compounds into the MS, reducing ionisation issues and adding orthogonal information for identification. m/z = mass to charge ratio, RT = retention time, PC = principal component

Investigating barley responses to drought: untargeted metabolomics as a tool

Since there are such a range of potential drought response mechanisms that may be involved in mycorrhiza-mediated drought alleviation, a holistic approach to the plant-fungal response to drought could provide important insights (Augé 2001). In vascular plants, which have developed a complex secondary metabolism to harness biochemistry for coping with life as sessile organisms, using chemical compounds to defend themselves, communicate and respond to external stimuli, the metabolome is of particular interest. Metabolomics is the study of the suite of chemical compounds present in cells or tissues. Compared to genomics or transcriptomics, metabolomics represents an insight into the state of an organism that is less removed from the phenotype and is more representative of the interaction between genetics and environment (Allwood & Goodacre 2009).

Targeted metabolomics, which investigates the changes in specific (classes of) chemical compounds, can provide detailed insight into cellular functioning and quantifiable effects of treatments (Allwood *et al.* 2021). However, the plant metabolome is so enormous, containing over 200 000 potential compounds, that untargeted approaches, which aim to qualitatively analyse a broad range of metabolite classes, are beneficial for exploratory analysis, understanding co-occurring changes in multiple biochemical pathways, and signposting directions for further targeted analyses (Allwood & Goodacre 2009).

For untargeted metabolomics approaches, sampling and immediately quenching biochemical reactions in the sampled tissue, followed by analysis using various mass spectrometry techniques and bioinformatic comparison of samples, permits a semi-quantitative “snapshot” assessment of the organism’s biochemistry at a particular moment in time, termed a “metabolomic fingerprint” (Kaur *et al.* 2022). Different mass spectrometry (MS) techniques may be more-or-less suited to detecting certain chemical classes and thus, no one MS can give fully untargeted data for all plant metabolites, so decisions have to be made about which types of compounds may be of interest (Garibay-Hernández *et al.* 2021).

Direct injection electro-spray ionisation mass spectrometry (DI-ESI-MS), is a fast and cost-effective high-throughput approach that can be used as a “first-pass” for investigating a new experimental system (Allwood & Goodacre 2009) and is used as such in chapter 3 of this thesis. Liquid chromatography coupled to electro-spray ionisation mass spectrometry (LC-ESI-MS) provides an orthogonal set of data (retention time in addition to mass-to-charge ratio), which improves the ease of metabolite annotation for a broad range of compounds (de Vos *et al.* 2007, Tautenhahn *et al.* 2012) and is thus used for detailed analysis of leaf metabolomes in chapter 5 of this thesis.

Untargeted metabolomics analysis to investigate stress-responses in plants requires a sophisticated bioinformatics workflow to reduce background noise; identify peaks in the data; align, group and normalise the data between samples before the MS data can be submitted to multivariate analysis to identify patterns in the metabolomic fingerprints (fig. 1.2. and Allwood *et al.* 2021). Data is typically acquired in a proprietary format using vendor software but efforts to improve the interoperability and reproducibility of downstream workflows has been identified as an important development required for future research (Allwood *et al.* 2021) and this is addressed in chapter 5 (and appendix I) of this thesis.

Current understanding of drought effects on the barley metabolome

Untargeted metabolomics has been employed by a number of studies to investigate the barley response to drought stress. Primary metabolites, such as the sugars fructose and glucose are found to accumulate under drought conditions in barley leaves from multiple cultivars, concurrent with reductions in accumulation of starch and amino acids such as serine, aspartate and glutamate (Templer *et al.* 2017, Chmielewska *et al.* 2016). The tricarboxylic acid (TCA) cycle is affected by drought in barley (Swarcewicz *et al.* 2017) with TCA cycle intermediates citrate and malate found to accumulate in droughted barley leaves while fumarate and succinate accumulate in non-stressed leaves (Chmielewska *et al.* 2016).

Other compatible solutes such as proline have been found to accumulate in the leaf in response to drought stress in barley (Chmielewska *et al.* 2016, Templer *et al.* 2017). Yuan *et al.* (2018) found accumulation of proline to be time dependent and influential in the difference between drought-tolerant and drought-susceptible hulless barley cultivars' metabolomic responses to simulated drought. Glycine betaine is another compatible solute involved in maintaining cellular homeostasis under osmotic stress, with steps in its biosynthesis highlighted as important in the drought-tolerance of barley by genomic studies (Guo *et al.* 2009, Ashoub *et al.* 2015, Harb *et al.* 2020).

Piasecka *et al.* 2017 found 135 compounds, the majority of which had potential antioxidant properties, to be affected by drought in a study of 100 recombinant inbred lines (RIL) of barley. These included flavonoid glycosides, hydroxycinnamic esters of flavones, ferulic acid derivatives and blumenol derivatives, and similar patterns have since been observed using phenolomics (metabolomics targeted to phenolic compounds) (Piasecka *et al.* 2020, Kowalczewski *et al.* 2020).

Studies have shown considerable differences in the leaf metabolomes of barley collected from different cultivars (Piasecka *et al.* 2015) or at different developmental stages (Lee *et al.* 2016, Brauch *et al*. 2018, Yan *et al.* 2022). It therefore remains challenging to speculate on

which metabolomic responses are common to the drought response of barley and which are cultivar specific or may be related to enhanced drought-tolerance.

Arbuscular mycorrhizal fungi and host metabolomics

As well as altering the root metabolome of plants upon colonisation (Hill *et al.* 2018), arbuscular mycorrhizal fungi have also been found to cause systemic metabolomic changes, with the range and levels of compounds in the leaf metabolome altered by having a symbiont partner (Schweiger *et al.* 2014, Adolfsson *et al.* 2017, Shtark *et al.* 2019). This thesis focuses on the metabolomic responses of barley leaves, since the effects of AM colonisation on root metabolomes is confounded by the presence of the fungal structures within the plant tissue (Toussaint *et al.* 2007).

The leaf metabolomic responses to AM colonisation remain little studied, particularly with regard to polyphenols (Balestrini *et al.* 2020). Whilst the effect of AM colonisation on leaf metabolomes in cereals has been somewhat studied (e.g. Wang *et al.* 2018), it is particularly important when considering the arbuscular mycorrhizal symbiosis not to extrapolate the effects of colonisation between plant hosts or systems, as leaf metabolomic responses can vary dramatically, even between closely related species (Schweiger *et al.* 2014). At the time of writing, no studies of the effect of AM colonisation on the metabolomic response of barley to drought stress are known. However, in a study investigating the effects of the root endophyte *Piriformospora indica* on barley drought stress response, *P. indica* colonised plants had a dramatically different leaf metabolome to uncolonised plants, demonstrating that it is possible to detect symbiont-mediated drought responses using untargeted leaf metabolomics (Ghaffari *et al.* 2019).

Aims of this thesis

The aim of this thesis was to address the following research questions:

- How does drought affect barley physiology and how does that change over the course of a drought period?
- Does the arbuscular mycorrhizal (AM) symbiosis alleviate drought symptoms in spring barley?
- Which biochemical pathways are affected by drought and by the AM symbiosis?

Three experiments using spring barley cv. Concerto (Limagrain, GBR) were conducted to address these questions. In each case, barley seedlings were grown under controlled glasshouse conditions for 6 weeks with either AM inoculation treatments (mycorrhizal AM;

non-mycorrhizal NM), drought treatment (well-watered WW; drought-stressed DS) or a fully factorial combination of both, depending on the objectives of each experiment. Physiological drought symptoms and responses were assessed at three sampling time points during an 18 day drought period, concurrent with harvest of leaf metabolite samples. Leaf metabolomic fingerprints were analysed using either DI-ESI-MS or LC-ESI-MS and an untargeted metabolomics workflow.

Hypotheses

An 18 day drought treatment was expected to reduce stomatal conductance and photosynthetic rate of plants (DS) compared to those that continued to receive regular irrigation (WW).

Leaf relative water content was used to assess the efficacy of the applied drought, and was expected to be significantly reduced in DS plants as compared to WW control plants.

Photosystem II efficiency as assessed by Fv'/Fm' was used to determine the onset of stress damaging to the photosynthetic machinery. Damage was expected to occur earlier in non-mycorrhizal (NM) plants, with mycorrhizal (AM) plants in the drought treatment maintaining an Fv'/Fm' closer to that of well-watered controls for longer.

It was hypothesised that leaf metabolomic fingerprints of spring barley would be distinguishable through multivariate analysis based on whether they had been colonised by AM fungi (AM) or not (NM), and based on whether they had been experiencing drought stress (DS) or not (WW). In addition, temporal differences in metabolomic fingerprints were expected associated with the developmental stage at the time of sampling.

It was expected that differences in metabolomic fingerprints would be observed at sampling time points in advance of damage to photosynthetic machinery (i.e. before Fv'/Fm' was significantly reduced in DS plants compared to WW).

Predictions of the biochemical pathways expected to be involved in barley response to drought stress, and detectable with an untargeted metabolomics approach, were:

- The **phenylpropanoid pathway** was predicted to be involved in barley drought-response based on evidence from Piasecka *et al.* 2017, Kowalczewski *et al.* 2020, and Piasecka *et al.* 2020 who observed differences in the glycosylation patterns of **flavonoids**, and the accumulation of **hydroxycinnamic acid derivatives** (such as hordatines) and **terpenoids** (such as blumenol C derivatives). **Antioxidant**

systems in general have been found to be upregulated in AM compared to NM plants under drought stress (e.g. in maize by Barzàna *et al.* 2015);

- **Amino acid biosynthesis pathways**, particularly those related to biosynthesis of proline, were expected to be affected by drought in barley based on evidence from Templer *et al.* 2017 who observed accumulation of **proline** under drought stress in multiple accessions of barley. However other studies have observed notable reductions in proline in response to drought and/or cultivar-dependent patterns (Chmielewska *et al.* 2016, Guo *et al.* 2018);
- Accumulation of other **compatible solutes** and/or osmolytes was expected under drought conditions based on evidence of accumulation of compounds such as **fructose, glucose, galactinol** and Krebs' citric acid cycle intermediates in droughted barley (Templer *et al.* 2017, Swarcewicz *et al.* 2017).

Compounds and pathways expected to be involved in the response to AM colonisation, and detectable in the leaves, were:

- **Glycosides of blumenol C and hydroxyblumenol C** as these have been proposed as leaf biomarkers for AM colonisation and have been observed as such in barley, amongst other species (Wang *et al.* 2018, Mahood *et al.* 2022);
- Krebs' citric acid cycle intermediates, accumulation of **sugars and starches**, since AM colonisation has been shown to affect sink strength and alter photosynthetic efficiency (e.g. in legumes Kaschuk *et al.* 2009);
- **Chlorophylls** and **carotenoids**/ chlorophyll precursors as chlorophyll content has been shown to increase for a number of species in response to AM colonisation (e.g. in wheat by Yaghoubian *et al*. 2014, Mathur *et al*. 2019 and Abdi *et al.* 2021; in castor bean by Zhang *et al.* 2018; in chicory by Langeroodi *et al.* 2020; and in soybean by Metwaly & El-Khateeb 2019).
- Compounds involved in biosynthesis of plant phytohormones, such as ABA, SLs, JAs and SAs (although the phytohormones themselves are unlikely to be detected with the untargeted methods employed) since AMF have been observed to have diverse effects on phytohormone signalling under various stress conditions (Pendranzani *et al.* 2016, Wang *et al.* 2016, Zhang *et al.* 2019).

Since many compounds and pathways hypothesised to be affected by AM colonisation in barley are also involved in drought response (e.g. blumenol C derivatives, Krebs' citric acid cycle sugars) metabolomes of AM plants in the droughted treatment were expected to be more similar to those of well-watered plants, and that AM colonisation could somewhat alleviate the physiological symptoms of drought.

Chapter 1 - Materials and Methods Used throughout this Thesis

A number of approaches and techniques were used consistently between the three experiments presented in this thesis. To avoid unnecessary repetition, these materials and methods are provided here for reference. Details of methodology specific to each experiment and subsequent analysis are provided in the relevant data chapters (2 to 5).

Plant material

Barley seed (*Hordeum vulgare* cv. Concerto) was supplied by Limagrain UK Ltd. Seeds were surface sterilised using 5% sodium hypochlorite for 3 minutes, rinsed with sterile water three times and then germinated on damp filter papers under sterile conditions for 5 days.

Planting and growth conditions

Seedlings displaying a shoot >10mm with three or more healthy roots were transferred to pots. Plants were grown in square pots of dimensions 110 x 110 x 190 mm. Pots were filled with 1.4l substrate and then adjusted to hold the same mass of substrate as each other.

Plants were grown in a controlled growth facility at the Arthur Willis Environment Centre at the University of Sheffield, UK (53°22'52.8"N 1°29'55.8"W). Temperature day length was 16 hours and day/ night temperatures were 20°C/15°C, while light day length was 12 hours. Ambient light levels were supplemented when ambient light fell below 1000mM, boosting the light level by 200mM.

Experiment dates:

- Experiment 1 (chapters 2 and 3) 27/03/2018 to 08/05/2018
- Experiment 2 (chapter 2) 25/09/2018 to 07/11/2018
- Experiment 3 (chapters 4 and 5) 05/03/2019 to 18/04/2019

Sampling time points

In experiment 1 and 3, three “sampling time points” were established at:

- T1 - 29DPI (5th day of drought)

- T2 - 35DPI (11th day of drought)
- T3 - 42DPI (18th day of drought)

Due to logistical and time constraints, analyses of stomatal conductance and photosynthetic rate are presented for T1 and T2 only in experiment 1 (chapters 2 and 3) and for metabolomic data at T2 and T3 from that experiment (chapter 3).

In experiment 2, metabolite samples were gathered at all three time points but not analysed.

In experiment 3, stomatal conductance, photosynthetic rate and metabolite samples were assessed and analysed at all three time points. For comparison of the timings of sampling in the three experiments, see table 1.1.

Chlorophyll fluorescence

Light-adapted quantum yield (Fv'/Fm') was assessed non-destructively on alternate days between 22 and 28DPI, and then daily there-after to give an estimate of the photosystem II efficiency (Oxborough & Baker 1997) using a Fluorpen FP100 ((Photon Systems Instruments, Drasov, Czech Republic) attached to the central third of the youngest expanded leaf on each plant.

Photosynthetic rate and stomatal conductance

Photosynthetic rate and stomatal conductance were non-destructively assessed in a subset (n=5 or 7) of plants from each treatment combination at sampling time points. Measurements were made using a Li 6400 portable gas exchange system (LI-COR, Lincoln, USA) on the youngest expanded leaf on the main stem. Measurements were performed from 2 hours after the start of the light period in the growth chamber and were completed within 7 hours. The IRGAs were matched every 30 minutes. Relative humidity inside the IRGA chamber was maintained at 45-55% using self-indicating desiccant, flow rate was set at 300μmol s^{-1}. Leaf temperature was set at 20°C, reference CO_2 at 400ppm and light intensity at 150μmol m^{-2} s^{-1}. Plants were allowed to equilibrate for 10 minutes in the IRGA chamber. Once readings were stable, measurements were taken every 20s for 5 minutes and were subsequently averaged (mean) and normalised for leaf area within the IRGA.

Table 1.1. Gantt chart of timing of drought treatment, metabolite sampling and assessment of physiological parameters in experiments 1, 2 and 3. * indicates that samples were taken but are not analysed in this thesis, “s” indicates that the assessment was performed in a subset of plants, “X” indicates that assessment was made for all plants.

EXPERIMENT 1 - axenic AM v NM (no colonisation), WW vs DS

Sampling time point																													T1									T2								T3
																													1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42
Transplant + inoculate	X																																											
Weighing pots		X		X			X		X		X			X		X		X			X		X		X			X	X	X	X	X		X	X	X	X	X	X	X	X	X	
Irrigation (WW)				X			X		X		X			X		X		X			X		X		X			X		X		X		X		X		X		X		
Irrigation (DS)				X			X		X		X			X		X		X			X		X		X										X		X		X				
Chlorophyll fluorescence (light- adapted)																									X			X	X	X		X	X	X	X	X	X	X	X	X		X		
Photosynthesis																														S						S									
Stomatal conductance																														S						S									
Metabolite sample (leaf)																														*						X									X
Leaf RWC																																					S								
Destructive harvest																																													X

EXPERIMENT 2 - AM vs NM

	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43
Transplant + inoculate	X																																											
Metabolite sample (leaf)																														*						*									*
Destructive harvest																																													X

EXPERIMENT 3 - AMDS, AMWW, NMDS, NMWW

Sampling time point																													T1									T2									T3		
Day of drought																													1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Days post inoculation	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44
Transplant + inoculate	X																																												
Weigh pots	X	X		X			X		X		X			X		X		X			X		X		X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	
Irrigation (WW)	X	X		X			X		X		X			X		X		X			X		X		X		X		X		X		X		X			X		X			
Irrigation (DS)	X	X		X			X		X		X			X		X		X			X		X		X											X								
Chlorophyll fluorescence (light- adapted)																							X		X		X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	
Photosynthesis																														S						S								S		
Stomatal conductance																														S						S								S		
Metabolite sample (leaf)																														X						X								X		
Leaf RWC																																					S									
Destructive harvest																																													X

Leaf relative water content

At 37DPI leaf relative water content was assessed in the same subsample of plants that was used for stomatal conductance and photosynthetic rate measurements (see above). A 6cm leaf tip sample was removed from the second youngest expanded leaf on the main stem and sealed in a pre-weighed ziplock bag (BW). The mass of the bag containing the leaf (BLW) was recorded and then 2ml of UHP water was added the bag resealed and suspended for 24 hours in the dark. The leaf sample was removed and reweighed to give turgid mass (TW) before being dried at 80°C for 48 hours and reweighed (DW). The leaf relative water content was calculated using equation 3:

Equation 3 $$RWC = ((BLW - BW) - DW)/(TW - DW) \times 100$$

Biomass measurements

Plants were destructively harvested (following leaf, root and substrate sample collection for metabolite extractions - see below). Aboveground (shoot) and belowground (root) biomass were separated, roots were rinsed with tap water and the fresh mass of shoot and root was recorded. Biomass was dried at 80°C for 72 hours before shoot and root dry masses were recorded. Root biomass was calculated using the total fresh mass (total FW) of the washed root biomass and the mass of the subsample removed for mycorrhizal staining (subsample FW) as well as the dried mass of the remaining roots (bulk DW) as per equation 2.

Equation 2 $$estimated\ total\ DW\ =\ (subsample\ FW\ /\ total\ FW\ +\ 1\ )\ \times\ bulk\ DW$$

Scoring mycorrhizal colonisation of roots

Following destructive harvest, root samples were stained using a method adapted from Vierheilig *et al.*1998. Root samples were rinsed with dH_2O and placed in 10% KOH at 80◦C for between 22 and 25 minutes. Samples were then rinsed with dH_2O and placed in the stain solution for 20 minutes at room temperature. The stain solution comprised 5% Pelikan 4001 black ink (Pelikan AG, Berlin, Germany), 5% acetic acid, 90% dH_2O. Samples were then rinsed with 1% acetic acid before being left for 1 hour in 1% acetic acid to de-stain. Root samples were further de-stained in 50% glycerol overnight at room temperature.

An adapted version of the grid-line intersect method (McGonigle *et al.* 1990) was used to assess % root colonisation. Twenty pieces of stained root 1cm in length were cut and mounted on each microscope slide and eight slides were prepared per root sample (160cm root total per plant). Assessment of root colonisation was made by examining slides at 200x magnification using a Zeiss primo star microscope (Zeiss, Oberkochen, Germany). Two

passes were made per slide and intersections were scored according to Brundrett *et al.* 1994 until 300 intersections had been scored per sample. Maximum mycorrhizal, arbuscular and vesicular colonisation were calculated according to Brundrett *et al.* 1994. This method meets the minimum requirements set out by Sun & Tang (2012) for scoring colonisation in this type of experiment.

In addition, photomicrographs of representative root sections containing fungal structures were taken at 200x or 400x magnification using an Olympus BX51 microscope with a connected DP71 digital camera (Olympus Optical Ltd, London, UK).

Metabolite sampling and extraction

At each sampling time point, a 2cm leaf tip sample was taken from the youngest expanded leaf on a tiller. The final leaf tip sample was taken immediately prior to destructive harvest, from the youngest expanded leaf on the main stem. 2ml sample of substrate was taken from substrate that initially clung to the roots but was shaken free (rhizosphere substrate) and an approximately $2cm^2$ section of roots was rinsed in UHP water. All samples were snap frozen in liquid nitrogen before being stored at -80°C. Rhizosphere substrate and root samples are not analysed here.

Leaf metabolites were extracted on ice using the water: methanol: chloroform method (Overy *et al.* 2005). Briefly, leaf samples were ground and homogenised with an extraction solvent mixture ($MeOH/CHCl_3/H_2O$) using ball bearings in a FastPrep-24 5g bead mill (MP Biomedicals, USA) then centrifuged and the supernatant removed, while the pellet was re-extracted with a second extraction solvent mixture ($MeOH/CHCl_3$). The resulting supernatant was combined with the existing extract. Phase separation was achieved by adding ice cold distilled H_2O and $CHCl_3$ and centrifuging. Phases were stored separately at -80°C and the aqueous phase was analysed within 3 days.

Statistical analyses

The R statistical computing environment (R version 3.5.1 for chapters 2 and 3 and version 4.1.2 for chapters 4 and 5) was used to perform all statistical analyses (R core team 2018). Packages used in data cleaning and analysis included readr (Wickham *et al.* 2021), tibble (Müller *et al.* 2021), ggplot2 (Wickham *et al.* 2016), ggpubr (Kassambara 2020), dplyr (Wickham *et al.* 2021), tidyr (Wickham *et al.* 2021), stringr (Wickham 2019), emmeans (Lenth 2022), Rmisc (Hope 2013), car (Fox & Weisberg 2019), pcaMethods (Stacklies *et al.* 2007), muma (Gaude *et al.* 2012), vegan (Oksanen *et al.* 2022).

Binomial GLMs were used to analyse the effect of AM inoculation and Drought treatment on the mass of water remaining in pots (as a % of pot capacity). Each of the three time points were analysed separately using the following model in which `percentPC` is the mass of water remaining in the pot expressed as a percentage of the initial pot capacity:

```
water.timepoint.model<-glm(percentPC ~ Drought * AMF,
family="binomial", data=irrigation_timepoint)
```

The effects of AM inoculation and drought on the mass of water remaining in pots (as a percentage of the initial pot capacity) over the experiment was analysed using the following generalised linear mixed effects model (GLMM), from the lme4 package (Bates *et al.* 2015), fitted by maximum likelihood:

```
water.model<-lmer(percentPC ~ Drought + AMF + (1|Pot) + (1|Time),
data=irrigation_table, REML=FALSE)
```

For assessing the effects of AM inoculation and drought on Fv'/Fm' over the drought period, the following GLMM was fitted by maximum likelihood:

```
lightQY.model<-lmer(QY_light ~ Drought + AMF + (1|Pot) + (1|Time),
data=QYtable, REML=FALSE)
```

Root colonisation by AM fungus (maximum colonisation and arbuscular colonisation), leaf relative water content, shoot and root dry weights and C, N, P and Si content were analysed using 2-way analysis of variance (ANOVA). 2-way ANOVAs were also used to analyse selected variables at the three sampling timepoints (Fv'/Fm', stomatal conductance and photosynthetic rate). In these analyses time points were treated separately. All analyses used the 95% confidence interval for estimating statistical significance.

Statistical analysis of metabolomic data is detailed in the relevant chapters.

Chapter 2 - Development of an experimental system to simultaneously investigate physiological and metabolomic response to drought and AM inoculation

ABSTRACT

Arbuscular mycorrhizal (AM) fungi are root symbionts that have been shown to alleviate the deleterious effects of drought in a number of crop plants. Studies exploring the beneficial effects of AM colonisation under drought are lacking. Here, two glasshouse experiments are presented which aimed to establish a suitable system for measuring both physiological and metabolomic responses of cereals to drought. An 18 day drought treatment from the 24th day post transplant was found to be sufficient to elicit a physiological drought response in spring barley cv. Concerto. Combining commercially available inoculum in a primarily sand substrate was found to result in a satisfactory level of AM colonisation in roots of treated plants. An otherwise similar non-mycorrhizal (NM) treatment was mixed in the primarily sand substrate using the manufacturer's carrier substrate and this was found to be satisfactory in producing barley plants lacking evidence of root colonisation by AM fungi.

INTRODUCTION

Between 2016 and 2017, the area of arable land used for spring barley production in the UK increased by 10.4% (DEFRA 2018). Concerto, the cultivar chosen for this study, is a spring barley released in 2009 and by 2016 had come to be considered the UK "market leader" (Limagrain 2016). It was recommended for cultivation across the UK for both brewing and malt distilling purposes (AHDB 2017). Currently, RGT-Planet (RAGT seeds) which was bred from TamTam x Concerto (Agrii 2022), is the second most sown barley in the UK (AHDB 2021).

Arbuscular mycorrhizal (AM) fungi have been shown to alleviate the physiological symptoms of drought, and maintain the yield of various crop species, under drought conditions (Jayne & Quigley 2014). However, few studies have investigated the effects of AM colonisation on barley production (Zhang *et al.* 2019) or the potential of AM colonisation to alleviate the negative effects of drought in barley (Jayne & Quigley 2014).

The aim of the current study was to compare metabolomic responses to drought of mycorrhizal and non-mycorrhizal barley. This chapter describes the work that was first necessary to establish an experimental system in which:

(a) A drought regime could be imposed that would result in physiological stress;
(b) Arbuscular mycorrhizal (AM) inoculum would result in root colonisation of barley and the control non-mycorrhizal (NM) inoculum would not lead to root colonisation;
(c) The substrate could support healthy plant growth in both the AM and NM treatments without inhibiting root colonisation.

The time taken for mycorrhizae to establish in the roots of cereal plants in pot experiments has varied between previous studies. Evidence of colonisation is sometimes observed from as early as 16 days post inoculation in barley (Vierheilig *et al.* 2000). Vierheilig & Ocampo (1990) found no difference between different AM fungal species in the percentage root colonisation achieved in two wheat cultivars at 6 weeks post inoculation (WPI). Stoner *et al.* (2014), on the other hand, found AM fungal species identity to be particularly important in affecting the speed, as well as the extent of colonisation: at 6 WPI wheat roots inoculated with *Gigaspora margarita* were not yet colonised whereas roots inoculated with *Rhizophagus irregularis* were (Stoner *et al.* 2014). By 16 WPI, both fungi had colonised wheat with *G. margarita*-colonised and *R. irregularis*-colonised plants showing 30% and 90% root colonisation respectively (Stoner *et al.* 2014).

In soils containing low levels of phosphorus (P), Graham & Abbott (2000) found that three cultivars of wheat had 40-60% root length colonisation by 2 weeks post inoculation (WPI), while three other cultivars had much lower levels of root colonisation (less than 20%). When the same cultivars were grown in high P soils, however, all 6 cultivars had low levels of root length colonisation (below 20% at 2, 4 and 6 WPI) (Graham & Abbott 2000). Other glasshouse pot studies in non-field soil substrates have demonstrated 40-80% root colonisation of barley cv. Salome by 5 weeks post inoculation (Maier *et al.* 1995, Fester *et al.* 1999, Vierheilig *et al.* 2000). This variability in results, highlights the importance of careful substrate choice and nutrient availability in designing experimental systems that promote mycorrhizal colonisation. Any alteration to the substrate, plant cultivar choice or AMF strain is predicted to alter root colonisation levels as well as the “benefit” of the symbiosis to the plant. This makes experimental design choices and reproducibility extremely challenging in mycorrhizal research and also limits the parallels that can be drawn between superficially similar studies (Sun & Tang 2012).

For the current study, plants were grown for 6 weeks post inoculation and a root colonisation of >20% root intersections containing arbuscules, vesicles or intraradical hyphae was deemed acceptable to confirm the plant as mycorrhizal as long as the root colonisation in plants treated with a non-mycorrhizal inoculum would result in <1% root colonisation.

Both axenically produced (from root organ cultures) and commercially available mycorrhizal inocula were trialled in the study presented here. Root organ cultures are produced using *Daucus carota* roots and Ri T-DNA transformation by *Agrobacterium rhizogenes* resulting in “hairy roots” that can proliferate on gel media containing sucrose (Fortin *et al.* 2002). This permits root growth without any photosynthetically active organs. Sterilised arbuscular mycorrhizal spores can be added to these root organ cultures so that the symbiosis occurs without the need for pot cultures. The root-only and root-AMF cultures can be propagated aseptically to produce a regular supply of NM and AM inocula in the laboratory (Cranenbrouck *et al.* 2005).

Commercial inoculum, on the other hand, is supplied without detailed information on the strains of AM fungi in the inoculum. For this study, the supplier also provided enough carrier substrate (granular clay) to produce a non-mycorrhizal control treatment.

Leaf relative water content (RWC) and the photosystem II efficiency (Fv’/Fm’) were used in this study to monitor the water status of the plants during the drought period and confirm that the imposed drought regime was eliciting a physiological response in the barley. Reduced photosystem II efficiency and leaf RWC have been used extensively as markers of plant drought stress when comparing drought-stressed to well-watered control plants (Maxwell & Johnson 2000, Jones 2007). Hand-held instruments mean data-collection is fast and makes it possible to track changes in photosystem II efficiency daily on large numbers of plants over the course of an experimental drought period. For example, Hughes *et al.* (2017) used this approach of monitoring Fv’/Fm’ throughout a drought period, along with measuring leaf RWC, in order to confirm the increased drought tolerance of barley mutants with reduced stomatal density compared to control plants.

AIMS AND OBJECTIVES

To establish a glasshouse pot set-up that is suitable for testing the effects of drought and mycorrhizal colonisation on barley physiology concurrent with collecting multiple sequential metabolite samples from the same plants.

Experiment 1 - To withhold water from barley plants (*Hordeum vulgare* cv. Concerto) in a way that causes a reduction in at least one of leaf relative water content or photosystem II efficiency (as compared to well-watered control plants). To check that AM and NM inoculation treatments result in root colonisation >20% and <1% respectively (including evidence of arbuscules).

Experiment 2 - To check that AM inoculation (with Plantworks Ltd. commercially available inoculum) of barley (*Hordeum vulgare* cv. Concerto) results in AM colonisation of roots after 6 weeks (with root colonisation >20% for AM and <1% for NM treatments). To choose a substrate that supports healthy growth combined with successful AM colonisation.

MATERIALS AND METHODS

Experimental design

Two experiments were carried out to establish a suitable system for measuring both physiological and metabolomic responses of barley (*Hordeum vulgare* cv. Concerto) to drought. Both were set up in pots under glasshouse conditions. Methods to measure physiological parameters (chlorophyll fluorescence and leaf relative water content) were conducted to monitor the effects of drought treatment in this system and leaf metabolite samples were collected during drought periods to ascertain whether combined collection of these measurements was possible from the same plants within the time-frame of the experiment. At destructive harvest, root samples were taken for assessment of mycorrhizal colonisation. Data on growth and biomass were also recorded.

Specific aspects of the experimental design of each trial were as follows:

Experiment 1 - A fully factorial trial in which barley plants were grown in a compost/ sand substrate and received one of two axenically-produced mycorrhizal inoculation treatments (inoculated with AM fungi or a mock inoculum) and then, after an establishment period, plants were subjected to one of two irrigation treatments for 2 weeks: well-watered or drought stressed.

Experiment 2 - Barley plants were grown in one of two substrates, with either a commercial mycorrhizal (AM) inoculum or the inoculum carrier lacking any fungal material (NM inoculum). Plants were harvested at 6 weeks to assess colonisation.

Mycorrhizal inoculum

For the axenic liquid inoculum used in experiment 3, arbuscular mycorrhizal fungi of the strain DAOM 197198 (*Rhizophagus irregularis*) were obtained from stocks in the Department of Animal and Plant Sciences at the University of Sheffield. Inoculum was grown on MSR medium using Ri T-DNA transformed carrot root according to the methods of Cranenbrouck

et al. (2005). Two plates of inoculum dense with hyphae and spores were liquified and diluted with UHP water under sterile conditions to give 250ml of liquid mycorrhizal (AM) inoculum with a spore density of 16 spores ml^{-1}, as assessed by a spore count at x200 magnification using a Zeiss primo star microscope (Zeiss, Oberkochen, Germany). An equivalent NM inoculum was produced by an identical method but using plates containing only roots. 8ml of AM (equivalent to 128 spores per plant) or NM inoculum was added to the pots when the seedlings were transplanted (2ml in the central planting hole and a further 1.5ml in each of 4 peripheral inoculating holes).

Experiment 2 - A mixed strain AM inoculum was supplied in carrier substrate from Plantworks, Kent, UK, in addition to sufficient carrier substrate (granular clay) to produce an NM inoculum. Inocula were incorporated in their supplied form as detailed under "Growth Substrates" resulting in a higher application rate than that recommended by the manufacturer.

Growth substrates

All growth substrates were autoclaved for 1 hour, left at room temperature for a week, and then re-autoclaved for a further hour. Prior to use, the substrate was left for 2 weeks to avoid the negative plant-growth effects of autoclaving (Rovira & Bowen 1966). Substrates were mixes (see table 2.1) of Levington's advanced M3 compost (ICL, Ipswich, UK) and silica sand.

In the case of substrate 5 and 6, compost and sand were mixed in a ratio of 1:2 or 2:1 and autoclaved as above. Just before filling pots, this substrate was mixed with commercial AM or NM inoculum in a ratio of 3:1 to produce substrates 5 and 6.

Estimating pot capacity

In experiment 1 the pot capacity (PC) was estimated using 10 pots filled in the same way as the experimental pots (see chapter 1). Pots were saturated with UHP water and allowed to drain by gravity for 1 hour (the point at which dripping stopped). The mass of the pot and substrate before saturation was subtracted from its mass at 1 hour after saturation to give the mass of water required to achieve pot capacity. An average of this measure was taken from 10 pots to give the "pot capacity" (PC) for this experimental setup. Three times per week, pots were weighed and then the mean mass required to return the pots to 80% PC was added so that all pots received the same mass of water on any given day, while % PC was still tracked.

In experiment 2, all pots received the same mass of water on any given day, and were watered three times per week.

Drought treatments

Experiment 1 - From 24 DPI (Zadok's scale Z15.23), plants in the well-watered treatment (WW) continued to be watered three times per week as described above. Plants in the drought stress (DS) treatment received only enough water to return the mean of 10% PC. As such, plants in the WW treatment received 2506g water in total over the course of the experiment while plants in the DS treatment received 1325g water. From 24DPI, gravimetric % PC was measured daily by weighing for all pots.

See also: Chapter 1 - Materials and Methods used throughout this Thesis

Table 2.1. Substrate compositions

Substrate	Ratio	Components	Used in:
Substrate 3	1:1	M3 compost: sand	Experiment 1
Substrate 5	1:1:2	(AM or NM) inoculum: M3 compost: sand **Primarily sand substrate**	Experiment 2
Substrate 6	1:2:1	(AM or NM) inoculum: M3 compost: sand **Primarily compost substrate**	Experiment 2

RESULTS

Mycorrhizal colonisation

Experiment 1 - Mycorrhizal structures were observed in both axenically produced AM and NM treated plants (fig. 2.1). Mycorrhizal colonisation of roots was low and did not exceed 4% in any plant. Maximum % mycorrhizal colonisation of barley roots was unaffected by both drought treatment ($z=0.944$, $p=0.345$), inoculation treatment ($z=-0.073$, $p=0.942$), or their interaction ($z=-1.120$, $p=0.263$) (table 2.2).

Experiment 2 - Treatment with commercial mycorrhizal (AM) inoculum significantly increased colonisation 6 weeks after transplanting seedlings (fig. 2.2.e) ($z=-7.408$, $p=1.29 \times 10^{-13}$). Plants grown in the primarily sand substrate (substrate 5) had maximum percentage root colonisation more than twice that of plants grown in the primarily compost substrate (substrate 6) ($z=10.958$, $p<2 \times 10^{-16}$). In the primarily sand substrate maximum colonisation was 49.0% while in the compost substrate a maximum colonisation of only 20.9% was observed. Plants from the primarily sand AM substrate had 33.1% and 12.1% root containing arbuscules and vesicles respectively. In the compost AM substrate, a lower percentage of roots contained arbuscules (12.1%) and vesicles (4.0%) than in the primarily sand AM substrate.

In the plants treated with non-mycorrhizal control inoculum (NM), only very minimal mycorrhizal colonisation was observed: 0.4% in the primarily sand substrate and 0.3% in the compost substrate. No arbuscules or vesicles were found in the roots of non-mycorrhizal control plants from either substrate.

Water remaining in pots

Experiment 1 - Inoculation treatment had no effect on water remaining in pots as a percentage of pot capacity ($\chi^2=0.353$, $df=1$, $p=0.553$). The well-watered treatment maintained % PC around 51.7% while drought treatment reduced the % PC ($\chi^2=29.262$, $df=1$, $p=0.632 \times 10^{-8}$) to 2.2% by the end of the drought period (fig. 2.3.a).

Leaf relative water content

Experiment 1 - Leaf relative water content was significantly reduced in droughted plants compared to well-watered plants ($F=37.516$, $df=1$, $p=1.46 \times 10^{-5}$) but was unaffected by inoculation treatment ($F=0.214$, $df=1$, $p=0.650$) or the interaction between drought and inoculation treatments ($F=0.384$, $df=1$, $p=0.544$) (fig. 2.3.c).

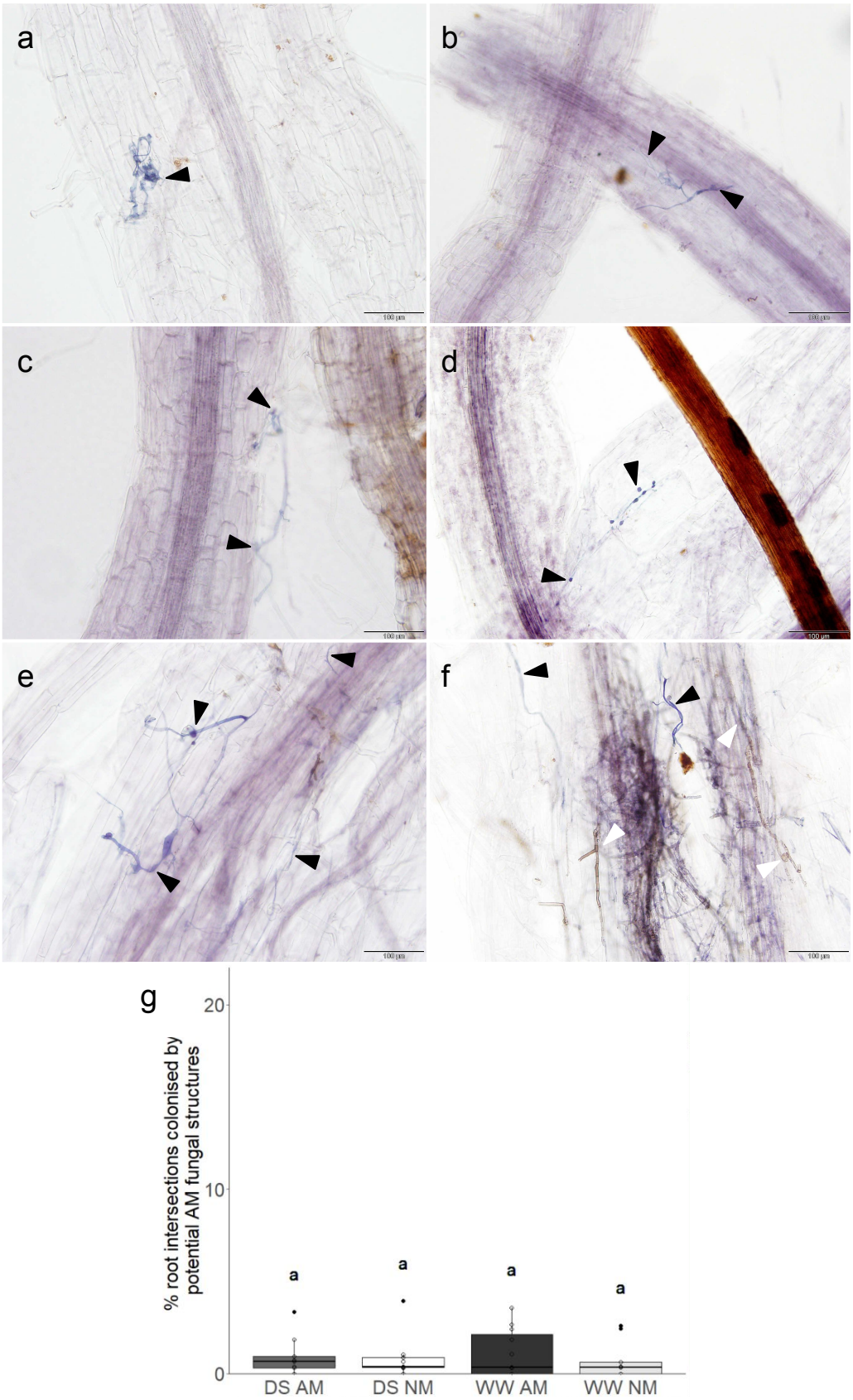

Fig. 2.1. Photomicrographs of *Hordeum vulgare* cv. Concerto roots grown with AM (*R. irregularis* DAOM197198) or NM inoculum in experiment 1. AM fungal structures are stained blue (ink and vinegar stain) and denoted by a black arrow. White arrows denote fungi unlikely to be mycorrhizal. (a) and (b) are rfrom the same AM inoculated plant in the well-watered treatment. (c) is from an NM plant in the well-watered treatment. (d) is from an NM inoculated plant under drought stress. (e) and (f) are from the same NM inoculated plant in the drought stress treatment. Mean max. % root colonised by hyphae, arbuscules or vesicles is shown in (g) for drought stressed (DS) and well-watered (WW) plants in either the AM (mycorrhizal inoculum) or NM (non-mycorrhizal) treatment. Boxes that share the same letter are not statistically significantly different at the 95% CI.

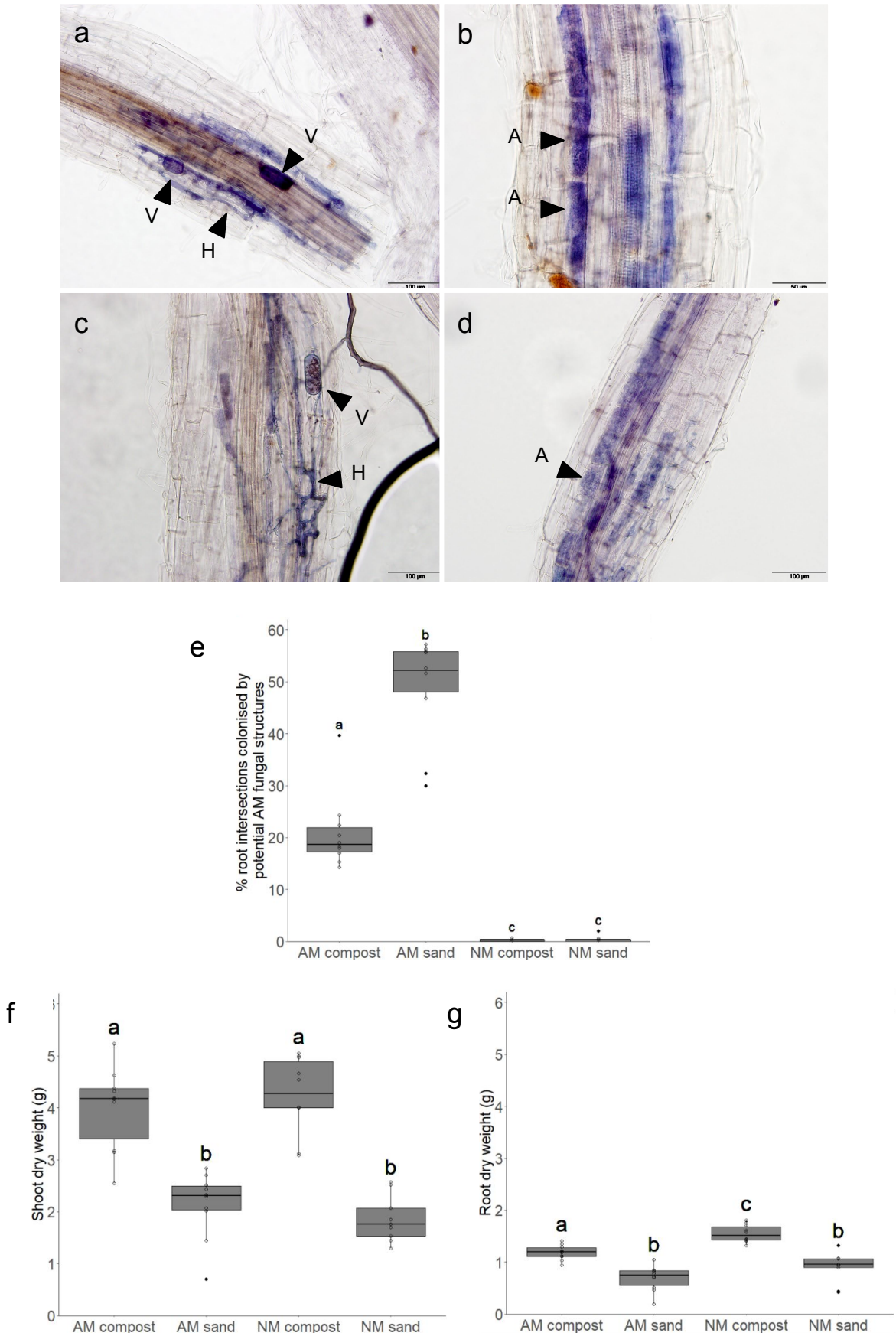

Fig. 2.2. Photomicrographs of *Hordeum vulgare* cv. Concerto roots in experiment 2 grown with AM or NM inoculum (Plantworks Ltd.). AM fungal hyphae (H), arbuscules (A) and vesicles (V) are stained blue (ink and vinegar stain) and denoted by a black arrow. Mean maximum % of root colonised by hyphae, arbuscules or vesicles (e) and shoot (f) and root (g) dry biomass are shown for plants from the two trialled substrates (primarily compost and primarily sand). Bars that share the same letter are not statistically significantly different at the 95% CI.

Table 2.2. Statistical summaries of the effects of AMF inoculation and Drought treatment or Substrate composition on AM colonisation, mass of water in pots, leaf relative water content (RWC), efficiency of photosystem II (Fv'/Fm'), shoot and root dry weights (DW). NS = non significant, * = significant at the 90% CI, ** = significant at the 95% CI, *** = significant at the 99% CI

	Experiment 1			Experiment 2		
	AM inoculation	**Drought treatment**	**AMF * Drought**	**AM inoculation**	**Substrate treatment**	**AMF * Substrate**
AM colonisation	z=0.073, p=0.942	z=0.944, p=0.345	z=-1.120, p=0.263	z=-7.408, p=1.29e-13	z=10.958, p=<2e-16	z=-0.760, p=0.447
Mass water remaining in p	χ2=0.353, df=1, p=0.553	χ2=29.262, df=1, p=0.632e-8	χ2=0.199, df=1, p=0.655	-	-	-
Leaf RWC	F=0.214, df=1, p=0.650	F=37.516, df=1, p=1.46e-5	F=0.384, df=1, p=0.544	-	-	-
Fv'/Fm'	χ2=0.0169, df=1, p=0.897	χ2=20.719, df=1, p=5.318e-6	χ2=0.0269, df=1, p=0.870	-	-	-
Shoot DW	F=7.656, df=1, p=0.009	F=188.548, df=1, p=7.02e-1	F=3.182, df=1, p=0.083	F=0.060, df=1, p=0.808	F=95.938, df=1, p=1.45e-11	F=1.489, df=1, p=0.231
Root DW	F=0.141, df=1, p=0.710	F=9.073, df=1, p=0.00479	F=0.245, df=1, p=0.623	F=18.740. df=1, p=1.19e-4	F=66.751, df=1, p=1.26e-9	F=1.305, df=1, p=0.261

	Experiment 1			Experiment 2		
	AM inoculation	**Drought treatment**	**AMF * Drought**	**AM inoculation**	**Substrate treatment**	**AMF * Substrate**
AM colonisation	NS	NS	NS	***	***	NS
Mass water remaining in p	NS	***	NS	-	-	-
Leaf RWC	NS	***	NS	-	-	-
Fv'/Fm'	NS	***	NS	-	-	-
Shoot DW	**	***	NS	NS	***	NS
Root DW	NS	**	NS	***	***	NS

Photosystem II efficiency

Experiment 1 - The drought treatment significantly reduced the photosystem II efficiency as measured by Fv'/Fm' (χ^2=20.719, df=1, p=5.318 x 10^{-6}) whilst the inoculation treatment had no effect on Fv'/Fm' (χ^2=0.0169, df=1, p=0.870). From the 11th day of drought, Fv'/Fm' diverged between droughted and well-watered plants (fig. 2.3.b).

Plant biomass

Experiment 1 - Drought treatment caused a significant reduction in the shoot dry biomass of barley plants (F=188.548, df=1, p=7.02 x10^{-16}) as did inoculation treatment (F=7.656, df=1, p<0.00888). In the drought-stressed treatment, there was no significant difference between the shoot dry biomass of plants from the AM and NM treatments (Tukey HSD p>0.05), whereas in the well-watered treatment, AM plants had a significantly larger shoot dry biomass than NM plants (Tukey HSD p<0.05). However the interactive effect of drought and inoculation treatment was not statistically significant (F=3.182, df=1, p=0.0829).

Inoculation treatment had no significant effect on root dry biomass (F=141, df=1, p=0.710), the drought treatment significantly affected the dry biomass of roots (F=9.073, df=1, p=0.00479) with droughted plants having smaller root systems than well-watered plants. There was no significant interactive effect of AMF and drought treatments on root dry biomass (F=0.245, df=1, p=0.623).

Experiment 2 - AM inoculation had no significant effect on barley shoot dry biomass (F=0.060, df=1, p=0.808) while the shoots of plants grown in the primarily compost substrate were significantly bigger than those grown in the primarily sand substrate (F=95.938, df=1, p=1.45 x 10^{-11}) (fig. 2.2.f). In fact, plants grown in the compost substrate had above-ground biomass approximately twice as big as that of plants grown in the sand substrate (3.99g and 4.24g in AM and NM compost and 2.13g and 1.86g in AM and NM sand substrates respectively). There was no interactive effect of AM inoculation treatment and substrate composition (F=1.489, df=1, p=0.231).

AM inoculation had a significant effect on root dry biomass (F=18.740, df=1, p=0.000119) (fig. 2.2.g). In the primarily compost substrate, root dry biomass was reduced in the AM treatment compared to the NM treatment (Tukey HSD: p<0.05), however in the primarily sand treatment, there was no difference between AM and NM treated plants (Tukey HSD >0.05).

Plants grown in primarily compost substrate had significantly larger root dry biomass than plants grown in primarily sand substrate (F=66.751, df=1, p=1.26 x 10^{-9}). However there was

no significant interactive effect of inoculation treatment and substrate composition (F=1.31, df=1, p=0.210).

DISCUSSION

In experiment 1, treatment with axenically produced arbuscular mycorrhizal (AM) inoculum had no effect on leaf relative water content (RWC) of spring barley. This lack of inoculation effect on plant physiology is unsurprising given that mycorrhizal colonisation was extremely low (below 4% in all plants) and did not differ significantly between NM and AM inoculation treatments or between drought and well-watered treatments.

The presence of mycorrhizal structures in the NM inoculated roots indicates a source of mycorrhizal inoculum other than the applied inoculum. The NM inoculum was produced from plates of carrot root organ culture that had not been inoculated with fungal spores and, prior to applying the inoculum, no spores were found in a subsample of the NM inoculum when examined at x200 magnification. Mycorrhizal hyphae are unlikely to have grown between pots in this instance since pots were given individual saucers and were repositioned daily in order to be weighed.

An estimated 128 spores per plant had been applied in the AM treatment. Other studies applying an *in vitro*-produced AMF inoculum have used anything from 350-450 spores per plant (Fellbaum *et al.* 2014) to 1500 spores per plant (Kiers *et al.* 2011). Though the spore count of inoculum used in experiment 1 was relatively low, it does not fully explain the lack of colonisation observed.

The substrate was a possible source of the AM fungi in experiment 1 in both AM and NM plants. Autoclaving, while an accepted method of reducing the inoculum potential of substrate, does not completely eliminate spores (Endlweber & Sheu 2006). Furthermore, the substrate in this experiment was left for two weeks following the double autoclave treatment in order to avoid the negative plant-growth effects of autoclaving (Rovira & Bowen 1966) but this will also have given time for microbial recolonisation, including by AM fungal spores. Root colonisation resulting from the applied AM inoculum would be expected to be morphologically similar, even between plants, since this inoculum was produced from a single strain, cultured axenically. However, whilst scoring root sections for mycorrhizal colonisation, a range of fungal morphologies were observed (see fig. 2.1.f).

Whilst it could not confirm successful AM colonisation, experiment 1 did confirm that the pot set-up would meet other objectives. In this experiment, withholding and then limiting irrigation allowed % pot capacity to be reduced to 12% on average across the drought period

compared to a % pot capacity of 51.7% in the well-watered treatment. By the end of the 18 day drought period, % pot capacity in the droughted pots had fallen to 2.2%. This drought period and regime was sufficient to elicit indications of drought stress from plants in the drought treatment. For example, light-adapted quantum yield of photosystem II (Fv'/Fm') diverged between droughted and well-watered plants from the 11th day of the drought period (fig.2.3.b). Over the course of the drought period, drought treatment significantly reduced Fv'/Fm' compared to well-watered controls.

The drought treatment used in experiment 1 also significantly reduced leaf RWC during the drought period compared to the well-watered treatment. Similar reductions have been observed in droughted versus well-watered barley for leaf RWC (Ghotbi-Ravandi *et al.* 2014). These results confirm that, not only was a drought stress imposed by withholding water, but that plants were indeed experiencing the physiological effects of drought (Jones 2007).

Since inoculation of barley roots using the axenically-produced liquid inoculum did not result in extensive colonisation of the AM treated plants, a commercially available mycorrhizal inoculum was trialled in experiment 2 instead.

In experiment 2, inoculation with commercial mycorrhizal inoculum (AM) resulted in successful colonisation of barley cv. Concerto roots at 6 weeks after transplant of seedlings. Percentage root colonisation was highest in the plants from AM substrate 5 (primarily sand substrate) with 49.0% root colonisation compared to 20.9% in AM substrate 6 (primarily compost substrate). This root colonisation included hyphae, arbuscules (33.1% and 12.1% in substrate 5 and 6 respectively) and vesicles (12.1% and 4.0% in substrate 5 and 6 respectively). Plants in the NM control treatment had such low levels of root colonisation as to be regarded as non-mycorrhizal (0.4% in substrate 5 and 0.3% in substrate 6). Thus the commercial inoculum trialled here meets the criteria for use in further experiments.

In experiment 2, plants grown in substrate 6 (primarily compost substrate) had significantly larger aboveground biomass than plants grown in substrate 5 (primarily sand substrate) . However, in both substrates, there was no effect of AM inoculation on aboveground biomass which is an advantage in studies aiming to compare the interactive effects of AM colonisation and drought on physiological parameters (Augé 2001).

AM treatment did affect the below ground biomass of plants grown in substrate 6 but not in substrate 5. There may be some inaccuracy associated with root biomass measurements in experiment 2 since there were differences in the ease of root washing between substrates and there will have been variation in the proportion of root taken as subsamples for

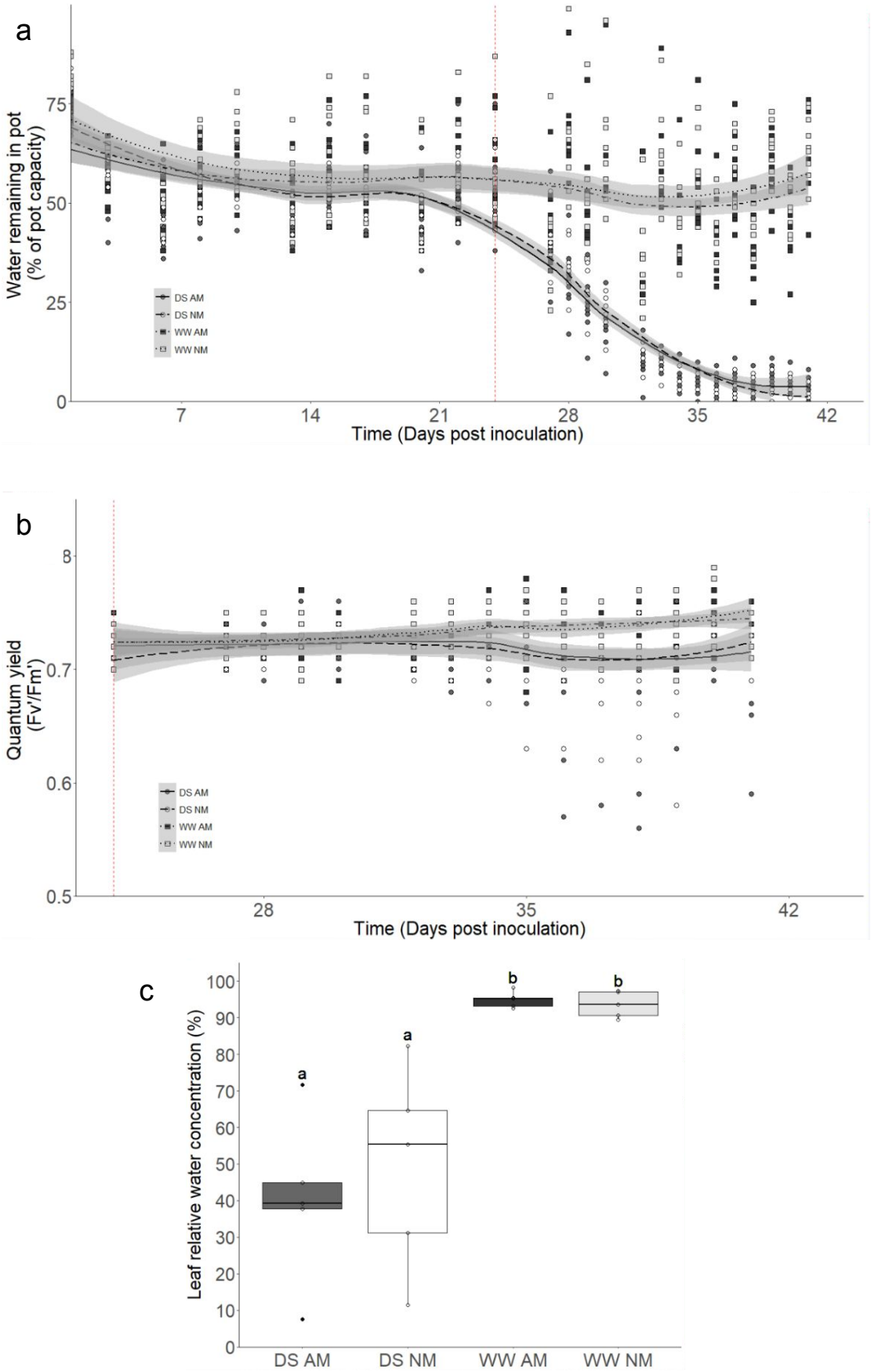

Fig. 2.3. Mass of water remaining in pots as a % of pot capacity (a) and Fv'/Fm' (b) of barley (*Horderum vulgare* cv. Concerto) during experiment 1 in which plants inoculated with *R. irregularis* DAOM197198 (filled points) and NM-inoculated plants (empty points) were subjected to well-watered (square points) or drought (circular points) conditions. Red dashed line indicates the initiation of water withdrawal. Leaf relative water content (RWC) towards the end of the drought period is shown in (c). Bars that share the same letter are not statistically significantly different at the 95% CI.

mycorrhizal scoring. It is worth noting that roots from plants in the primarily sand substrate were much easier to wash than those from the primarily compost substrate, making processing faster and likely improving the reliability of subsequent analysis (e.g. root staining and nutrient analysis) due to reduced contamination with compost.

While a substrate primarily composed of sand (substrate 5) may require more regular watering to avoid extreme drying-rewetting cycles and maintain a stable percentage pot capacity of water, the higher root colonisation of barley achieved in substrate 5 and the relative ease of root washing suggest substrate 5 would be most suitable for use in subsequent experiments.

CONCLUSIONS

A drought commencing at 24 days post inoculation and lasting 18 days in which water is withheld to maintain pots at 10% of pot capacity is sufficient to elicit a strong physiological drought stress response in barley cv. Concerto. Including commercially available inoculum (Plantworks Ltd., Sittingbourne UK) in a substrate composed of inoculum: M3 compost: sand in a ratio of 1:1:2 results in extensive root colonisation of barley cv. Concerto (49% in AM-treated plants compared to <1% in NM-treated control plants), is satisfactory for use in a pot set-up and optimises root analysis.

Chapter 3 - Investigating the barley response to drought using an untargeted metabolomics approach

The results presented here use data that has been pooled from plants treated with mycorrhizal (AM) and non-mycorrhizal inoculum from experiment 1 in which AM inoculation did not result in mycorrhizal colonisation. Analyses of the unpooled physiological data can be found in chapter 2 and unpooled metabolomic analyses can be found in appendix XI.

ABSTRACT

Untargeted metabolomics is a tool increasingly used to form hypotheses on which metabolites, and thus which biochemical pathways, may be involved in responses to abiotic stresses such as drought. To date, a significant effort has been made to characterise the metabolomic responses of drought-tolerant and drought-sensitive cereal crops such as barley. However, since the metabolomic response of plants is so dynamic, the number of plant metabolites so large (in excess of 200 000), and the available technologies so numerous, drawing conclusions between species, cultivars or studies remains challenging. This study aimed to form hypotheses on which plant secondary metabolites might be most altered by drought in the metabolomic fingerprints of spring barley cv. Concerto. Direct injection mass spectrometry (DI-ESI-MS) was used as a “first pass” technique and an untargeted analysis of polar leaf metabolites at the 11th and 18th day of drought was used to compare between droughted (DS) and well-watered (WW) plants. Metabolomic features of interest were putatively identified to the level of chemical class, though many features of interest remain un-annotated. Flavonoids and flavonoid glycosides were identified as important in distinguishing the metabolomic fingerprints of droughted and well-watered barley plants.

INTRODUCTION

In order to identify biochemical pathways involved in cereal responses to drought, recent efforts have sought to understand metabolomic changes in a number of crops during drought events using mass spectrometry techniques (Kaur *et al.* 2021). Metabolomics provides insight into the “fine-tuning” of the phenotype (Ullah *et al.* 2017), taking into account transcriptomic, post-transcriptomic and allosteric alterations to the biochemical response of plants to stress (Sanchez-Martin *et al.* 2015).

A popular approach has been to compare cultivars of the same crop, one of which is known to be drought-tolerant and the other drought-susceptible. For example, Sanchez-Martin *et al.* (2015) highlighted contrasting metabolomic responses of drought-tolerant and -susceptible cultivars of oat (*Avena sativa L.*) to reduced water availability. The drought-tolerant cultivar had early but fine control of stomatal closure via an early and sustained accumulation of salicylic acid (SA) as well as an increase in the ascorbate pathway associated with the scavenging of reactive oxygen species (ROS) (Sanchez-Martin *et al.* 2015). In contrast, the drought-susceptible cultivar exhibited rapid and tight stomatal closure with a low or late induction of antioxidant pathways leading to accumulation of ROS (Sanchez-Martin *et al.* 2015).

A significant effort has been made in recent years to characterise the biochemical mechanisms involved in the barley drought response. In the UK, barley is the second most important crop after wheat, accounting for 19% of crop production (FAO STAT 2016). Summer rainfall in the UK is predicted to decrease over the coming century with the severity and frequency of future droughts remaining uncertain (Watts *et al.* 2015). Drought not only reduces the yield of barley but also affects grain quality, which can be a particular problem for the brewing and distilling industry (Morgan & Riggs 1981, Coles *et al.* 1991). Barley is a useful experimental model for studying cereal responses to drought since, relative to wheat, it has a short life cycle and small diploid genome (Harwood 2019).

Some apparently conserved metabolomic responses to drought stress amongst barley cultivars and accessions include a reduction in starch, aspartate, glutamate and serine accumulation (Templer *et al.* 2017); an increase in sugars such as fructose and glucose accumulation (Templer *et al.* 2017); and increases in the accumulation of galactinol and Krebs' cycle intermediates (TCA cycle) (Swarcewicz *et al.* 2017). Sugars and sugar alcohols act as compatible solutes in osmoregulation and this is thought to be an important mechanism of drought response in barley (Templer *et al.* 2017).

Other metabolites are of interest because they have shown an accumulation pattern that is associated with differences in drought tolerance between barley accessions under drought stress. These include: sucrose and malate (Templer *et al.* 2017); glycosides of flavones, hydroxycinnamic esters of flavones, metabolites containing ferulic acid, blumenol derivatives (Piasecka *et al.* 2017). Flavonoids, cinnamate derivatives and terpenoids have been shown to act as non-enzymatic antioxidants in barley (Sallam *et al.* 2019) which may explain their importance in relation to drought-tolerance in some cultivars.

Against this background of research into the metabolomic response of barley to drought stress, a study was carried out using the spring barley cultivar Concerto. This cultivar was

the UK market leader at the time of the experiment, holding over 50% of the market share for spring barley (AHDB 2015). In 2019, Concerto remained in the top 6 barley cultivars in the UK in terms of area planted (AHDB 2019).

AIMS & HYPOTHESES

The aim of this study was to trial a drought regime and untargeted metabolomics workflow to test whether it would be suitable for investigating the metabolomic response of barley leaves to drought.

In chapter 2, the imposed drought regime was confirmed to effectively stress the plant. Photosystem II efficiency (Fv'/Fm') declined gradually in droughted plants over the course of the drought treatment. Drought-stressed plants had reduced photosynthetic rate, stomatal conductance and leaf relative water content (RWC) compared to well-watered control plants and drought had a negative effect on biomass and nutrient concentrations of barley plants. In chapter 2, the AM-inoculation treatment did not result in AM colonisation and so pooled results from the AM and NM treatment are presented here.

Metabolite samples were taken from barley seedlings at 11 and 18 days into the drought treatment. It was expected that drought would elicit changes in leaf polar metabolites and that it would be possible to detect this response using an untargeted mass spectrometry approach.

MATERIALS AND METHODS

Experimental design

The experiment was set up with 40 barley plants in pots under glasshouse conditions. Twenty-four days after seedlings were transplanted into pots, half the plants underwent an 18 day drought treatment, while the other half continued to receive adequate irrigation. Several plant physiological techniques were used to assess drought severity and shoot and root biomass was recorded at the end of the experiment. Leaf samples were removed at two time points for extraction of polar metabolites so that metabolome profiles could be compared using mass spectrometry.

Watering regimes and drought treatments

See chapter 2 - Development of an experimental system to simultaneously investigate physiological and metabolomic response to drought and AM inoculation (*Experiment 1*)

Metabolite sampling, extraction and analysis

At 35DPI, a 2cm leaf tip sample was taken from the youngest expanded leaf on a tiller. At 42DPI, immediately prior to destructive harvest, leaf samples were taken from the youngest expanded leaf on the main stem. Samples were immediately snap frozen in liquid nitrogen before being stored at -80°C.

Leaf metabolites were extracted on ice using the water: methanol: chloroform method (Overy *et al.* 2005) (details in chapter 1). For analysis, the aqueous phase was diluted to 10% using 1:1 UHP water and methanol before being run in triplicate through a Waters Synapt G2 Mass Spectrometer (Waters Ltd, Manchester, UK) with automatic injection using Waters Alliance 2695 HPLC (no column used). The MS had an electrospray sample introduction system and data were acquired in positive ionisation mode (64 samples), followed by negative ionisation mode (61 samples) using Waters MassLynx data system (Waters, Massachusetts, USA). Detailed settings are available in appendix VI.

Spectra were extracted using MassLynx (Waters, Massachusetts, USA) software. For each sample, three technical replicates were combined using Visual Basic macro 216 (Overy *et al.* 2005) which required peak detection in all three of the technical replicates to be treated as a true peak. Mass-to-charge ratios (m/z) were rounded to 0.2 Da bins and the % total ion count (TIC) (a measure of abundance) for mass numbers within that bin was summed.

Using SIMCA software (Umetrics, Geottingen, Germany), samples were compared with an untargeted principal component analysis (PCA), in which binned mass number was the primary variable and the drought treatments and timepoints were observational variables. Timepoints were subsequently analysed separately. In order to ascertain which m/z might be responsible for divergence between metabolomes, a directed analysis, orthogonal projections of latent structures (OPLS-DA) modelling, was applied to combinations of treatments in which PCA had revealed difference between metabolomes. M/z bins that had the largest significant effects on the model were taken as potential masses of interest (at the 99% confidence interval).

To annotate masses of interest, the median value of detected masses in the bin was treated as the mass of interest for searching the METLIN Metabolite and Chemical Entity Database (Scripps Institute, accessed 18/02/2022 see Xue *et al.* 2020) for compounds with KEGG IDs

whose expected m/z values fell within 30ppm of an experimentally detected mass. METLIN includes the option to also search for matches that may occur due to a range of adducts; here, only the most commonly formed $[M+K]^+$, $[M+H]^+$ and $[M+Na]^+$ adducts were considered in positive mode, while all available adduct information was considered in negative mode. The literature was also searched using Google Scholar and the search term "m/z" followed by the integer value of the m/z of interest. Reference literature are cited in table 3.1. Kyoto Encyclopaedia of genes and genomes (KEGG) Compound and KEGG Pathway (Kanehisa Laboratories, accessed 18/02/2022 see Kanehisa *et al.* 2000, 2019 and 2021) were used to exclude biologically unlikely matches to create shortlists of potential compounds of interest.

See also: Chapter 1 - Materials and Methods used throughout this Thesis

RESULTS

Effect of drought on plant physiology

The mass of water in the pots of the drought-stress treatment was significantly lower than in the well-watered pots over the course of the drought period (χ^2X=91.209, df=1, $p<2.2 \times 10^{-16}$)(fig. 3.1.a).

The photosystem II efficiency (Fv'/Fm') of plants in the drought-stressed and well-watered treatments began to diverge from the 9th day of the drought treatment. Drought treatment was associated with a statistically significant reduction in Fv'/Fm' over the course of the drought period (F=10.68, df=1, p=0.00115) (fig.3.1.b).

Photosynthetic rate on the 11th day of drought (35DPI) was reduced by half in the drought treatment (3.82μmol m^{-2} s^{-1}) compared to the well-watered control plants (8.36μmol m^{-2} s^{-1}) (t=-4.741, df=11.17, p=0.0005833) (fig.3.1.c). Stomatal conductance was also significantly reduced by drought treatment (t=-10.898, df=14.564, $p=2.148 \times 10^{-8}$) on the 11th day of drought (35DPI) (fig.3.1.d).

The leaf relative water content of droughted plants on the 14th day of drought was less than half of that of well-watered plants (t=-6.3785, df=9.2512, p=0.0001138) (fig.3.1.e).

Effect of drought on plant biomass

Shoot and root dry biomass were both significantly reduced by drought treatment (shoot: t=-12.368, df=22.53, p=1.604 x 10^{-11} and root: t=-8.4375, df=24.595, p=9.998 x 10^{-09}) after 18 days of drought treatment (fig. 3.2).

Comparison of droughted and well-watered barley metabolomes

In order to compare the global metabolomes of droughted and well-watered barley plants, principal component analysis (PCA) was applied to mass spectra of polar leaf metabolites. PCA visualisation showed separation of samples from 35DPI and 42DPI in the positive ionisation mode along the second principal component (PC2 explained 9.21% of the variance) and by drought treatment along the first principal component (PC1 explained 19.5% of the variance) (fig. 3.3.a and 3.3.b respectively). Samples also clustered in the negative ionisation mode but the separation was not so defined. In the negative ionisation mode, PC1 explained 15.2% of the variance while PC2 explained 10.4% of the variance (fig. 3.3.c and 3.3.d).

Samples were subsequently split by time point for further analysis. At 35DPI (11th day of drought period), samples separated clearly by drought treatment along PC2 in the positive ionisation mode (which explained 16% of the variance) while PC1 explained 19.4% of the variance (fig. 3.4.a). In the negative ionisation mode, samples from the two drought treatments separated along PC1 (which explained 18.8% of the variance) while PC2 explained 9.43% of the variance (fig.3.4.c). To find which compounds might be responsible for the differences in metabolome fingerprints between droughted and well-watered plants, OPLS-DA models were applied to the data from 35DPI. Strong separation by defined class (drought treatment) was observed in both the analysis of positive and negative mode data (fig. 3.4.b and 3.4.d).

A similar pattern was observed at 42DPI (18th day of drought period): samples separated clearly along PC1 in the positive ionisation mode (which explained 25.8% of the variance) while PC2 explained 8.16% of the variance (fig. 3.5.a). In the negative ionisation mode, samples from drought-stressed and well-watered treatments clustered separately along PC1, with PC1 explaining 20.4% of the variance and PC2 explaining 7.56% of the variance (fig. 3.5.c). As in the analysis of timepoint 1, subsequent OPLS-DA models allowed investigation of m/z bins that were responsible for the divergence between the droughted and well-watered plants' metabolome fingerprints. Strong separation by defined class (drought treatment) was observed in both the analysis of positive and negative mode data for timepoint 3 (fig. 3.5.b and 3.5.d).

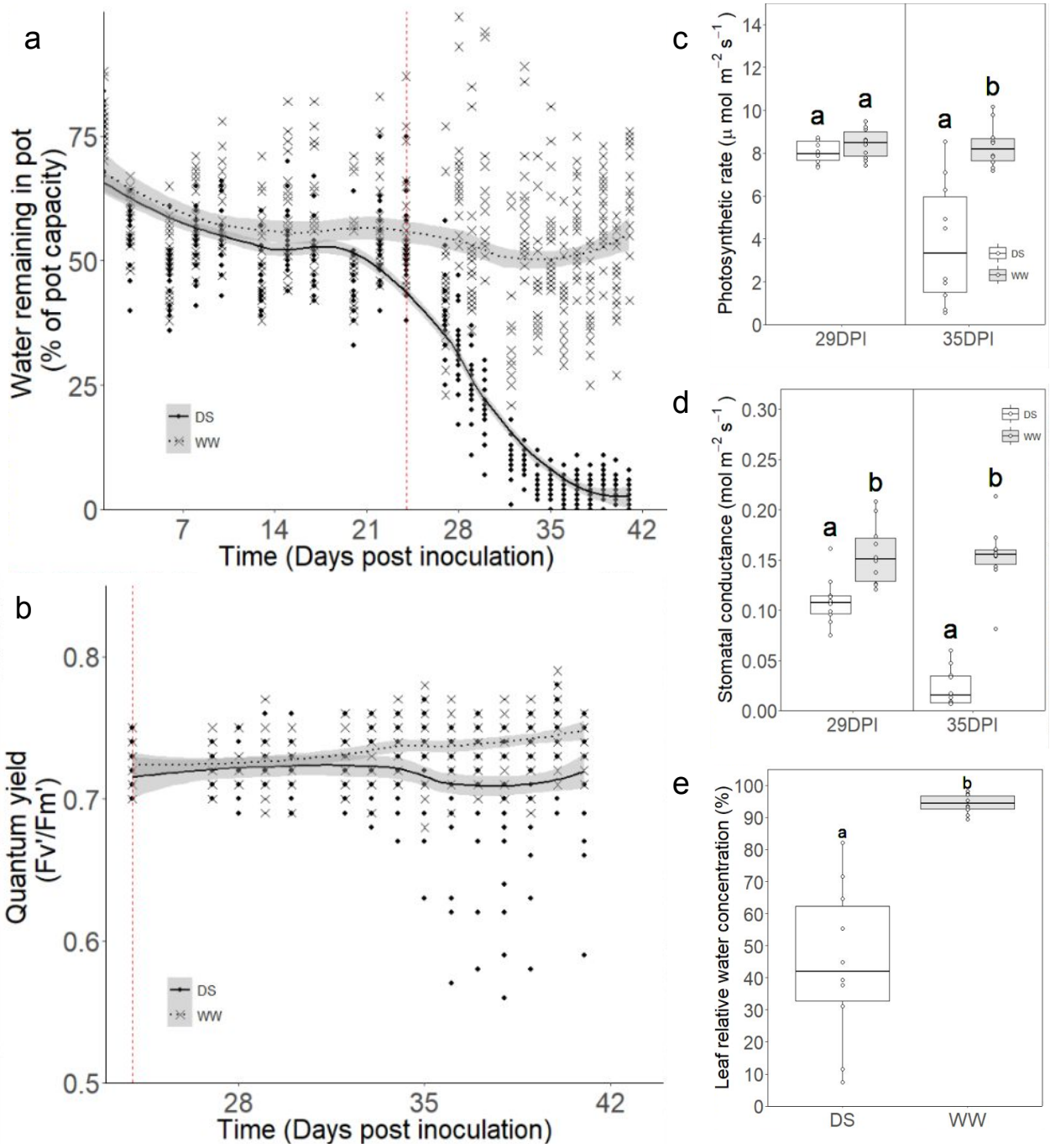

Fig. 3.1. Mass of water remaining in pots as a percentage of pot capacity across the experiment (a). Efficiency of photosystem II (Fv'/Fm') of barley plants across the drought treatment (b). Points and dotted line represent plants in the drought treatment while crosses and solid line represent plants in the well-watered treatment. Lines represent locally fitted loess regression with 95%CI represented by grey shading. Red vertical dashed lines represent first day of drought treatment.

The photosynthetic rate (c) and stomatal conductance (d) are shown on the 5th (29DPI) and 11th days of drought (35DPI). Leaf relative water content (e) is shown on the 13th day of drought (37DPI). Points and white boxes represent plants in the drought treatment while crosses and grey boxes represent plants in the well-watered treatment. Boxplots show median, 25th and 75th percentiles, with data points >1.5*IQR plotted as outliers. Boxes sharing a letter are statistically similar according to Student's t test at 95%CI (within facets).

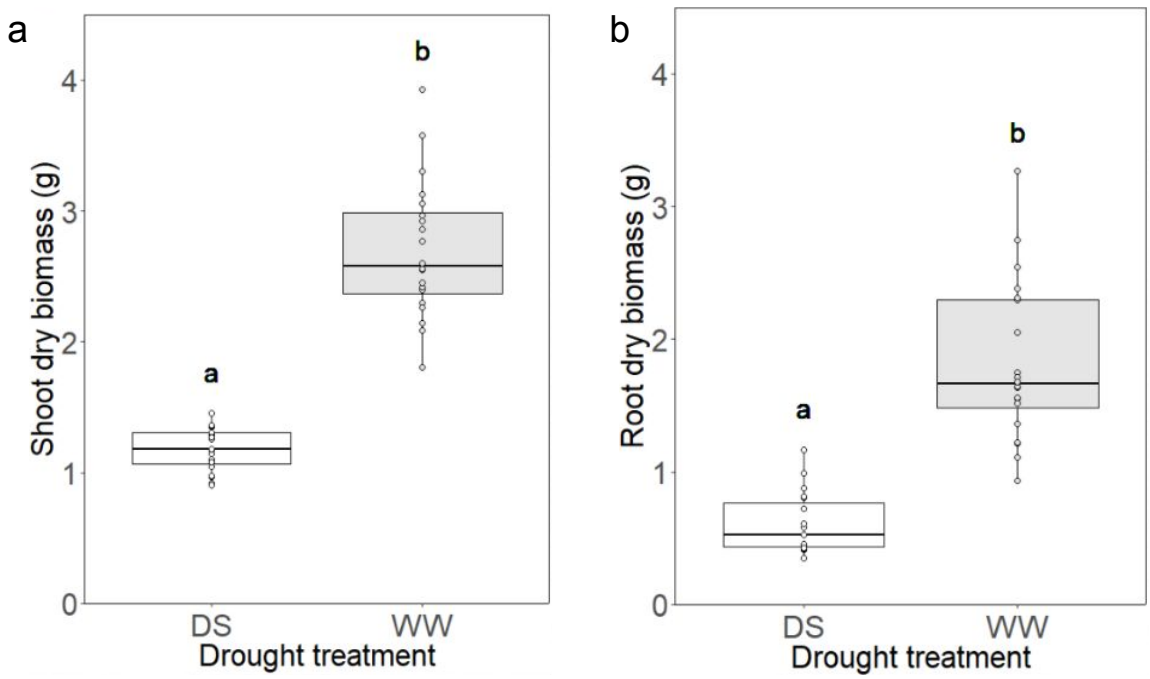

Fig. 3.2. Shoot (a) and root (b) dry biomass harvested at 42DPI after 18 days of drought treatment or well-watered conditions. Boxplots show median, 25th and 75th percentiles, with data points >1.5*IQR plotted as outliers. Boxes sharing a letter are statistically similar according to Student's t test at 95%CI (within facets).

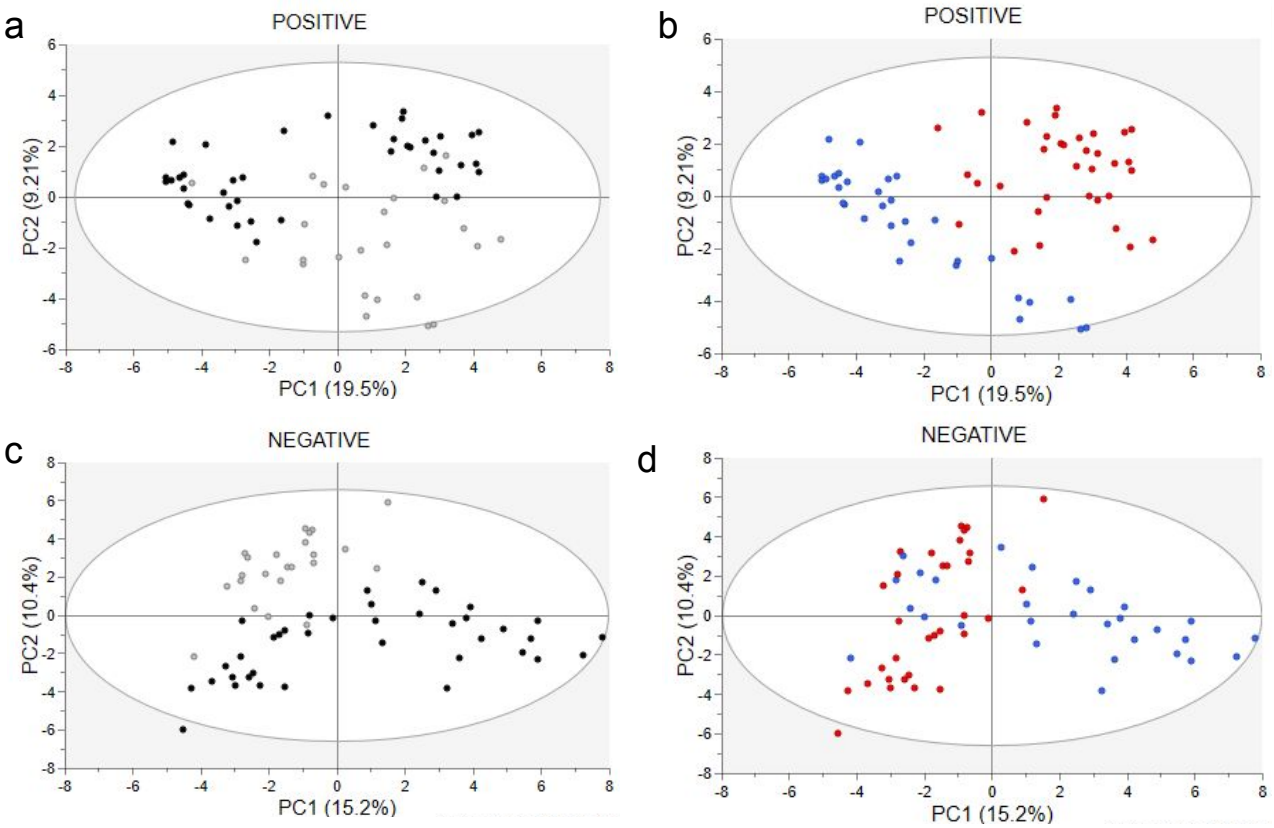

Fig. 3.3. Principal components 1 (x) and 2 (y) from PCA of barley leaf polar metabolite samples run in positive (a) and (b) and negative mode (a) and (d). In (a) and (c) black points represent samples collected early in the drought period (35DPI) and pale grey points represent samples collected late in the drought period (42DPI). In (b) and (d) red points represent samples from plants subjected to drought and blue points represent samples from well-watered plants. Ellipses represent hotelling at 95% CI and % values on axes represent proportion of variance explained by relevant principal component.

The next step was to ascertain which m/z bins (and therefore potentially which compounds) were responsible for divergence between well-watered and droughted plants' metabolome fingerprints. To do this, the loadings values and jack-knifed uncertainties at 95% CI from the OPLS-DA models were used to identify the m/z bins most reliably associated with each drought treatment at each timepoint (i.e. the m/z having the largest effects on the outcome of the models), creating a long-list of m/z of interest. Putative annotations for twenty-one m/z bins most reliably associated with well-watered controls are shown in table 3.1, including three that were reliably associated with the well-watered treatment at both 35 and 42DPI. On comparison with the literature, the co-occurence of the m/z bin at 609Da and 611Da in negative and positive ionisation modes respectively is suggestive of a group of flavonoid di-glycosides including hesperidin, rutin, lutonarin (isoorientin-7-O-glucoside) and meloside L (isoorientin-2"-O-glucoside). Other compounds putatively identified as associated with the well-watered treatment were myo-inositol triphosphate and deamino-NAD+. Six of the m/z bins remained un-annotated.

Seventeen of the m/z bins most reliably associated with the drought-treatment were investigated, including five that were associated with drought at both 35 and 42 DPI. Putative annotations for the m/z bins most associated with drought are shown in table 3.1. Comparison of the m/z bins associated with DS were ambiguous in some cases, but a number of them were identified as cinnamate and hydroxycinnamic acid derivatives. Other potential identities included flavonoids, with the m/z bin at 341Da in negative ionisation mode being widely referenced in the literature as indicative of isoscoparin- and iosvitexin-derivatives or fragments thereof (Ferreres *et al.* 2008, Piasecka *et al.* 2015, Tang *et al.* 2021). Other tentatively identified compounds included indole and 2'-deoxyuridine 5'-diphosphate (dUDP). Six of the m/z bins associated with drought treatment remained un-annotated.

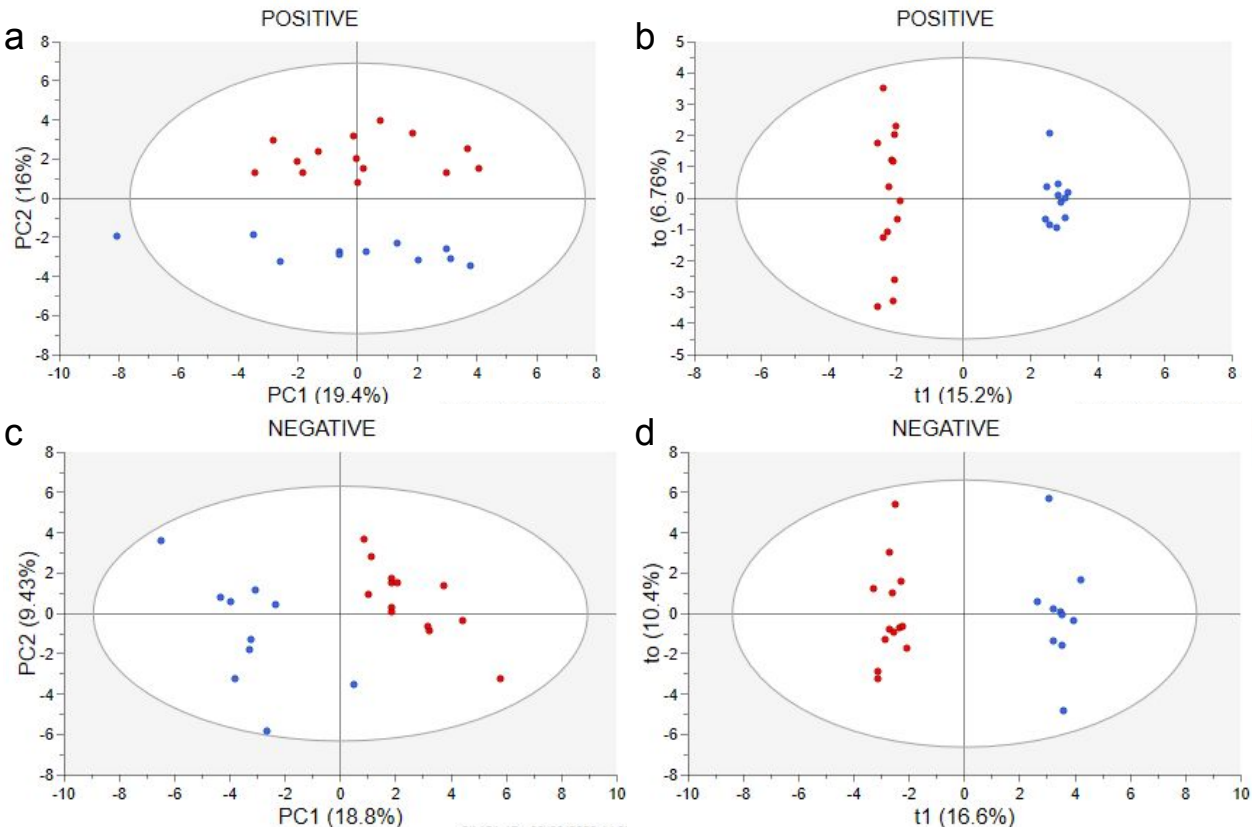

Fig. 3.4. Multivariate analysis of barley leaf polar metabolite samples from 35DPI (11th day of drought). Principal components 1 (x) and 2 (y) from PCAs of samples run in positive (a) and negative modes (c). OPLS-DAs show separation between defined well-watered (blue) and droughted (red) classes for samples run in the positive (b) and negative (d) modes. Ellipses represent hotelling at 95% CI and % values on axes represent proportion of variance explained by relevant principal component (a and c) or projection (b and d).`

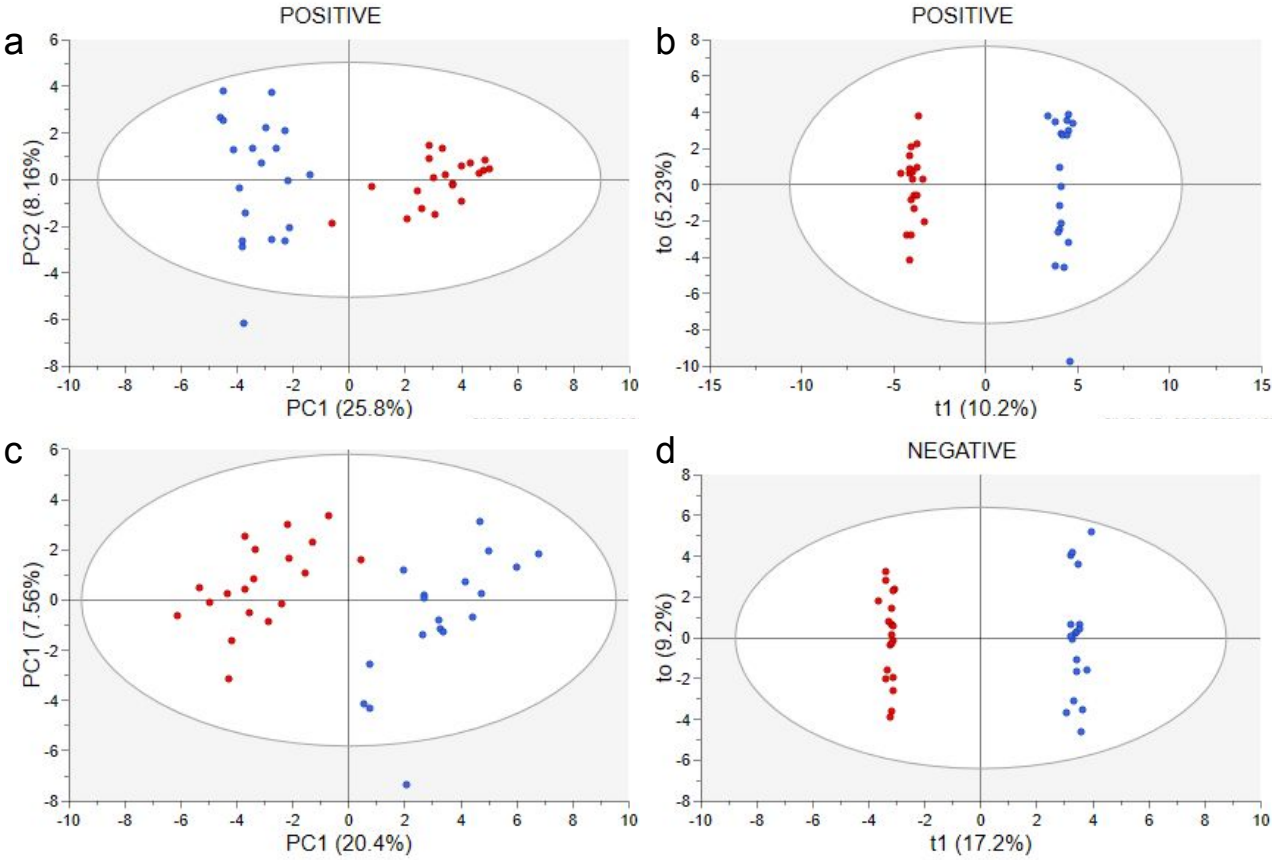

Fig. 3.5. Multivariate analysis of barley leaf polar metabolite samples from 42DPI (18th day of drought). Principal components 1 (x) and 2 (y) from PCAs of samples run in positive (a) and negative modes (c). OPLS-DAs show separation between defined well-watered (blue) and droughted (red) classes for samples run in the positive (b) and negative (d) modes. Ellipses represent hotelling at 95% CI and % values on axes represent proportion of variance explained by relevant principal component (a and c) or projection (b and d).

Table 3.1. Tentative class annotations of metabolites most influential in discriminating drought stress (DS) and well-watered (WW) polar leaf metabolomes of barley at 11th (T2) and 18th (T3) days of drought treatment. ^ Metabolomics standards intitiative level 3 identification (Sumner et al. 2007) a = Piasecka et al. 2015; b = Seeram et al. 2006; c = Lee et al. 2016; d = Alesenko 2013; e = Tang et al. 2021; f = Zhang et al. 2017; g = Li et al. 2003; h = Kang et al. 2016; i = Prassain et al. 2003; j = Cuyckens et al. 2001; k = Sanchez-Rabaneda et al. 2003; l = Davidson et al. 2020; m = El-Sakka et al. 2009; n = Ferreres et al. 2008; o = Brauch et al. 2018

					Compounds from literature and repositories				
Ionisation mode	Time point	Bin	Detected m/z	Putative class annotation^	Ion m/z	ppm	Fragment m/z	Compound	Source
Associated with well-watered barley plants (WW)									
negative	2	97.0	96.9039	-					
negative	3	111.0	110.9469	Carboxylic acid	[M+K-2H]−	110.9490	19	Glycoxylic acid	METLIN
positive	2	162.0	161.9749	Heterocyclic organic compound					
							162	Fragments of triazino[1,2-b]-phthalazine-5,10-dione	m
							162	phthalazine-1,4- dione	m
							162	Pyrrolidine ring	l
negative	2	227.0	226.9115	Heterocyclic organic compound	[M+K-2H]−	226.9115	0	Bromobenzene-2,3-dihydrodiol/ Bromobenzene-3,4-dihydrodiol	METLIN
							227	Isomer of Naringenin	h
							227	Isoscoparin hexoside (Chrysoeriol hexoside)	h
							227	Naringenin	h
negative	2	232.8	232.8010	-					
							233	Unknown dihydroxy-octadecenoic acid	h
							233	Unknown flavonoid or polyphenol	h
positive	2*	365.0	365.0157	Pyrimidine ribonucleoside	[M+Na]+	365.0157	0	5-Fluorouridine monophosphate	METLIN
					[M+K]+	365.0147	3	2,4-Dioxotetrahydropyrimidine D-ribonucleotide	METLIN
negative	2	431.0	430.9676	Flavone *O*-glucoside or derivative	[M-H]−	431		1,3-O-Feruloyl-dihydrocaffeoylglycerol	h
							431	Apigenin 7-O-glucoside	k
							431	Apigenin 7-O-rhamnoside 4′-O-glucoside	a
					[M-H]−	431		Apigenin-4' -O-hexoside	h
					[M-H]−	431		Apigenin-7-O-hexoside	h
					[M-H]−	431		Cimicifugic acid E and F	g
					[M-H]−	431	431	Isovitexin (Apigenin-8-C-glucoside)	a, c, e, f, k, n
								Isovitexin 2"-O-[X-sinapoyl]-hexoside	o
								Isovitexin 7-O-[6"-caffeoyl]-glucoside	n
								Isovitexin 7-O-[6"-sinapoyl]-glucoside	n
							431	Isovitexin 7-O-[6″-feruloyl]-glucoside	a, n
							431	Isovitexin 7-O-[6″-feruloyl]-glucoside 4′- O-glucoside	a, e
							431	Isovitexin 7-O-[6″-hydroxyferuloyl]- diglucoside	a
							431	Isovitexin 7-O-[6″-hydroxyferuloyl]- diglucoside	a
							431	Isovitexin 7-O-[6″-hydroxyferuloyl]- glucoside	a
							431	Isovitexin 7-O-[6″-hydroxyferuloyl]- glucoside	a
							431	Isovitexin 7-O-[6″-p-coumaroyl]-glucoside	a
							431	Isovitexin 7-O-[6″-sinapoyl]-glucoside 4′- O-glucoside	a, e

Table 3.1. (continued …) Tentative class annotations of metabolites most influential in discriminating drought stress (DS) and well-watered (WW) polar leaf metabolomes of barley at 11th (T2) and 18th (T3) days of drought treatment. ^ Metabolomics standards intitiative level 3 identification (Sumner et al. 2007) a = Piasecka et al. 2015; b = Seeram et al. 2006; c = Lee et al. 2016; d = Alesenko 2013; e = Tang et al. 2021; f = Zhang et al. 2017; g = Li et al. 2003; h = Kang et al. 2016; i = Prassain et al. 2003; j = Cuyckens et al. 2001; k = Sanchez-Rabaneda et al. 2003; l = Davidson et al. 2020; m = El-Sakka et al. 2009; n = Ferreres et al. 2008; o = Brauch et al. 2018

					Compounds from literature and repositories				
Ionisation mode	Time point	Bin	Detected m/z	Putative class annotation^	Ion m/z	ppm	Fragment m/z	Compound	Source
								Isovitexin 7-O-[X-feruloyl]-hexoside	o
								Isovitexin 7-O-[X-sinapoyl]-hexoside	o
							431	Isovitexin 7-O-[X″-feruloyl]-glucoside	a
							431	Isovitexin 7-O-diglucoside 4′-O-[6″- sinapoyl]-glucoside	a
							431	Isovitexin 7-O-glucoside (Saponarin)	a, e, n, o
							431	Isovitexin 7-O-glucoside 4′-[6″-feruloyl]- O-glucoside	a
							431	Isovitexin 7-O-glucoside malonylated	a
							431	Isovitexin 7-O-rhamnoside	a
							431	Isovitexin 7-O-rhamnosylglucoside	a
							431	Isovitexin 7, 4′-di-O-glucoside	a
							431	Isovitexin 7, 4′-di-O-glucoside	a
							431	Isovitexin caffeate	a
							431	Isovitexin O-glucoside ferulate	a
							431	Isovitexin O-glucoside sinapate	a
							431	Isovitexin-7-O-rutinoside	e, n
					[M-H]−	431		*O*-glycosides of isoflavonoids	i
					[M-H]−	431		Piscidic acid ester of ferulic acid/ isoferulic acid	g
							431	Rhoifolin	j
					[M-H]−	431	431	Vitexin (Apigenin-6-C-glucoside)	d, f, h, k
negative	2	439.0	438.9519	Myoinositol triphosphate	[M+F]−	438.9608	20	1D-myo-Inositol 1,3,4-trisphosphate	METLIN
					[M+F]−	438.9608	20	D-myo-Inositol-1,4,5-triphosphate (potassium salt)	METLIN
positive	2 & 3	455.0	454.9683	-					
positive	2	463.0	462.9744	Flavonol glucoside	[M-H]+	462.9636	23	Quercetin 3,3'-di-O-sulfate	METLIN
					[M-H]+	462.9636	23	Quercetin 3,4'-di-O-sulfate	METLIN
							463	Quercetin-glucoside	b
positive	2	527.0	527.0540	Flavonoid diglycoside			527	Naringin	j
							527	Narirutin	j
positive	2	543.0	543.0258	Flavonoid diglycoside			543	Isorhoifolin	j
							543	Rhoifolin	j
negative	2 & 3	609.0	608.9905	Flavonoid diglycoside	[M-H]−	609		Hesperidin	j
							609	Isoorientin 7-O-[X-feruloyl]-hex	o
							609	Isoorientin 7-O-[X-sinapoyl]-hex	o
					[M-H]−	609		Isoorientin-2″-O-glucoside (Meloside L)	n
					[M-H]−	609.1463		Isoorientin-2″-O-hex	o

Table 3.1. (continued …) Tentative class annotations of metabolites most influential in discriminating drought stress (DS) and well-watered (WW) polar leaf metabolomes of barley at 11th (T2) and 18th (T3) days of drought treatment. ^ Metabolomics standards intitiative level 3 identification (Sumner et al. 2007) a = Piasecka et al. 2015; b = Seeram et al. 2006; c = Lee et al. 2016; d = Alesenko 2013; e = Tang et al. 2021; f = Zhang et al. 2017; g = Li et al. 2003; h = Kang et al. 2016; i = Prassain et al. 2003; j = Cuyckens et al. 2001; k = Sanchez-Rabaneda et al. 2003; l = Davidson et al. 2020; m = El-Sakka et al. 2009; n = Ferreres et al. 2008; o = Brauch et al. 2018

					Compounds from literature and repositories				
Ionisation mode	Time point	Bin	Detected m/z	Putative class annotation^	Ion m/z	ppm	Fragment m/z	Compound	Source
					[M-H]−	609		Isoorientin-7-O-glucoside (Lutonarin)	c, n
					[M-H]−	609.1458		Isoorientin-7-O-hex	o
					[M-H]−	609	609	Rutin	b, d, f, j, k
negative	3	610.0	609.9939	-					
positive	3	611.0	611.0474	Flavonoid diglycoside	[M+H]+	611		Hesperidin	j
							611	Isoorientin 2″-O-glucoside (Meloside L)	a
							611	Isoorientin 7-O-glucoside (Lutonarin)	a
					[M+H]+	611.1635		Isoorientin-2″-O-hex	o
					[M+H]+	611.1618		Isoorientin-7-O-hex	o
							611	Isorhamnetin O-arabinosylglucoside	a
							611	Luteolin 7-O-gentobioside	a
					[M+H]+	611		Rutin	j
positive	3	703.0	703.0616	Nicotinic acid dinucleotide	[M+K]+	703.0563	8	Deamino-NAD+	METLIN
negative	2 & 3	707.0	706.9473	-					
positive	3	731.0	731.0079	-					
positive	3	747.0	747.0761	-					
							747	Isovitexin 7-O-[6″-sinapoyl]-glucoside	a
					[M+H]+	747.0540	30	Adenylated molybdopterin	METLIN
Associated with drought-stressed barley plants (DS)									
negative	2	115	114.9403	-					
positive	2 & 3	116	116.0148	-					
positive	2 & 3	118	118.0069	Heterocyclic organic compound	[M+K]+	118.0054	13	Pyridine	METLIN
positive	2	119	118.9834	Hydroxycinnamic acid					
							119	Caffeic acid derivative	a
							119	p-Coumaric acid	e
positive	2 & 3	120	120.0236						
negative	2	133	132.9483	Flavonoid					
							133	7,30 ,40 -Trihydroxyflavone	h
							133	Dihydroxyflavone	h
							133	Luteolin	h, k
							133	Kaempferol	k
					[M+Cl]−	132.9463	15	Phosphoric acid	METLIN
positive	2	156	156.0173		[M+K]+	156.0210	24	Indole	METLIN
positive	2 & 3	188	188.0591						
							188	Fragments of triazino[1,2-b]-phthalazine-5,10-dione	m
					[M+H]+	188.0553	20	2-(Acetamidomethylene)succinate	METLIN
					[M+Na]+	188.0543	26	3-Methylguanine/ 7-Methylguanine	METLIN
negative	2 & 3	245	244.9507	Flavanol					
							245	Catechin	h, k

Table 3.1. (continued …) Tentative class annotations of metabolites most influential in discriminating drought stress (DS) and well-watered (WW) polar leaf metabolomes of barley at 11th (T2) and 18th (T3) days of drought treatment. ^ Metabolomics standards intitiative level 3 identification (Sumner et al. 2007) a = Piasecka et al. 2015; b = Seeram et al. 2006; c = Lee et al. 2016; d = Alesenko 2013; e = Tang et al. 2021; f = Zhang et al. 2017; g = Li et al. 2003; h = Kang et al. 2016; i = Prassain et al. 2003; j = Cuyckens et al. 2001; k = Sanchez-Rabaneda et al. 2003; l = Davidson et al. 2020; m = El-Sakka et al. 2009; n = Ferreres et al. 2008; o = Brauch et al. 2018

					Compounds from literature and repositories				
Ionisation mode	Time point	Bin	Detected m/z	Putative class annotation^	Ion m/z	ppm	Fragment m/z	Compound	Source
							245	Epicatechin	k
					[M+K-2H]−	244.9494	5	Stipitatonate	METLIN
negative	2	267	266.9762				267	Hordatine B glucoside	a
							267	Hordatine B	a, o
							267	Hordatine C	a
							267	1,3-O-Diferuloylglycerol	h
					[M+Na-2H]−	266.9805	16	Isopentenyl pyrophosphate (IPP)/ Dimethylallyl pyrophosphate (DMAPP)	METLIN
negative	2	333	332.9539	-					
positive	3*	365.0	365.0157		[M+Na]+	365.0157	0	5-Fluorouridine monophosphate	METLIN
					[M+K]+	365.0147	3	2,4-Dioxotetrahydropyrimidine D-ribonucleotide	METLIN
negative	3	341	341.0026	Flavonoid			341	Isoscoparin	a, n
							341	Isoscoparin 7-O-glucoside	a, n
							341	Isovitexin 7-O-rhamnosylglucoside	a
							341	Isoscoparin 7-O-rhamnosylglucoside	a
							341	Isoscoparin 6″-O-glucoside	a
							341	Isoscoparin 7, 6″-di-O-glucoside	a
							341	Isoscoparin 7-O-[6″-hydroxyferuloyl]- glucoside	a
							341	Isoscoparin 7-O-[6″-sinapoyl]-glucoside	a, n
							341	Isoscoparin 7-O-[6″-hydroxyferuloyl]- glucoside 4′-O-glucoside	a
							341	Isoscoparin 7-O-[6″-hydroxyferuloyl]- glucoside 4′-O-glucoside	a
							341	Isovitexin (Apigenin-6-C-glucoside)	c, e, h, k
							341	Isovitexin-7-O-[6-sinapoyl]-glucoside	c, n
							341	Isovitexin-7-O-[6-feruloyl]-glucoside	c, n
							341	Isovitexin-7-O-rutinoside	e, n
							341	Isovitexin-7-O-glucoside (Saponarin)	e, n
							341	Apigenin-6-C-arabinoside-8-C-glucoside	e
							341	Isovitexin-7-O-[6-sinapoyl]-glucoside	e
							341	Isovitexin-7-O-[6-feruloyl]-glucoside	e
							341	Penoidin-malonilglucoside	e
					[M-H]−	341		Caffeic acid hexose	f, h
							341	Isoscoparin-7-O-hexoside(Chrysoeriol hexoside I)	h, o
							341	Vitexin (Apigenin-8-C-glucoside)	k
							341	Isoscoparin-7-O-rutinoside	n

Table 3.1. (continued …) Tentative class annotations of metabolites most influential in discriminating drought stress (DS) and well-watered (WW) polar leaf metabolomes of barley at 11th (T2) and 18th (T3) days of drought treatment. ^ Metabolomics standards intitiative level 3 identification (Sumner et al. 2007) a = Piasecka et al. 2015; b = Seeram et al. 2006; c = Lee et al. 2016; d = Alesenko 2013; e = Tang et al. 2021; f = Zhang et al. 2017; g = Li et al. 2003; h = Kang et al. 2016; i = Prassain et al. 2003; j = Cuyckens et al. 2001; k = Sanchez-Rabaneda et al. 2003; l = Davidson et al. 2020; m = El-Sakka et al. 2009; n = Ferreres et al. 2008; o = Brauch et al. 2018

					Compounds from literature and repositories					
Ionisation mode	Time point	Bin	Detected m/z	Putative class annotation^	Ion m/z		ppm	Fragment m/z	Compound	Source
								341	Isoscoparin-2"-O-glucoside	n
								341	Isovitexin-7-O-[6-p.coum]-glc	n
								341	Isoscoparin-7-O-[6-fer]-glc	n
								341	Isovitexin	n
					[M+K-2H]−	341.0069	13		(±)-Taxifolin	METLIN
					[M+Cl]−	341.007	13		2-Protocatechoylphloroglucinolcarboxylate (Quercetin derivative)	METLIN
					[M+K-2H]−	341.0069	13		Pentahydroxyflavanone	METLIN
negative	3	377	376.9683	-						
negative	3	379	378.9659	-						
positive	3	381	381.0565	Cinnamate derivative				381	Isoscoparin 7-O-[6"-feruloyl]-glucoside	a
					[M+K]+	381.0582	4		Caffeic acid 3-glucoside	METLIN
					[M+K]+	381.0582	4		1-Caffeoyl-beta-D-glucose	METLIN
negative	3	387	386.9961					387	Pelargonidin-3-O-glucoside	e
								387	Kaempferol-7-O-neohesperidoside	j
						387.0000	10		2'-Deoxyuridine 5'-diphosphate (dDUP)	METLIN
negative	3	439	438.9519	-						

DISCUSSION

Untargeted metabolomics analysis of barley at the vegetative growth stage showed that leaf polar metabolite fingerprints of droughted and well-watered plants were divergent by the 11th day of water-restriction, as hypothesised. Differences in the metabolite fingerprints of droughted and well-watered plants were detected by DI-ESI-MS concurrent with a drought-induced reduction in photosynthetic rate. Prior to this, at the 5th day of drought, stomatal conductance had already begun to decrease in droughted plants as compared to well-watered controls, confirming that plants were experiencing sufficient water stress to elicit a physiological response but photosynthetic rate had not yet been significantly reduced by drought treatment. By the 14th day of the drought period, the drought-treatment was confirmed to have reduced relative leaf water content.

Photosystem II efficiency had also begun to diverge between droughted and well-watered plants at the 11th day of drought. Decreases in light-adapted quantum yield here signify damage to the photosynthetic machinery of the newest fully expanded leaves and suggest the plant is experiencing persistent stress (Maxwell & Johnson 2000). Hughes *et al.* (2017) observed that it took 7-8 days post-watering before a significant reduction in Fv'/Fm' was apparent in spring barley, similar to the 8-9 days post-watering observed here.

The latter sampling time point (18th day post-watering), showed the progression of drought with photosynthetic rate, stomatal conductance and chlorophyll fluorescence dramatically declining in drought-treated plants by this point. The divergence between droughted and well-watered metabolite fingerprints was exacerbated at this latter sampling time point, and some of the m/z most reliably causing the divergence were the same as at the earlier time point.

A number of spectral peaks that differentiated between droughted and well-watered barley plants in this chapter have been putatively annotated at Metabolomics Standards Initiative (MSI) level 3, that is, identification of chemical class by matching to reference spectral features in the literature but not confirmed by internal standards or orthogonal data such as chromatographic separation or tandem MS (Sumner *et al.* 2007). While the untargeted metabolome fingerprinting approach used in this study only permits tentative annotation of compounds responsible for differences in metabolomes, some compounds from pathways expected to be involved in drought response were highlighted by the analysis.

Flavonoids and their glycosides are affected by drought in barley

Flavonoids and glycosides of flavonoids appear repeatedly in the potential annotations of the m/z bins of interest causing differentiation between droughted and well-watered barley metabolomic fingerprints. Flavonoids are involved in, and produced by, the phenylpropanoid pathway in barley and have antioxidant properties, scavenging reactive oxygen species (ROS), particularly under water stress (Agati *et al.* 2012), with glycosylation patterns being important in the fast and dynamic response of barley tissues to oxidative stress (Kumar & Pandey 2013). Lutonarin (isoorientin-7-O-glucoside) and saponarin (isovitexin-7-O-glucoside) are flavonoid di-glycosides that have been previously shown to be the most abundant phenolic compounds in barley (Ferreres *et al.* 2008).

While identifications of these flavonoid glycosides in the data presented here are tentative, it is consistent with previous studies that have found the glycosylation patterns of flavonoids (particularly flavones) to be altered by drought (Piasecka *et al.* 2017, Kowalczewski *et al.* 2020, Piasecka *et al.* 2020). Furthermore, other compounds and derivatives of the phenylpropanoid pathway were highlighted by the analysis presented in this chapter, such as cinnamate and hydroxycinnamic acid derivatives, potentially including the hordatines, which is in line with the findings of previous studies in barley (Piasecka *et al.* 2017, Piasecka *et al.* 2020).

Further work

In this study, physiological measurements and leaf samples for metabolomic analysis were only taken at two time points during the drought period. However, collecting these samples weekly during the drought period would provide one extra, earlier time point whilst still allowing sufficient time for fresh leaves to expand for harvesting. This would provide a better estimate of when plants in the drought treatment first start to exhibit physiological stress symptoms and whether untargeted metabolomics can identify drought stress symptoms earlier than physiological measurements such as Fv'/Fm' can. In the results presented here, barley plants had begun to reduce stomatal conductance by the 5th day of water-restriction but had not yet reduced their photosynthetic rate or suffered significant damage to photosystem II so it would be advantageous to analyse the leaf metabolome at this point in the drought.

Direct infusion electro-spray ionisation time-of-flight mass spectrometry (DI-ESI-ToF-MS), which was used to fingerprint leaf polar metabolites in this study, offers a medium resolution, wide coverage of plant secondary metabolites and can be considered useful as a "first pass" for untargeted metabolomics with a new system due to it's high throughput (Allwood &

Goodacre 2009). With any mass spectrometry approach for untargeted metabolomics, there is a tradeoff between resolution (i.e. capturing information to allow better identification of compounds with similar m/z ratios) and speed of analysis, and thus cost and throughput (Goodacre & Allwood 2008). The bin size used for data pre-processing in this workflow was relatively wide by modern standards (Allwood *et al.* 2021) but considering the speed of the mass spectrometry, a relatively high proportion of overlapping peaks would be expected and a smaller bin size could not resolve this. Furthermore, direct infusion methods of mass spectrometry are unable to differentiate between isomeric forms of a molecule and can be subject to adduct formation and ion suppression (de Vos *et al.* 2007). Database searching tools such as METLIN do allow tentative annotation of the data presented here, but coverage of plant metabolites using various MS technologies for comparison still remains relatively sparse due to the enormous number of metabolites produced by plants (Allwood *et al.* 2021).

An alternative approach would be to couple (ultra high performance) liquid chromatography with electro-spray ionisation time-of-flight mass spectrometry (LC-ESI-ToF-MS). LC-ESI-MS allows detection of a wider range of plant secondary metabolites than DI-ESI-MS (de Vos *et al.* 2007), which is particularly useful for investigating the mechanisms involved in responses to abiotic stresses (e.g. drought) (Allwood *et al.* 2021). Though chromatography filters some compounds out of the sample, LC-ESI-MS gives retention times associated with the different mass to charge (m/z) ratios of ions, which, in conjunction with widely-available, open-source software for data processing and large, better annotated, cloud-based data repositories, permits better peak annotation than DI-ESI-MS and better comparison of data with other studies (Tautenhahn *et al.* 2012).

CONCLUSIONS

Taken together the results in this chapter show that metabolomic fingerprinting can be an effective tool for characterising crop responses to drought stress and that it is possible to combine physiological and metabolomic assessment of drought responses within the same plants. This approach may be able to help identify specific compounds and pathways that are important in drought responses, which would pave the way towards a better understanding of drought tolerance mechanisms in barley.

Chapter 4 - AM inoculation only subtly alters the physiological response of barley to drought stress

ABSTRACT

Arbuscular mycorrhizal (AM) fungi have been promoted as biostimulants which can provide protection against biotic and abiotic stresses, as well as potentially reducing reliance on inorganic phosphate fertilisers. In contrast to other cereal crops, in which AM inoculation has been shown to generally benefit growth and yield (Zhang *et al.* 2019), few studies have investigated the effects of AM inoculation on barley physiology. The glasshouse experiment presented here assesses the growth and physiological response of barley (*Hordeum vulgare* cv. Concerto) to a commercially available AM inoculum under both well-watered and drought stress conditions. Drought caused a small but non-significant reduction in mycorrhizal colonisation of barley plants while AM inoculation reduced barley root biomass under well-watered conditions but had no effect on above-ground biomass. Drought dramatically reduced the stomatal conductance of barley plants after 5 days of drought and their photosynthetic rate after 11 days of drought but AM inoculation had no effect on photosynthetic rate or stomatal conductance in either the well-watered or drought-stressed treatments. AM colonisation slightly delayed the drought-induced decline in photosystem II efficiency (Fv'/Fm'). This study found evidence of a subtly beneficial effect of AM inoculation on young barley plants under drought conditions.

INTRODUCTION

Benefits of inoculation with arbuscular mycorrhizal (AM) fungi are by no means guaranteed under field conditions: Hijri *et al.* (2016) found that, compared to un-inoculated controls, AM inoculation resulted in yield reductions of 14.7% in potato field trials. Benefits of resident or applied AM fungi to crop plants are not uniform or ubiquitous across crop species (Coccina *et al.* 2019, Tran *et al.* 2019) or even across cultivars of the same crop (Watts-Williams *et al.* 2019) and are particularly dependent on management practices and AM fungal identity and diversity (Dai *et al.* 2014, de Novais *et al.* 2014, Turrini *et al.* 2018). Meta-analyses investigating effect sizes of AM inoculation on crop growth or stress responses note that the field still suffers from lack of open data availability, and potential publication bias towards positive growth responses with under-reporting of negative and neutral results due to a plant-centric view of the symbiosis and its applications in agriculture (Jayne & Quigley 2014, Zhang *et al.* 2018).

While inoculation with AM fungi in the field has been found to have a positive effect on grain yields of cereals in general (Zhang *et al.* 2018), relatively few studies have investigated this in barley, and meta-analysis has shown a neutral effect of AM inoculation on barley grain yield (Zhang *et al.* 2018), though the literature does include examples of AM directly exchanging nutrients such as P and N with barley (Thirkell *et al.* 2021).

There is evidence to demonstrate that under specific conditions AM inoculation can benefit barley plants. Inoculating barley with arbuscular mycorrhizal fungi has been found to alleviate the effects of low-temperature and freezing shocks on barley seedlings (Hajiboland *et al.* 2019). Arbuscular mycorrhiza have been shown to improve P uptake, as well as micronutrient uptake, of barley under salinity stress (Mohammad *et al.* 2011). Similarly under drought conditions, AM inoculation increased barley shoot P content compared to uninoculated controls (Al-Karaki & Clark 1999).

While meta-analysis showed a general alleviation of drought stress across crop species by inoculation with AM fungi, very few studies have explored the potential alleviation of drought symptoms by AM fungi in barley (Jayne & Quigley 2014). Though drought reduced biomass and shoot P content of barley, inoculation with Glomus mosseae[1] was shown to somewhat mitigate those reductions, with AM plants in the drought treatment having higher biomass and shoot P content than non-inoculated controls (Al-Karaki & Clark 1999). Khalvati and colleagues (2005) found that under successive drying cycles, barley inoculated with *Glomus intraradices*[2] was able to maintain turgor pressure, water potential, photosynthetic rate and stomatal conductance closer to that of well-watered plants than uninoculated control plants exposed to the same drought cycles.

Most recently, Sendek and colleagues (2019) have explored the effects of varying AMF (*Rhizoglomus intraradices*[3]*, Claroideoglomus claroideum*[4] and *Funneliformis mosseae*[5]) and barley cultivar diversity on crop yield and drought response. Being colonised by multiple AMF species was found to have a negative effect on shoot biomass under non-drought conditions, and increasing colonisation rate increased shoot biomass. However under drought, the trends were more complicated: at low colonisation rates, being colonised by a single species was less beneficial to shoot biomass while at high colonisation rates, being colonised by multiple species was least beneficial to shoot biomass. Neither altering AMF

[1] As reported in the study - now known as *Funneliformis mosseae*
[2] As reported in the study - now known as *Rhizophagus intraradices*
[3] As reported in the study - previously known as Glomus intraradices
[4] As reported in the study - previously known as *Glomus claroideum*
[5] As reported in the study - previously known as *Glomus mosseae*

species richness between 1 and 3, nor varying the colonisation rate affected the decrease in seed biomass or root biomass observed under drought conditions (Sendek *et al.* 2019).

AIMS & HYPOTHESES

The aim of this experiment was to characterise the effects of AM colonisation on barley (*Hordeum vulgare* cv. Concerto) seedling physiological responses to drought.

Photosystem II efficiency as measured by Fv'/Fm' was expected to decline more rapidly in non-mycorrhizal (NM) than in mycorrhizal (AM) plants over the drought period. AM inoculation was predicted to alleviate the negative effects of drought on photosynthetic rate and stomatal conductance compared to NM plants. It was hypothesised that droughted AM plants would maintain a leaf relative water content closer to their well-watered controls than droughted NM plants would. No difference in shoot or root biomass was expected between plants under well-watered conditions (based on results from chapter 2 in the same primarily sand substrate) but under drought conditions, AM plants were expected to have higher shoot biomass than NM plants.

MATERIALS AND METHODS

Experimental design

The experiment was set up in pots under glasshouse conditions with a fully factorial design. Plants received one of two mycorrhizal inoculation treatments (a commercially available AM inoculum or the inoculum carrier lacking the AM propagules) and then, after 24 days, half the plants were droughted, while the other half continued to receive irrigation. Photosystem II efficiency was assessed daily during the drought period. Stomatal conductance and photosynthetic rate were assessed at the 5th, 11th and 18th day of drought, and leaf relative water content at the 13th day of drought. Plants were harvested, and biomass and mycorrhizal colonisation of roots assessed, at 43 days at which point they had experienced a 19 day drought period.

Growth substrate and mycorrhizal inoculum

Primarily sand growth substrate was prepared as per chapter 2. Briefly, Levington's advanced M3 compost (ICL, Ipswich, UK) was mixed in a 1:2 ratio with silica sand. This substrate was then autoclaved for 1 hour, left at room temperature for a week, and then re-autoclaved for a further hour. Prior to use, the substrate was left for 2 weeks to avoid the negative plant-growth effects of autoclaving (Rovira & Bowen 1966).

Commercially available inoculum containing a mix of 5 species of arbuscular mycorrhizal fungi (AM inoculum) was obtained from Plantworks Ltd. (Sittingbourne, UK) who also supplied the inoculum carrier without the fungus (NM inoculum). Prior to potting up, the substrate was mixed in a 3:1 ratio with AM or NM inoculum.

Watering regimes and drought treatments

Square pots of dimensions 110 x 110 x 190 mm were used. Pot capacity (PC) for the substrate and pots used was determined for 10 pots with the following method.

Pots were filled with 1605g substrate (1.4litres) and saturated with UHP water. The pots were allowed to drain by gravity for 1 hour to give an estimate of the pot capacity. The mass of the pot and substrate before saturation was subtracted from its mass at 1 hour after saturation to give the mass of water required to achieve pot capacity. An average of this measure was taken from 10 pots to give the standard pot capacity (PC) for this experimental setup.

Pots containing 1605g substrate were initially given 100% of this calculated mass required for PC (521g). Subsequently, pots were weighed three times per week to calculate the mass of water remaining in the pot. The average mass of water required for pots to be returned to 80% PC was calculated and applied to the base of each plant. Thus all pots within a treatment received the same mass of water throughout the experiment.

After the 24th day post inoculation (24DPI), plants in the well-watered treatment (WW) continued to be watered three times per week as described above. Plants in the drought stress (DS) treatment received only enough water to return the mean soil moisture content to 20% PC on day 10 and 12 of the drought (34 and 36DPI). As such, plants in the WW treatment received 2995g water in total over the course of the experiment while plants in the DS treatment received 1754g water.

See also: Chapter 1 - Materials and Methods used throughout this Thesis

RESULTS

Root colonisation by arbuscular mycorrhizal fungi

The maximum root colonisation (including arbuscules, vesicles and all potential AM hyphae) of barley plants was significantly increased by the AM inoculation in this experiment after 6 weeks of growth (F=77.604, df=1 p=4.58e-10) (fig. 4.1). For both sets of NM inoculated plants, colonisation was below 1%. In AM-inoculated plants, maximum root colonisation of well-watered controls was 33.5% while for drought-stressed plants it was 28%. Drought treatment did not affect maximum root colonisation (F=0.840, df=1, p=0.366) and there was no interactive effect between AM inoculation and drought treatment (F=0.761, df=1, p=0.389).

No arbuscules were found in the roots of NM inoculated plants. AM inoculated plants in the well-watered control and drought-stressed groups had arbuscules in 16.8% and 9.9% of root intersections respectively. Drought-treatment did not significantly reduce the proportion of root containing arbuscules in AM-inoculated plants (Mann-Whitney U=29, p=0.072).

Water remaining in pots

Comparing the generalized linear mixed effects model (GLMM) used to analyse the mass of water remaining in pots (as a % of pot capacity) to null models showed no significant effect of the AM inoculation treatment ($\chi^2\chi$=0.405, df=1, pr=0.524) (fig. 4.2.a). Adding the interactive term between AM inoculation and Drought treatment did not significantly affect the model ($\chi^2\chi$=0.940, df=1, pr=0.332) and so this term was not included in the final model. There was, however, a significant effect of including Drought treatment ($\chi^2\chi$=84.6, df=1, pr<2.2e-16). Since the same plants were measured on repeated days, two random effects were included: the pot identifier ($\chi^2\chi$=316.5, df=1, Pr=2.2 x 10^{-16}) and the day of measurement ($\chi^2\chi$=366.27, df=1, Pr<2.2 x 10^{-16}). The model fitted the data well (R^2m (fixed effects)=0.63, R^2c (random effects)=0.846).

On the 5th day of drought, there was no effect of drought on the mass of water remaining in pots (z=1.73, df=42, Pr=0.0839) but by the 11th and 18th days of the drought period there was significantly less water remaining in the pots of plants in the drought treatment compared to well-watered controls (11th day: z=2.066, df=32, Pr=0.0388; 18th day: z=2.38, df=32, Pr=0.0173).

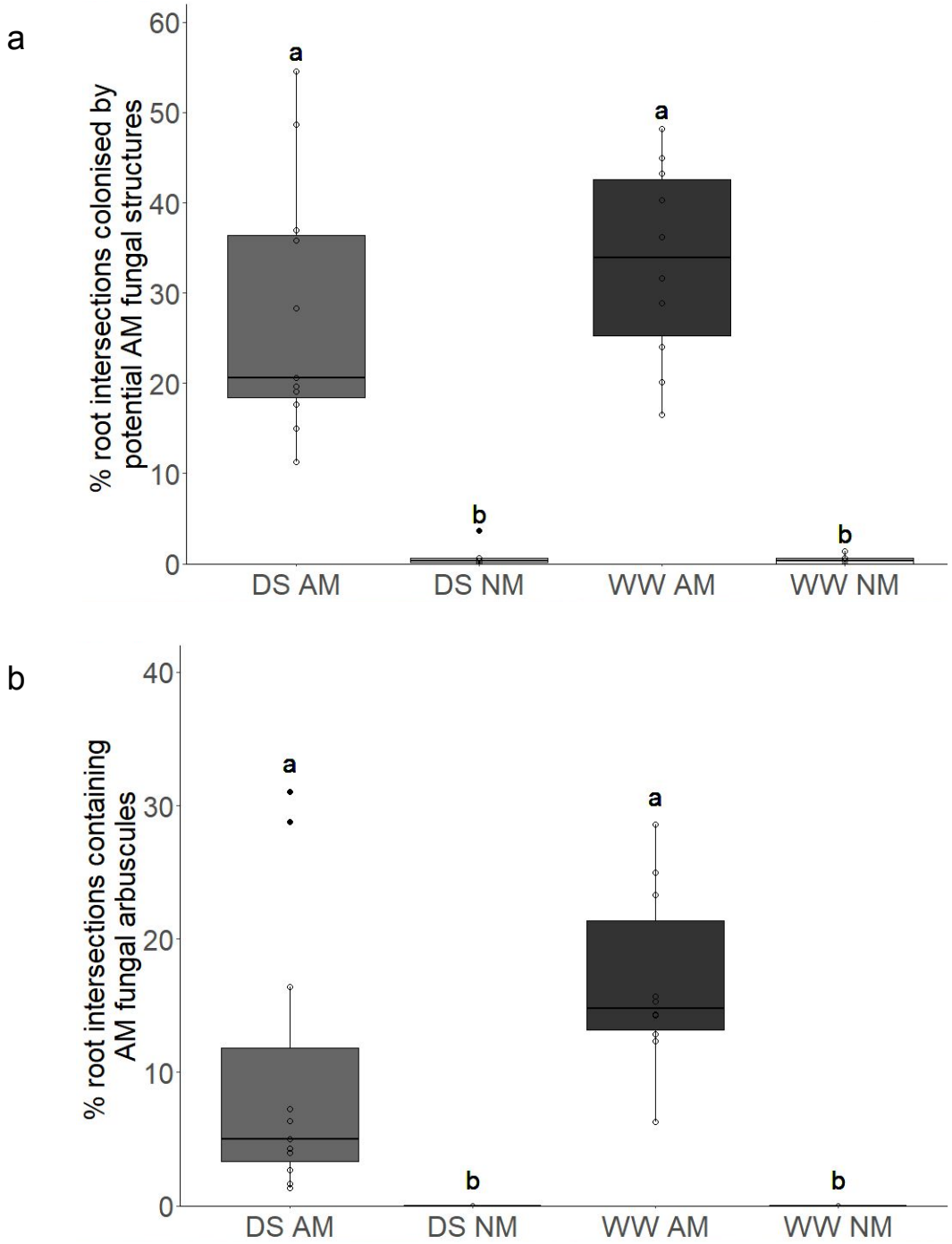

Fig. 4.1. Colonisation of 6 week old barley roots (*Hordeum vulgare* cv. Concerto) by arbuscular mycorrhizal fungi. Maximum colonisation (percentage of root intersections in which arbuscules, vesicles or potential AM hyphae were observed) (a) and colonisation by arbuscules only (b) are shown for AM-inoculated and NM-inoculated plants in drought-stressed (DS) and well-watered (WW) conditions. Boxes sharing the same letter are statistically similar at the 95% CI using pairwise post-hoc tests.

Table 4.1. Leaf relative water content, C, N, P and Si concentration and C/N ratio in shoots of barley plants inoculated with mycorrhizal (AM) or non-mycorrhizal (NM) inoculum and subjected to drought stress or well-watered conditions. Values within columns that share a letter are not significantly different at the 95% CI according to pair-wise comparisons (TukeyHSD).

	Leaf RWC (%)	Shoot C (%)	Shoot N (%)	Shoot C/N ratio	Shoot P (%)	Shoot Si (%)
AM DS	27.7 a	38.01 ab	1.84 a	22.37 a	0.426 a	1.05 a
NM DS	29.7 a	37.95 a	1.60 a	24.66 a	0.414 a	1.00 a
AM WW	91.2 b	38.74 b	0.99 b	39.44 b	0.300 b	1.17 a
NM WW	95.6 b	38.60 ab	0.97 b	40.45 b	0.338 b	1.10 a

Table 4. 2. Root/ shoot ratio, C, N, P and Si concentration and C/N ratio in roots of barley plants inoculated with mycorrhizal (AM) or non-mycorrhizal (NM) inoculum and subjected to drought stress or well-watered conditions. Values within columns that share a letter are not significantly different at the 95% CI according to pair-wise comparisons (TukeyHSD).

	Root/ shoot ratio	Root C (%)	Root N (%)	Root C/N ratio	Root P (%)	Root Si (%)
AM DS	0.491 a	40.76 a	1.62 a	26.01 a	0.222 a	1.34 a
NM DS	0.459 a	41.35 a	1.50 a	28.34 a	0.207 a	1.02 a
AM WW	0.272 b	41.08 a	1.11 b	37.20 b	0.327 b	1.25 a
NM WW	0.412 a	37.92 b	0.70 c	54.69 c	0.203 a	2.59 b

Leaf relative water content

There was no significant effect of AM inoculation treatment on leaf relative water content on the 13th day of drought (F=1.595, df=1, p=0.225) (table 4.1). AM well-watered plants had a leaf RWC of 91.2% while NM well-watered plants had a leaf RWC of 95.6%. Drought treatment significantly reduced the leaf RWC of barley plants (F=658.246, df=1, p=2 x 10^{-14}) to 27.7% in AM droughted plants and 29.7% in NM droughted plants. There was no interactive effect of AM inoculation and drought treatment on leaf RWC (F=0.233, df=1, p=0.636).

Chlorophyll fluorescence

When the GLMM used to analyse Fv'/Fm' was compared to null models, the inclusion of drought treatment had a significant effect ($\chi^2\chi$=26.506, df=1, Pr=2.627 x 10^{-7}) as did the inclusion of AM inoculation treatment ($\chi^2\chi$=10.205, df=1, Pr=0.0014) (fig. 4.2.b). There was no significant effect of adding an interactive term to the model ($\chi^2\chi$=0.0082, df=1, Pr=0.9279) and so this was not included in the GLMM. Since the same plants were measured on repeated days, two random effects were included: the pot identifier ($\chi^2\chi$=3.7777, df=1, Pr=0.05194) and the day of measurement ($\chi^2\chi$=90.629, df=1, Pr<2.2 x 10^{-16}). However, the model did not fit the data particularly well (R^2m (fixed effects)=0.083, R^2c (random effects)=0.263) and due to the low number of degrees of freedom in the whole model it was not possible to incorporate a random slope which may have better explained the data.

Stomatal conductance

At no time point did AM inoculation treatment have any effect on stomatal conductance (g_{sw}) (5th day of drought: F=2.282, df=1, p=0.142; 11th day: F=0.259, df=1, p=0.615; 18th day: F=0.633, df=1, p=0.434) (fig. 4.3.b).

Drought treatment reduced stomatal conductance (g_{sw}) to 72% of that of well-watered plants on the 5th day of the drought period (F=16.4, df=1, p=0.000368). By the 11th day of the drought, g_{sw} of droughted plants was 46% that of well-watered plants (F=61.417, df=1, p=4.54 x 10^{-8}). On the 18th day of the drought, g_{sw} of droughted plants was reduced to only 6.4% of the g_{sw} of well-watered plants (F=173.6, df=1, p=1.76 x 10^{-12}).

There was no interactive effect of drought and AM inoculation treatments on g_{sw} at any time point (5th day of drought: F=F=0.138, df=1, p=0.713; 11th day: F=F=0.197, df=1, p=0.661; 18th day: F=F=0.614, df=1, p=0.441).

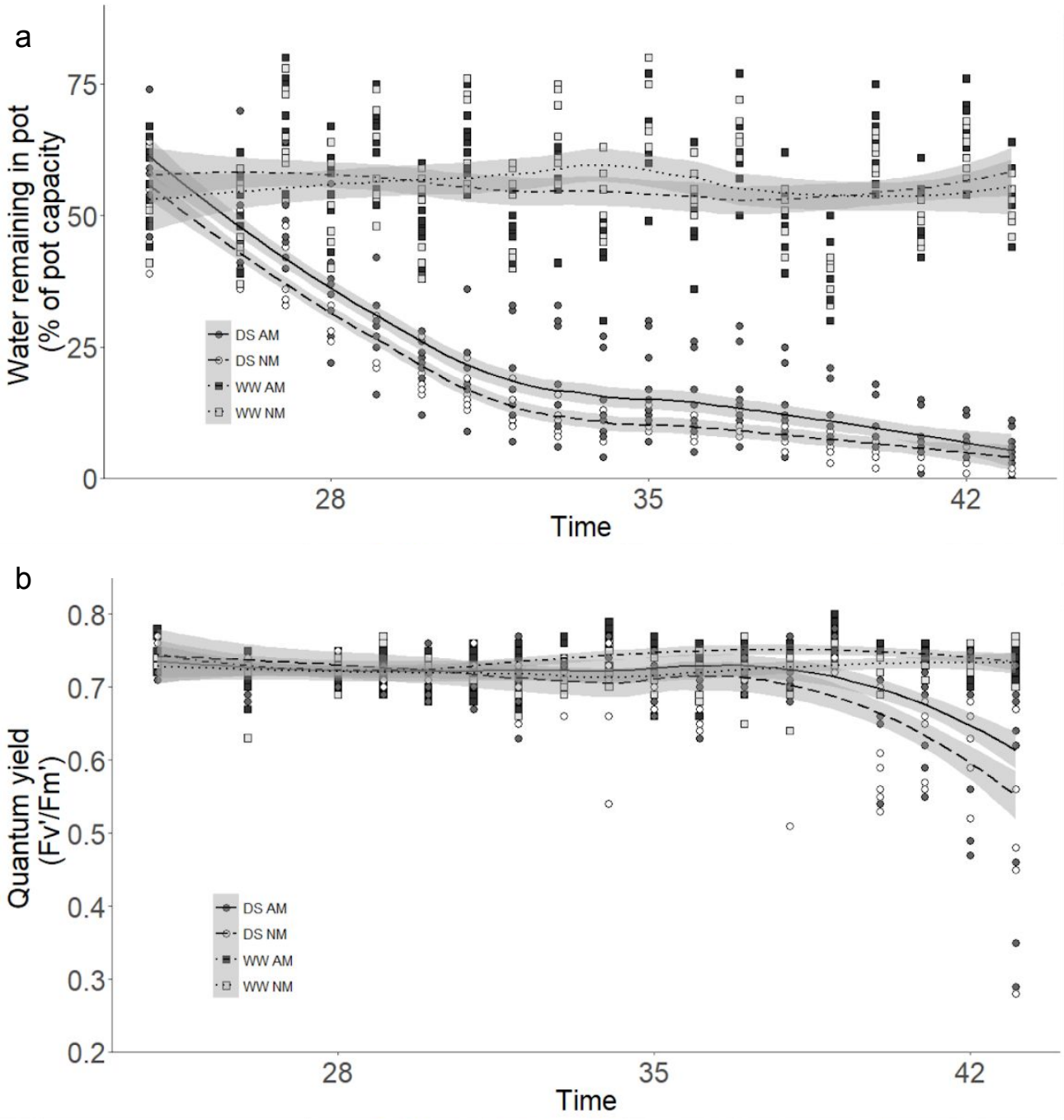

Fig. 4.2. Tracking the water remaining in pots during the drought period (as a percentage of pot capacity) (a) and the maximal efficiency of photosystem II (Fv'/Fm') (b). Well-watered plants (square symbols) received water three times per week during this time while droughted plants (circular symbols) only received water at 34 and 36 days post inoculation. Filled points represent AM inoculated plants while unfilled points represent NM inoculated plants. Grey shaded area represents 95% CI.

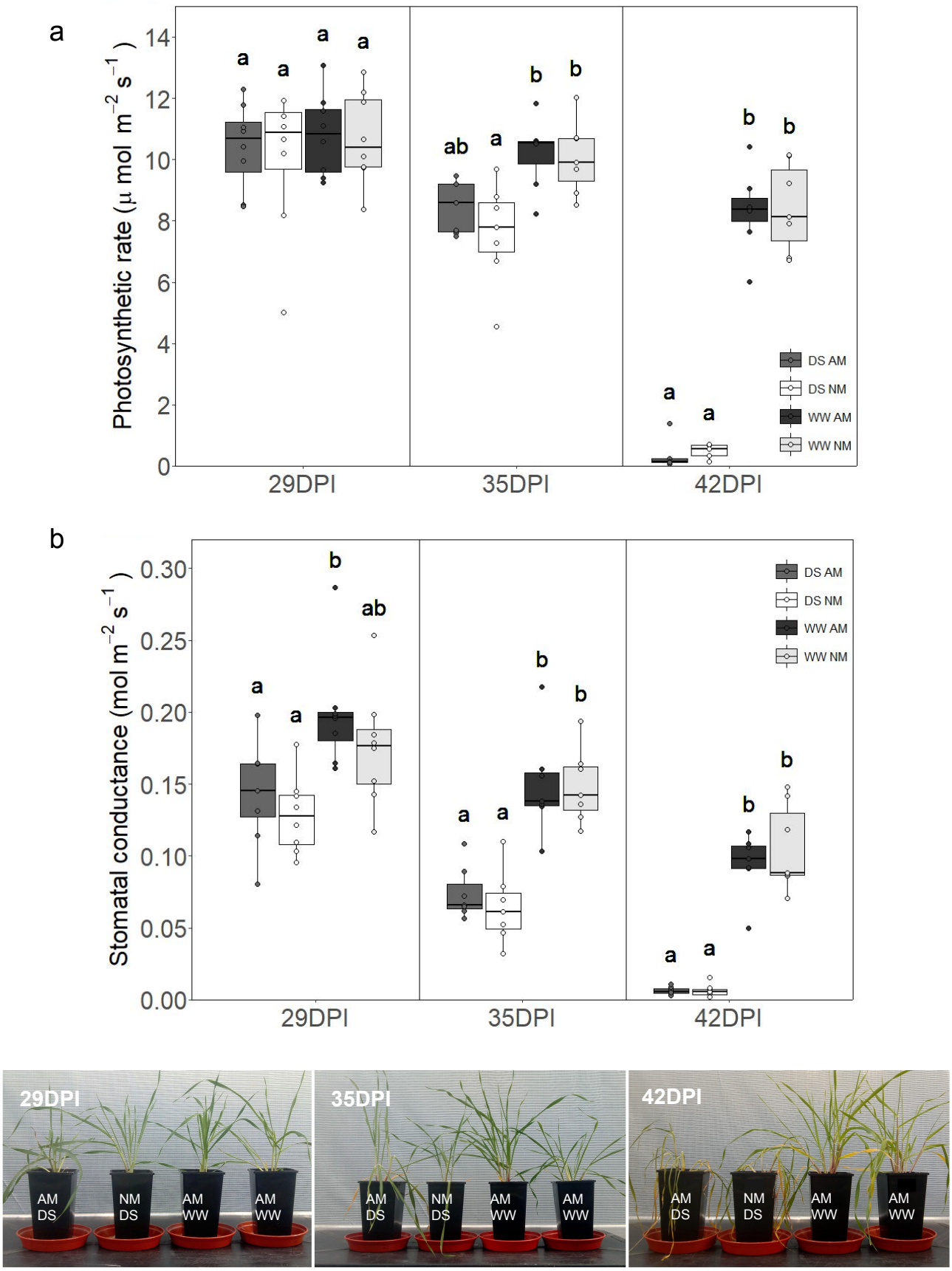

Fig. 4.3. Photosynthetic rate (*A*) (a) and Stomatal conductance to water (g_{sw}) (b) at three sampling time points during the drought period. At 29DPI n=8 plants were sampled per treatment, while at 35 and 42 DPI n=7 plants per treatment were sampled. Droughted plants (DS) and well-watered plants (WW) were inoculated with either arbuscular mycorrhizal inoculum (AM) or a non-mycorrhizal inoculum (NM). Boxes with the same letter (within facets) are statistically similar at the 95% CI using pairwise post-hoc tests.

Photosynthetic rate

At no time point did AM inoculation treatment have a significant effect on net assimilation rate (*A* - photosynthetic rate) (5th day of drought: F=0.168, df=1, p=0.685; 11th day: F=1.138, df=1, p=0.297; 18th day: F=0.818, df=1, p=0.054) (fig. 4.3.a).

On the 5th day of the drought period, there was also no significant effect of drought treatment on *A* (F=0.732, df=1, p=0.399). However, drought treatment caused a 20% reduction in *A* after 11 days of drought compared to well-watered plants (F=19.739, df=1, p=0.000171). On the 18th day of the drought, *A* had been reduced to <3% of the *A* of well-watered plants (F=425.052, df=1, $p<2 \times 10^{-16}$).

At no time point was there any interactive effect of the treatments on *A* (5th day of drought: F=0.049, df=1, p=0.827; 11th day: F=0.575, df=1, p=0.456; 18th day: F=0.002, df=1, p=0.968).

Biomass

Shoot dry biomass was unaffected by AM inoculation treatment (F=2.81, df=1, p=0.103) but was significantly reduced by drought treatment (F=136.625, df=1, $p=4.37 \times 10^{-13}$) (fig. 4.4.a). There was no interactive effect of AM inoculation treatment and drought treatment (F=2.201, df=1, p=0.148).

Drought treatment significantly reduced root dry biomass of barley plants (F=85.209, df=1, $p=1.55 \times 10^{-10}$) (fig. 4.4.b). There was a significant effect of AM inoculation on root dry biomass (F=5.171, df=1, p=0.0298) as well as a significant interactive effect of drought treatment and AM inoculation treatment (F=6.147, df=1, p=0.0186). In the well-watered condition, root dry biomass of AM inoculated plants was significantly less than that of NM plants (TukeyHSD p<0.05) however in the drought-stressed treatment, there was no difference between AM and NM plants' root dry biomass (TukeyHSD p>0.05).

AM inoculation did not have a significant effect on root/ shoot ratio (F=2.52, df=1, p=0.122) but there were significant effects of drought treatment (F=25.8, df=1, $p=1.56 \times 10^{-5}$) and the interaction between AM inoculation and drought treatment (F=8.57, df=1, 0.00624). The root/ shoot ratio of well-watered AM inoculated plants was significantly lower than that of the other treatment groups (TukeyHSD p<0.05) (table 4.2).

For further detailed nutrient analysis (C, N, P, Si content) see appendix XII.

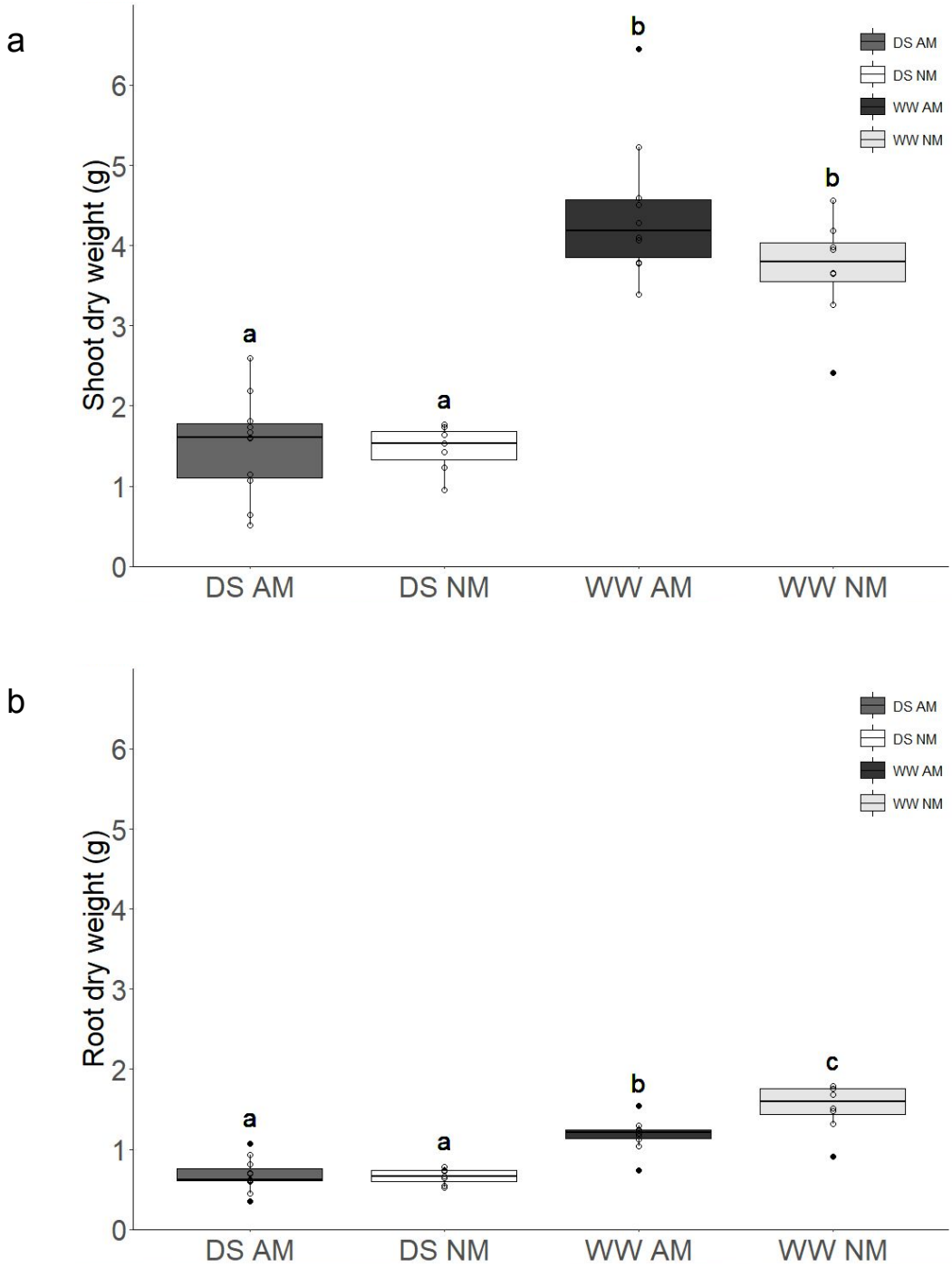

Figure 4.4. Shoot (a) and root (b) dry biomass of 6 week old barley seedlings. Droughted plants (DS) and well-watered plants (WW) were inoculated with either arbuscular mycorrhizal inoculum (AM) or an non-mycorrhizal inoculum (NM). Boxes sharing the same letter are statistically similar at the 95% CI using pairwise post-hoc tests.

DISCUSSION

The ultimate aim of homeostasis in plants is to protect the photosynthetic machinery so that the plant may continue to sustain its energy supply, even if this requires a temporary reduction in photosynthetic activity (Maxwell & Johnson 2000). As part of an arbuscular mycorrhizal (AM) symbiosis, this energy supply is also of utmost importance to the fungal partner, an obligate biotroph, and thus maintaining photosynthesis can be considered a common interest of the two organisms (Forczek *et al.* 2022). The hypothesis therefore follows that, under unfavourable external conditions that induce oxidative stress, AM fungi in an active symbiotic relationship with a plant will "boost" the plant's ability to protect its photosynthetic machinery (for example, AM rice was able to maintain stomatal conductance, photosystem II efficiency and photosynthetic rate under salt stress (Porcel *et al.* 2015)).

AM colonisation delays drought-induced decline in photosystem II efficiency

In this study, photosystem II efficiency (Fv'/Fm') was used to assess the damage to photosynthetic machinery sustained by barley seedlings under drought. AM colonisation was found to delay the reduction of Fv'/Fm' that resulted from drought by approximately one day (fig. 4.2.b). Despite this evidence of a protective effect of the symbiosis under drought conditions, mycorrhizal colonisation had no significant effect on stomatal conductance (g_{sw}) or photosynthetic rate (*A)* after 5, 11 or 18 days of drought. Similarly, the delay in damage to photosystem II did not translate into an amelioration of the negative effect of drought on above-ground biomass. At the field scale, above-ground biomass of barley at this early vegetative stage has been shown to have a strong correlation with the eventual yield (Křen *et al.* 2014) but without data on yield in this study we cannot conclude whether AM colonisation would ultimately benefit crop production for this cultivar of barley.

AM colonisation reduced root biomass under well-watered conditions only

Notably, AM colonisation reduced the size of below-ground biomass of barley seedlings, but in plants subjected to drought, no AM-related reduction was observed. As a consequence, AM barley plants in the well-watered treatment also had a root/shoot ratio that was approximately 60% that of the NM plants in both well-watered and water-restricted conditions and AM plants in the water-restricted treatment. This effect of AM colonisation reducing below-ground biomass has been noted previously in barley, for both mixed commercial inocula and for mixed species inocula produced from field soil (Frew 2020).

A larger root system in field-grown barley has been linked to a better maintenance of yield quantity and quality in dry years/ environments (Chloupek *et al.* 2010). It appears that the

AM symbiosis permitted barley seedlings to maintain the same rate of photosynthesis and aboveground growth as NM plants with a lower investment in growing roots under favourable conditions. It could be that any benefits of the AM symbiosis under favourable conditions are negated in this set-up by the smaller size of the AM root system, which would explain the lack of effect of AM colonisation on A or g_{sw}. Under drought conditions, we cannot know from this study whether AM and NM plants' root systems achieved the same biomass through a difference in rate of growth or through cessation of growth at different times, but by the 18th day of drought, AM and NM barley plants had the same below-ground biomass.

Stomatal conductance, photosynthetic rate and leaf relative water content were unaffected by AM colonisation (but reduced by drought)

A meta-analysis of 438 studies in which AM and NM plants were subjected to drought and well-watered control conditions concluded that AM colonisation increased stomatal conductance in general, with the increase being more exaggerated under severe drought stress (Augé *et al.* 2015). This was not the case in the present study. In a study by Khalvati *et al.* (2005) which measured net photosynthetic rate (A) and stomatal conductance (g_s) of barley at the end of a seven day drying cycle, a similar pattern was found to the present study with A and g_s of well-watered AM and NM plants being similar, significantly higher than those of AM and NM plants subjected to drought stress. In their study, plants were given a recovery period before being subjected to further drying and recovery cycles, after the second of which, AM plants began to show relatively higher A and g_s compared to NM plants in the drought treatment. It may therefore be that AM colonisation does not protect barley from the physiological symptoms of drought stress when the water deficit is prolonged, as in the experimental set-up presented in the current study, but that AM colonisation can provide some alleviation from, or priming for, recurrent drought, as presented by Khalvati and colleagues (2005).

The nature of the drought in this study was quite extreme (prolonged drought rather than drying-rewetting) with the leaf relative water content on the 13th day of drought reduced by 65% points compared to plants in the well-watered treatment. Since no recovery was included in the present study, it is not possible to confirm whether AM plants would have a better ability to "bounce back" than NM plants. However, the slight amelioration of photosystem II efficiency as measured by Fv'/Fm', indicates that AM plants could have been at a slight advantage had re-wetting occurred. Some of the theoretical benefits of the AM symbiosis under drought rely on the ability of AM extraradical hyphae to explore a greater soil volume, and smaller soil pores, than the roots (Augé 2001) which may be compromised in a pot experiment in which plants are grown bounded by plastic in a homogenised

substrate (Ryan & Graham 2018). In this experiment the possibility of plant-plant interactions via a common mycelial network was removed by growing individual plants in their own pots. Results from this experiment cannot be directly extrapolated to agronomic situations since studies on cereals performed in the lab or glasshouse generally show greater benefits of AM inoculation to growth and yield compared to field studies (Zhang *et al.* 2019), despite the use of commercially available inoculum.

Drought did not cause strong reduction in AM colonisation

While very little evidence for the amelioration of physiological drought symptoms by AM fungi was found in this study, observation of arbuscules by root staining and microscopy and assessment of the extent of root colonisation by AM fungal structures provided evidence that a functional symbiosis was established (appendix IV). By 6 weeks old, barley seedlings had root colonisation of 33.5% in the well-watered treatment and 28% under drought stress, however the difference was not significant. Studies in forage sorghum (Sun *et al.* 2017) and wheat (Yaghoubian *et al.* 2014, Mathur *et al.* 2018) have found drought treatment to reduce mycorrhizal colonisation. Drought treatment even reduced AM colonisation of sorghum when plants were inoculated with AM strains isolated from drought-tolerant environments (Symanczik *et al.* 2018). When barley was grown in microcosms with varying AM fungal diversity and barley genotypic diversity, drought reduced mycorrhizal colonisation frequency and the abundance of arbuscules and vesicles (Sendek *et al.* 2019). The results presented here are contrary to these findings in the literature: though there was a slight reduction in maximum colonisation and in proportion of arbuscules, drought did not significantly reduce AM root colonisation or the proportion of root containing arbuscules in this study.

Further work

To better understand how AM plants were able to delay the drought-induced decline in photosystem II efficiency presented here, leaf metabolomic fingerprints from the same plants are compared in chapter 5. An untargeted metabolomics approach can elucidate biochemical pathways relating to photosynthesis, respiration, antioxidant activity or osmoregulation (Allwood *et al.* 2021), and builds on the comparison of droughted and well-watered leaf metabolomic fingerprints of non-mycorrhizal barley plants presented in chapter 3.

CONCLUSIONS

AM colonisation delayed drought-induced decline in photosystem II efficiency in young barley plants by approximately one day. Drought reduced the stomatal conductance of

young barley plants 5 days into the drought period and reduced their photosynthetic rate and chlorophyll fluorescence by the 11th day of drought, with an ultimately negative effect on their biomass. This study found no effect of AM inoculation on barley stomatal conductance, photosynthetic rate or above-ground biomass under well-watered or drought stress conditions. AM inoculation reduced root biomass in the well-watered treatment but this effect was not evident in the drought-stressed treatment. These results suggest there is only a slight beneficial effect of AM inoculation on barley at the vegetative stage.

Chapter 5 - Effects of drought and AM fungal colonisation on leaf metabolomic fingerprints of barley

ABSTRACT

Arbuscular mycorrhizal fungi form symbiotic relationships with barley and have been shown to improve the response of many crop species to drought. Using LC-ESI-MS in an untargeted metabolomics approach, differences between mycorrhizal and non-mycorrhizal barley leaf polar metabolites were compared between well-watered and drought-stressed plants at 29, 35 and 42 days old. Growth stage at time of sampling was the strongest factor distinguishing leaf metabolomes. Flavonoids, alkaloids and amino acid derivatives were associated with barley leaves at 29 days old (tillering stage), while cellulose and a chlorophyll precursor were associated with barley leaf metabolomes at 42 days old (stem elongation stage). By the 18th day of drought, non-mycorrhizal plants showed accumulation of putatively annotated fatty acid derivatives, various sesquiterpenoids and phenylpropanoids in response to drought. In contrast, the effect of drought on mycorrhizal plants was weaker but mycorrhizal plants specifically accumulated putatively annotated chlorophyll precursors, ethanolamine, lactate and 12-OPDA in response to drought, suggesting involvement of the tetrapyrrole, glycerophospholipid and jasmonic acid biosynthesis pathways and methylglyoxal detoxification in the AM-mediated drought response of barley.

INTRODUCTION

Untargeted metabolomic fingerprinting is a mass-spectrometry approach that can be used to compare snapshots of the biochemical activity of a cell or organ over time or between treatments, and can provide insights into the metabolic pathways that are altered in response to environmental conditions such as drought (Kaur *et al.* 2022).

Existing literature suggests that, in response to drought, barley accumulates sugars and sugar alcohols such as fructose, glucose, galactinol and tricarboxylic acid cycle (TCA) intermediates, whilst reducing starch, aspartate, glutamate and serine accumulation in its leaves (Templer *et al.* 2017, Swarcewicz *et al.* 2017). Sugars act as compatible solutes, helping to maintain the cellular homeostasis during osmotic stress (Martinez *et al.* 2004). Glycosides of flavones (flavonoids), hydroxycinnamic acid amide (HCAA) derivatives such as hordatines, ferulic and sinapic acids, and blumenol derivatives (terpenoids) have also been associated with leaf drought response in some barley cultivars (Piasecka *et al.* 2017,

Kowalczewski *et al.* 2020, Piasecka *et al.* 2020). These compounds have been linked to antioxidant activity in response to drought stress (Agati *et al.* 2012) and some have also been shown to be accumulated in response to AM colonisation in barley and *Brachypodium distachyon* (Wang *et al.* 2018, Mahood *et al.* 2022).

No single mass-spectrometry technique can as yet analyse all the 200,000+ potential known plant metabolites so decisions about what kind of metabolites will provide insight into the biological question to be answered are required (Garibay-Hernández *et al.* 2021). In chapter 3, DI-ESI-MS was used as a "first pass" to compare barley plants subjected to drought against well-watered control plants. That study showed that it is possible to combine physiological and metabolomic sampling within the same set of plants, and that drought had a significant effect on the polar leaf metabolome of spring barley cv. Concerto. Putative compound identification suggested that the phenylpropanoid pathway was affected by drought in this cultivar, as well as other tentatively annotated compounds such as indole and 2'-deoxyuridine 5'-diphosphate (dUDP). However, although it favours high speed and high throughput, identification capabilities are limited in that DI-ESI-MS has no ability to differentiate between structural isomers (e.g. hexose sugars) and commonly has issues with ion suppression (ionisation energy being used to ionise some compounds at the expense of others) (Overy *et al.* 2005).

Liquid chromatography coupled to electrospray ionisation mass spectrometry (LC-ESI-MS) is widely used for untargeted metabolomic fingerprinting as it provides two sets of orthogonal data (retention time and m/z ratio) which aids in metabolite identification (Tautenhahn *et al.* 2012) whilst also permitting detection of a broader range of metabolites than ESI-MS alone (de Vos *et al.* 2007). Chromatic separation of metabolites before they are ionised can also ameliorate issues with ion suppression (Overy *et al.* 2005). Online data pre-processing (noise reduction, peak picking, alignment) via tools such as XCMS online allow optimised processing of large data sets, which, when coupled with well-maintained online reference data repositories (METLIN, MassBank), facilitates improved annotation of compounds of interest when using LC-ESI-MS (Tautenhahn *et al.* 2012).

The leaf metabolome has been shown to be dynamic, with transient metabolite accumulations having long-term effects on physiology (Jasiński *et al.* 2009). So while strong metabolomic responses to drought have been observed in barley leaves (Templer *et al.* 2017), the timing of accumulation during development and the alteration of this in response to drought remains complex and under-researched (Yuan *et al.* 2018). In this study, "snapshot" assessments of the metabolomic fingerprint of leaves were taken at multiple sampling time points in conjunction with physiological assessments of drought response.

The metabolomic fingerprints of plants at tillering and stem-elongation stages were compared. Previous research in barley has shown a strong effect of developmental stage on the above-ground metabolome profile. Specific phenolic compounds, such as flavonoids, and their glycosylation patterns in particular, show compound-specific accumulations at particular growth stages with phenolic compound accumulation peaking during the tillering stage (Lee *et al.* 2016, Yan *et al.* 2022), and different abundant phenolics such as saponarin and lutonarin, having different accumulation patterns to each other (Brauch *et al.* 2019).

Liquid chromatography electro-spray ionisation mass spectrometry (LC-ESI-MS) was used in this study to obtain polar metabolomic fingerprints of mycorrhizal (AM) and non-mycorrhizal (NM) barley leaves subjected to either drought-stress (DS) or well-watered (WW) conditions. Leaf samples were harvested from all plants at three time points during the drought period representing the 5th, 11th and 18th days of drought (29, 35 and 42 day post inoculation respectively).

In chapter 4, the effectiveness of the drought regime was evaluated as sufficient to induce drought stress in the plants subjected to this treatment. Briefly, a significant reduction in leaf relative water content by the 13th day of drought (37DPI) in both AM DS and NM DS plants was observed, and significant reductions in stomatal conductance (g_{sw}) and photosynthetic rate (A) were apparent in DS treated plants from the 5th (29DPI) and 11th (35DPI) days of drought respectively. Both above- and below-ground biomass were significantly reduced by DS treatment.

Arbuscular mycorrhizal (AM) colonisation of barley roots in AM treated plants, and, as far as is possible, its absence in non-mycorrhizal (NM) treated plants was also assessed and the efficacy of the treatments confirmed in chapter 4. Drought treatment was not found to significantly reduce root colonisation by AM fungi. AM colonisation did, however, reduce the root biomass of barley but only under WW conditions.The photosystem II efficiency (as measured by Fv'/Fm') was significantly reduced by DS treatment by the 12th day of drought (36DPI), however, the reduction was delayed by approximately one day in AM DS plants compared to NM DS plants, an indication that AM colonisation was, to some extent, ameliorating the effects of drought on barley. However, this did not translate to significant improvements in A *or* g_{sw} under DS conditions when these were assessed at the 5th (29DPI), 11th (35DPI) or 18th (42DPI) days of drought.

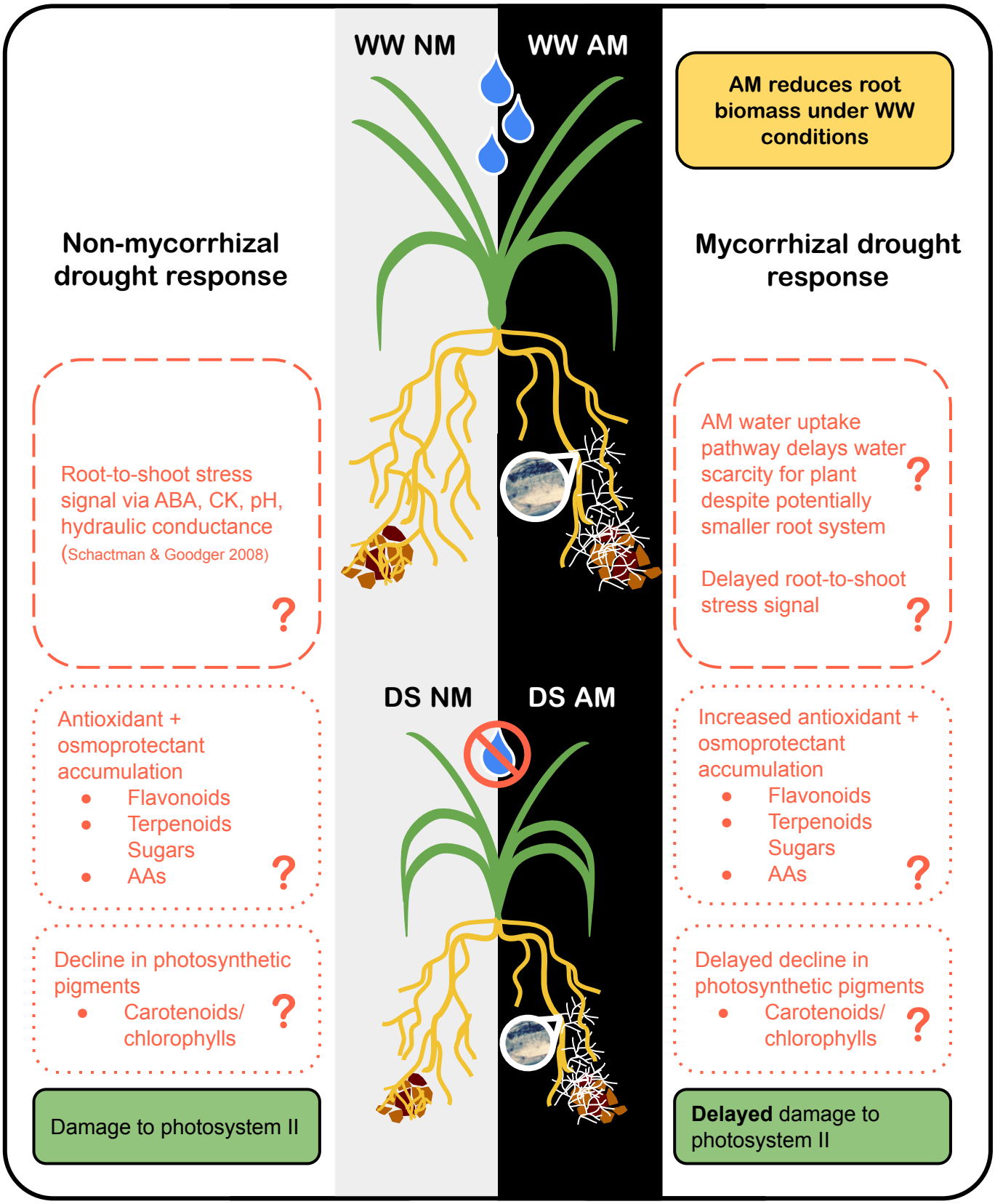

Fig. 5. 1. Expected effects of drought stress and arbuscular mycorrhizal fungal (AMF) colonisation on shoot metabolomic fingerprints of barley. Dashed outlines represent hypotheses not tested in this study; solid black outlines represent results from chapter 4; dotted outlines represent hypotheses for the metabolomic responses of barley leaves to drought stress for AM colonised or un-colonised (NM) plants.

AIMS & HYPOTHESES

In the preceding chapters of this thesis, it has been shown that detecting metabolomic differences in the leaves of droughted and well-watered barley plants is possible using an untargeted metabolomics approach (chapter 3). Furthermore, AM colonisation was shown to delay the drought-induced reduction of photosystem II efficiency by one day (chapter 4). The aim of this chapter was to investigate whether mycorrhizal colonisation alters the leaf metabolomic response of barley cv. Concerto to drought (fig. 5.1).

Metabolomic fingerprints of barley leaves were expected to differ based on their developmental stage at harvest (tillering vs. stem elongation), particularly in their accumulation of phenolic compounds such as flavonoids (Lee *et al.* 2016, Yan *et al.* 2022).

Drought stress was expected to have a clear effect on the metabolomic fingerprints of barley leaves, as was AM colonisation. Leaf metabolomic fingerprints from plants inoculated with commercially available arbuscular mycorrhizal inoculum and exposed to the drought treatment (AM DS) were expected to be more similar to well-watered controls (AM WW) than those receiving a non-mycorrhizal inoculum (NM DS vs NM WW). Differences between AM DS and AM WW plants could provide insights into the mechanism by which AM colonisation delayed reduction in photosystem II efficiency in these plants (as observed in chapter 4).

The following metabolomic differences were anticipated to account for distinctions between leaf metabolomes:

- Increased accumulation of terpenoid compounds in AM WW compared to NM WW plants in line with accumulation of blumenol C glycosides in response to AM colonisation (Wang *et al.* 2018, Mahood *et al.* 2022);
- Accumulation of compatible solutes and osmoregulatory compounds such as glycine betaine, proline, and sugars, sugar alcohols and amino acids under drought (Templer *et al.* 2017), with earlier or increased accumulation of these by AM colonised plants (Langeroodi *et al.* 2020);
- Accumulation of phenylpropanoid pathway intermediates and antioxidant compounds in response to drought stress (chapter 3), with an AM-mediated association, or earlier accumulation of these compounds;
- Phenylpropanoid association with tillering stage metabolomes in contrast to the stem elongation stage (Lee *et al.* 2016);
- Association of chlorophylls and their precursors with those plants observed to have higher photosystem II efficiency (chapter 4): NM WW and AM WW plants and to a lesser extent, AM DS in contrast to NM DS plants.

Table 5. 1. Plan of irrigation, sampling and duration of drought period in experiment 3, presented in chapters 4 and 5.

X = all plants in each relevant treatment; s = subset of plants in each relevant treatment; pale grey = drought period; dark grey = destructive harvest.

Day of drought																									1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Days post transplant + inoculation	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43
Weigh pots	X	X		X			X		X		X			X		X		X			X		X		X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Irrigation (WW)	X	X		X			X		X		X			X		X		X			X		X		X		X		X		X		X		X			X		X			
Irrigation (DS)	X	X		X			X		X		X			X		X		X			X		X		X									x		x								
Chlorophyll fluorescence (Fv'/Fm')																							X		X		X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Photosynthetic rate (*A*)																														S						S							S	
Stomatal conductance (*gsw*)																														S						S							S	
Metabolite sample (leaf)																														X						X							X	
Leaf RWC																																						S					S			

MATERIALS AND METHODS

Experimental design

The experiment was set up in pots under glasshouse conditions (as described in chapter 4) with a fully factorial design. Barley (*Hordeum vulgare* cv. Concerto) plants received one of two inoculation treatments (a commercially available AM inoculum or the inoculum carrier lacking the AM propagules) and then, after 24 days, half the plants underwent 19 days of water-restriction (confirmed as inducing drought stress in chapter 4, DS treatment), while the other half continued to receive irrigation (well-watered, WW, treatment). Plant physiological techniques were used to assess severity of drought stress and biomass was recorded at the end of the experiment (see chapter 4). Leaf samples were harvested from the same plants at three time points (5th, 11th and 18th day of drought, as per table 5.1) during the drought period polar and non-polar metabolites were extracted. Metabolomic fingerprints of polar extracts were obtained using liquid chromatography coupled to mass spectrometry (LC-ESI-MS). Multivariate data were processed using open source bioinformatics tools, analysed with PCA and OPLS-DA models, and then peaks (as variables) of interest were annotated with reference to publicly available databases, in order to highlight potential compounds associated with samples harvested at different growth stages and samples from the different treatment groups.

Growth stage was assessed at 29, 35 and 42 DPI according to Tottman *et al.* 1974 and was recorded as the number of leaves, number of tillers and number of nodes that could be felt on the main stem (dissection was not used to assess nodes).

Untargeted metabolomic analysis

Reproducible workflow

Considerable effort went into developing the analytical workflow that was used to investigate this comparative metabolomic dataset, and efforts were made to make the methodology freely available to future users. With the support of “Unleash your data and software” funding from the University of Sheffield Library, a user guide to this untargeted metabolomics workflow has been made available as an interactive web guide (https://untargeted-metabolomics-workflow.netlify.app/ accessed 24 March 2023) and in Parker *et al.* 2023 (included as chapter 6 of this thesis).

Data from this experiment are available at DOI: 10.15131/shef.data.19362002 and at MetaboLights MTBLS5918 (in curation 24 March 2023).

Interoperable R code required to repeat the analysis, or for use with other datasets, is available at:

https://github.com/LizzyParkerPannell/Untargeted_metabolomics_workflow.git

Metabolite sampling, extraction and LC-ESI-MS

At the 5th, 11th and 18th days of the drought period (29, 35 and 42 DPI respectively), metabolite samples were taken following gas exchange measurements (detailed in chapter 4). A 2cm leaf tip sample was taken from the youngest expanded leaf on a tiller at 29 and 35DPI and from the youngest expanded leaf on the main stem at 42DPI. All samples were snap frozen in liquid nitrogen before being stored at -80°C.

Metabolites were extracted using the water: methanol: chloroform method (Overy *et al.* 2005) (details in chapter 1). For data acquisition in positive mode, 1µl of the extract was run through a Waters Acquity C18 column coupled to a Waters Synapt G2 Mass Spectrometer (Waters Ltd, Manchester, UK). A linear gradient system using 95% water/ 5% acetonitrile (v/v), changing to 65% water/ 35% acetonitrile (v,v) at 3 minutes and 0% water/ 100% acetonitrile (v/v) at 6 minutes at a constant flow rate of 0.4 ml min^{-1}. The MS had an electrospray sample introduction system and data were acquired using Waters MassLynx data system. Settings are detailed in appendix VI.

LC-ESI-MS data processing

Data files were converted to the open .mzML format using MSConvert from Proteowizard (Chambers *et al.* 2012). Peak picking, alignment and retention time correction (using the obiwarp method of Prince & Marriott 2006) were performed in XCMS online to produce a peak table of intensities in each sample for each detected feature (feature here meaning m/z and retention time (RT) combination present in at least 50% of the samples) (Tautenhahn *et al.* 2012, Gowda *et al.* 2014). Detailed XCMS online parameters are available in appendix VII.

The following were treated as independent datasets beginning with the peak picking stage (i.e. separate XCMS jobs):

- All data from the experiment were compared across three time points (29, 35 and 42 DPI) to compare the metabolomes of mycorrhizal (AM) and non-mycorrhizal (NM) plants in both droughted (DS) and well-watered conditions (WW) and understand which factors had the biggest impact on the leaf metabolome (age at sampling time point, AM inoculation or Drought condition) *(multigroup comparison)*;

- Non-mycorrhizal well-watered (NMWW) plants were compared between 29DPI and 42DPI to compare the effect of developmental stage at time of sampling on the barley metabolome, independent of drought and AM treatments *(pairwise comparison)*;

- Samples from 42DPI (18th day of drought) were compared across four treatment groups to understand which factor was more influential on the leaf metabolome (AM inoculation or drought treatment) *(multigroup comparison)*;

- Non-mycorrhizal (NM) plants from all three time points (29, 35 and 42 DPI) to understand when a drought response was evident in the NM barley metabolome *(multigroup comparison)*;

- Mycorrhizal (AM) plants from all three time points (29, 35 and 42 DPI) to understand when a drought response was evident in the AM barley metabolome (and whether the timing was similar to the NM drought response) *(multigroup comparison)*;

- Non-mycorrhizal drought-stressed (NM DS) plants from 42DPI (18th day of drought) were compared to NM well-watered (NM WW) plants to understand the metabolomic drought response in NM plants *(pairwise comparison)*;

- Mycorrhizal drought-stressed (AM DS) plants from 42DPI (18th day of drought) were compared to AM well-watered (AM WW) plants to understand the metabolomic drought response in AM plants (and whether this was similar to the NM drought response) *(pairwise comparison)*.

Undirected analysis

Processed data were downloaded from XCMS online and analysed in R. Pareto scaling was applied and principal component analysis (PCA) was performed (specifically Nipals PCA from the *pcaMethods* package (Stacklies *et al.* 2007)) to develop hypotheses of which classes of samples differed. PERMANOVA (adonis from the vegan package in R) using 999 permutations was used to corroborate trends observed in the PCA scores plots (Oksanen *et al.* 2022) and summary statistics are provided in appendix VIII.

Directed analysis

Pairs of classes that clustered separately in the un-directed PCA (and/ or those between which differences in specific physiological parameters had been observed in chapter 4) then underwent directed analysis (specifically OPLS-DA from the *muma* package (Gaude *et al.* 2012)) in which the model is provided with class information and determines the variables

(peaks) that distinguish them. Variables were deemed to be “of interest” (i.e. strongly associated with one class) if their loadings had high reliability and high influence on the discrimination of the two classes from the OPLS-DA (of the top 20 loadings for each class, those with pcorr1 > 0.4 or pcorr1 < -0.4).

Compound annotation

To investigate potential identities of m/z ratios of interest from the OPLS-DA, the METLIN Metabolite and Chemical Entity Database (Scripps Institute, USA) was consulted using a “Simple search”. Briefly, compounds with an expected peak within 15ppm of the experimentally detected mass that also had a KEGG (Kyoto Encyclopedia of Genes and Genomes) identifier were returned as hits. All adducts were considered in the METLIN search, though where alternative identities were possible, matches with the ions [M+H]+, [M+Na]+ and [M+K]+ were given priority. Additional identification information was obtained from the MassBank data repository (Horai *et al.* 2010) using their online search function for each m/z of interest, with search criteria of intensity of 50 and tolerance of 0.05 in LC-ESI-MS and LC-ESI-MS/MS spectra in positive mode. The mass error of MassBank hits was subsequently calculated manually using the MassBank m/z ratio as “reference” and the m/z of interest as “detected” in equation 4.

Equation 4 $$ppm = ABS((reference - detected)/reference \times 10^6)$$

PubChem, BarleyCyc, KEGG Pathway and KEGG compound databases were consulted to discount METLIN and MassBank hits that were unlikely to be of plant origin and to add chemical class and pathway information.

See also: Chapter 1 - Materials and Methods used throughout this Thesis

RESULTS AND DISCUSSION

The leaf metabolomic fingerprint gives a snapshot of the biochemical status of the plant at the time of sampling. Here, the metabolite fingerprints of polar leaf extracts, obtained by LC-ESI-MS were subjected to a range of sequential analyses to form hypotheses of the compounds and biochemical pathways involved in barley cv. Concerto response to drought stress and to evaluate whether AM drought response occurs by the same mechanisms as NM drought response.

An initial analysis including leaf polar metabolomic fingerprints from all treatments and all timepoints was undertaken. A total of 6904 peaks (m/z ratios coupled with retention time information) were included in this analysis, following grouping and alignment of between 3131 and 6025 features from individual samples processed using XCMS online. Of these features, annotation was only attempted for those causing discrimination in directed pair-wise comparisons of treatment groups where exploratory analysis of metabolomic fingerprints (unsupervised PCA) revealed differences or where specific physiological and plant growth differences had been observed in chapter 4.

Barley leaf polar metabolomes differ between plants at different growth stages

When all samples were included in analysis, leaf metabolomic fingerprints of barley seedlings could be distinguished by plant age at sampling (fig. 5.2.a). Samples harvested at the same time clustered together and clear separation between 29DPI and 42DPI was observed, with samples from 35DPI overlapping the earlier and later sampling time points (fig. 5.2.b) (PERMANOVA F_2=20.29, R^2=0.28, p=0.001). Although drought had a significant effect on the leaf metabolome (PERMANOVA F_1=3.80, R^2=0.03, p=0.003), little clustering by treatment group was observed in the PCA scores plot (fig.5.2.a) other than a slight separation of treatment groups at 42DPI, which is congruous with an interactive effect of sampling time point and drought treatment in the PERMANOVA model (PERMANOVA F_2=2.66, R^2=0.04, p=0.004). The PERMANOVA model found no significant effect of AMF on the leaf metabolome (PERMANOVA F_1=1.61, R^2=0.01, p=0.144) nor of significant interactions between AMF*Time and AMF*Drought (for summary statistics see appendix VIII).

The effect of age at sampling/ growth stage on the leaf metabolomic fingerprint being greater than that of other experimental treatments is in line with experiments on pea (*Pisum sativum*), in which authors noted a much greater effect of plant age on the leaf metabolome than of mycorrhizal colonisation (Shtark *et al.* 2019).

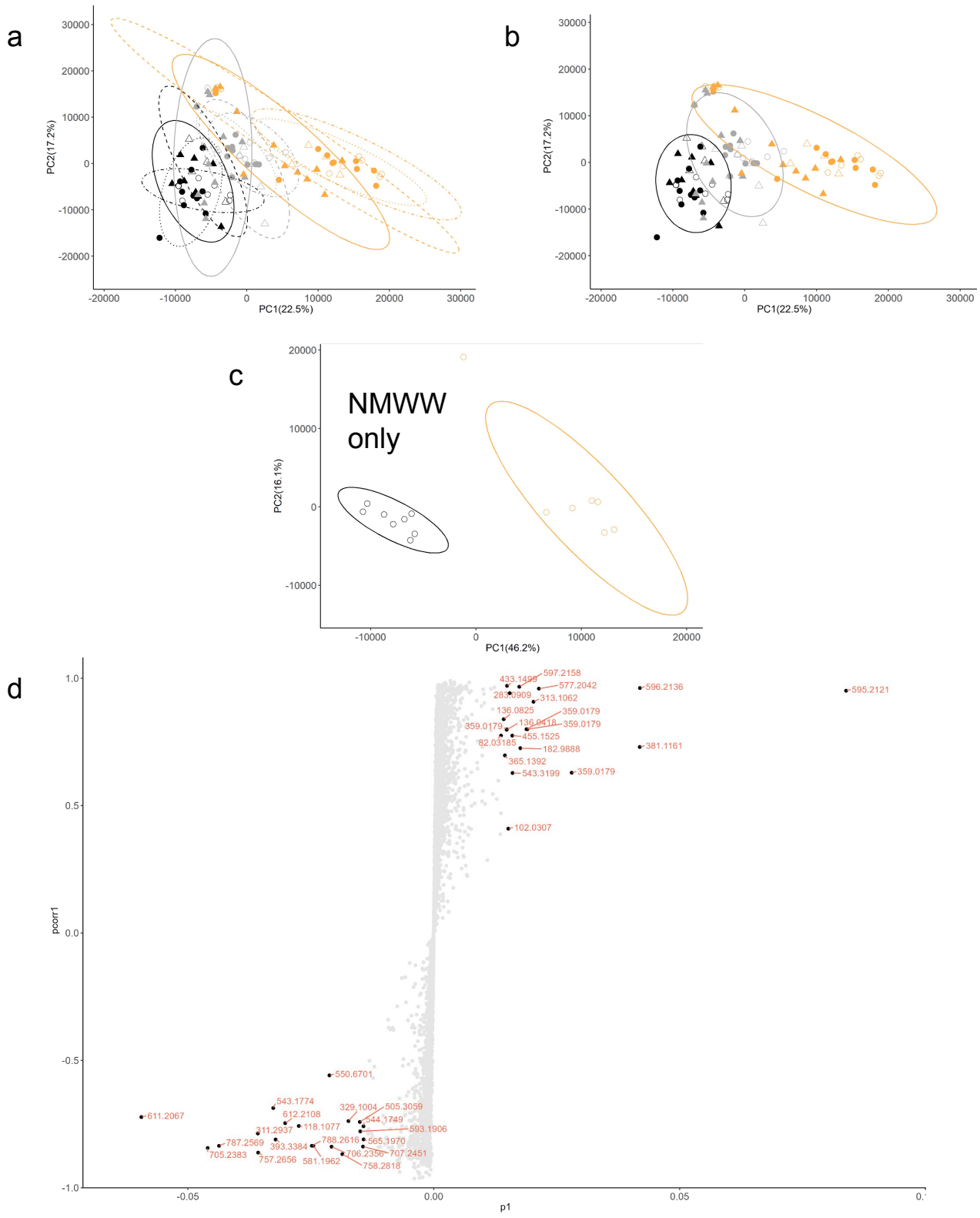

Fig. 5. 2. Leaf metabolomic fingerprints could be distinguished by age at sampling time point - separation between 29DPI and 42DPI was observed with samples from 35DPI overlapping the earlier and later time points. (a) and (b) show the same principal component analysis (PCA) scores plot for LC/MS of aqueous extracts in positive mode showing PC1 and PC2. (c) shows a separate analysis comparing NMWW samples between 29DPI and 42DPI which subsequently underwent directed analysis to understand which m/z values (red annotations in d) were attributable to growth stage/ ageing. Black = 29DPI/ 5th day of drought period; grey = 35DPI/ 11th day of drought period; gold = 42DPI/ 18th day of drought. NMWW = open circles; AMWW = filled circles; NMDS = open triangles; AMDS = filled triangles. Ellipses represent the 95% confidence interval for a given treatment group.

Table 5. 2. Metabolomic features associated with the leaves of well-watered non-mycorrhizal (WW NM) barley plants (H*ordeum vulgare* cv. Concerto) at tillering (29DPI) and stem elongation (42DPI) stages. Chemical class annotations are based on comparison with METLIN, MassBank, KEGG and BarleyCyc databases. Mass error is given for annotations in ppm (parts per million).

						Database (METLIN) m/z matches					
RT (secs)	**Detected m/z**	**Putative class annotation**	**METLIN ID**	**KEGG ID**	**Compound**	**Chemical class**	**Formula**	**ppm**	**Ion**	**Reference m/z**	**Accurate mass**
Associated with sampling time point 1 (29DPI)											
99	595.2121	Sesquiterpenoid	67437	C09113	Ingenol 3,20- dibenzoate	Sesquiterpenoid	C34H36O7	4	[M+K]+	595.2093	556.2461
99	596.2136	-									
23	381.1161	Flavonol	51421	C10030	5,4'-Dihydroxy-3,6,7,3'-tetramethoxyflavone	Flavonol	C19H18O8	0	[M+Li]+	381.1162	374.1002
395	359.0179	Dihydroflavonol	3450	C02906	Dihydromyricetin	Dihydroflavonol	C15H12O8	4	[M+K]+	359.0164	320.0532
99	577.2042	Lignan	71863	C17686	Lappaol C	Lignan	C30H34O10	0	[M+Na]+	577.2044	554.2152
99	313.1062	Flavonoid	49525	C10024	Baicalein 5,6,7-trimethyl ether	Flavone	C18H16O5	2	[M+H]+	313.1070	312.0998
			50069	C10045	Galangin 3,5,7-trimethyl ether	Flavonol	C18H16O5	2	[M+H]+	313.1070	312.0998
441	359.0179	Dihydroflavonol	3450	C02906	Dihydromyricetin	Dihydroflavonol	C15H12O8	4	[M+K]+	359.0164	320.0532
451	359.0179	Dihydroflavonol	3450	C02906	Dihydromyricetin	Dihydroflavonol	C15H12O8	4	[M+K]+	359.0164	320.0532
20	182.9888	-									
99	597.2158	Flavonone	52625	C09828	Pinocembrin 7-rhamnosylglucoside	Flavonone	C27H32O13	3	[M+CH3OH+H]+	597.2178	564.1843
233	543.3199	Alkaloid	68625	C10845	Belladonnine	Alkaloid	C34H42N2O4	3	[M+H]+	543.3217	542.3145
23	455.1525	Flavonone	53041	C17391	Sakuranin	Flavonone	C22H24O10	0	[M+Li]+	455.1530	448.1369
99	283.0909	Amino acid derivative	362	C01118	O-Succinyl-L-homoserine	Homoserine derivative	C8H13NO6	2	[M+ACN+Na]+	283.0901	219.0743
217	102.0307	Alkaloid	1457	C13747	1,7-Dimethylxanthine	Purine alkaloid	C7H8N4O2	0	[M+H+Na]2+	102.0306	180.0647
99	433.1499	Flavone	49925	C14942	Agecorynin C (Heptamethoxyflavone)	Flavone	C22H24O9	1	[M+H]+	433.1493	432.1420
			49916	C14953	5,6,7,8,3',4',5'-Heptamethoxyflavone	Flavone	C22H24O9	1	[M+H]+	433.1493	432.1420
4	359.0179	Dihydroflavonol	3450	C02906	Dihydromyricetin	Dihydroflavonol	C15H12O8	4	[M+K]+	359.0164	320.0532
398	136.0418	Amino acid	3256	C00155	L-Homocysteine	Amino acid	C4H9NO2S	6	[M+H]+	136.0427	135.0354
23	365.1392	Isoflavonoid	47589	C17765	Licoricone	Isoflavonoid	C22H22O6	0	[M+H-H2O]+	365.1389	382.1416
23	136.0825	-									
19	82.03185	-									

Table 5. 2. (continued …) Metabolomic features associated with the leaves of well-watered non-mycorrhizal (WW NM) barley plants (H*ordeum vulgare* cv. Concerto) at tillering (29DPI) and stem elongation (42DPI) stages. Chemical class annotations are based on comparison with METLIN, MassBank, KEGG and BarleyCyc databases. Mass error is given for annotations in ppm (parts per million).

						Database (METLIN) m/z matches					
RT (secs)	**Detected m/z**	**Putative class annotation**	**METLIN ID**	**KEGG ID**	**Compound**	**Chemical class**	**Formula**	**ppm**	**Ion**	**Reference m/z**	**Accurate mass**
Associated with sampling time point 3 (42DPI)											
88	611.2067	Alkaloid	68459	C10644	Atalanine	Acridone alkaloid	C34H30N2O9	7	[M+H]+	611.2024	610.1951
23	705.2383	Triterpenoid	67172	C08753	Bruceoside A	Triterpenoid	C32H42O16	2	[M+Na]+	705.2365	682.2473
117	787.2569	-									
349	311.2937	-									
88	757.2656	-									
23	543.1774	Cellulose	58599	C00760	1,4-beta-D-Glucan	Cellulose	C18H32O18	4	[M+Li]+	543.1749	536.1589
397	393.3384	-									
88	612.2108	Flavonone	70969	C15579	Flavanone 7-O-[alpha-L-rhamnosyl- (1->2)-beta-D-glucoside]	Flavonone	C27H32O12	9	[M+ACN+Na]+	612.2051	548.1894
23	118.1077	-									
117	788.2616	-									
92	581.1962	-									
386	550.6701	Fatty ester	63381	C16169	Docosahexaenoyl-CoA	Fatty ester	C43H66N7O17P3S	1	[M+H+Na]2+	550.6707	1077.3449
23	706.2356	-									
88	758.2818	-									
88	329.1004	Chlorophyll precursor	63924	C02880	Protochlorophyllide	Chlorophyll precursor	C35H32MgN4O5	0	[M+2Na]2+	329.1004	612.2223
349	505.3059	-									
88	593.1906	Alkaloid	68459	C10644	Atalanine	Acridone alkaloid	C34H30N2O9	3	[M+H]+	593.1924	610.1951
23	707.2451	-									
23	544.1749	-									
101	565.197	Isoprenoid	41175	C11663	Loganin pentaacetate	Isoprenoid	C27H36O15	7	[M+H-2H2O]+	565.1927	600.2054

Phenylpropanoids, cellulose and a chlorophyll precursor differentiate barley metabolomes at tillering and stem elongation stages

To investigate the effect of age/ growth stage at sampling time on the leaf metabolomic fingerprint of barley independently of drought and AM colonisation, raw data from NM WW plants sampled at 29DPI and 42DPI were resubmitted to the untargeted metabolomics workflow beginning with the peak picking step. Plants at 29DPI were at the tillering stage (Zadok's growth stage Z15.26) and by 42DPI were at the stem elongation stage (Zadok's growth stage Z18.28.31) (appendix IX).The PCA scores plot from this second analysis confirmed the separation observed between 29DPI and 42DPI in the first analysis (fig. 5.2.c). A directed model (OPLS-DA) was subsequently used to ascertain which features of the metabolome were responsible for the distinction between NM WW samples from these two growth stages (table 5.2).

The directed analysis indicated that metabolomes at the tillering stage (29DPI) were distinguished by accumulation of phenylpropanoids (flavonoids, lignans), alkaloids and amino acid derivatives. Accumulation of cellulose and protochlorophyllide (a chlorophyll precursor), as well as different flavonoids and alkaloids were associated with the metabolomes at the stem elongation stage (42DPI)).

Phenylpropanoids include coumarins, flavonoids, isoflavonoids, lignans and many other derivative compounds such as hydroxy-cinnamic acids. They are synthesised from phenylalanine and tyrosine via coumarate and trans-cinnamic acid. Phenylpropanoids are associated with antioxidant properties both for the plant and upon human consumption (Agati *et al.* 2012, Dwivedi *et al.* 2016).

Lee *et al.* 2016 used a more targeted mass-spectrometry approach than that presented in this chapter to identify ten phenolics (including flavonoids and their precursors) in barley seedlings and found that their accumulation was time dependent and that the pattern of accumulation varied between specific flavonoids (for example lutonarin accumulated early in seedling development and saponarin later during vegetative growth). The findings presented in this chapter are in line with reports of the highest accumulation of phenolic compounds at the tillering stage of development (Lee *et al.* 2016, Yan *et al.* 2022).

In the untargeted LC-ESI-MS analysis presented in this chapter, it is not possible to reliably distinguish specific flavonoids from their glycosides but the results strongly indicate detection of flavonones, flavonols and dihydroflavonols, as well as other phenylpropanoids, such as lignans. The analysis supports the hypothesis that specific flavonoid derivatives are accumulated at different stages of vegetative development and is in line with changes in

glycosylation patterns of flavonoids observed at different growth stages in barley (Brauch *et al.* 2019).

The untargeted analytic procedures employed here were sensitive enough to distinguish between the metabolic contents of the tip of the youngest expanded leaf from barley plants that were just two weeks apart in age.

Drought has a strong effect on the barley leaf metabolome

Amongst leaf metabolite samples collected at 42DPI, those from AM and NM plants overlapped but there was a tendency toward separation between droughted and well-watered plants in the first two principal components (fig. 5.3.a). This significant effect of drought, and lack of AMF effect, was confirmed in the PERMANOVA model (Drought: F_1=4.31, R^2=0.12, p=0.005; AMF: F_1=0.85, R^2=0.02, p=0.449; Drought*AMF: F_1=0.38, R^2=0.01, p=0.854).

In order to compare the metabolomic drought-response of AM and NM barley, the data from 42DPI were split into two subsets and analysed separately, using PCA and OPLSDA. Effects of drought stress on the leaf metabolome were only evident at the 18th day of drought (42DPI), while at the 5th and 11th days of drought (29 and 35DPI) there was no separation of WW and DS samples for either non-mycorrhizal (fig. 5.4) or mycorrhizal plants (fig. 5.5). This was confirmed by the significant interactive effect of sampling time and drought treatment in PERMANOVA models (NMonly Time*Drought: F_2=2.23, R^2=0.08, p=0.012; AMonly Time*Drought: F_2=2.11, R^2=0.05, p=0.016).

Only samples from the 18th day of drought (42DPI) were used for the subsequent analysis. In undirected PCA of metabolomic fingerprints from NM plants, samples from droughted and well-watered treatments could be clearly distinguished (fig. 5.3.c) (PERMANOVA F_1=7.16, R^2=0.39, p=0.001). This confirms results from chapter 3, which found a clear distinction between metabolomes of well-watered and droughted non-mycorrhizal barley plants at the same age using DI-ESI-MS.

In AM plants, leaf metabolomes of droughted and well-watered barley did not clearly separate, though well-watered samples clustered together (fig. 5.3.b) and a significant effect of drought was observed in the PERMANOVA model (F_1=4.43, R^2=0.19, p=0.01). A lack of clear distinction, in contrast to the distinction in the PCA scores plot between NMWW and NMDS plants, suggests drought did not have as strong an effect on the leaf metabolome of AM plants as it did on NM plants. This is congruent with the results from chapter 4, in which

AM plants maintained photosystem II efficiency (Fv'/Fm') for one day longer than NM plants, but showed few other differences in physiological drought response.

Phenylpropanoids, chlorophyll precursors, fatty acid derivatives, a dipeptide, sesquiterpenoids and terpenoids characterise the non-mycorrhizal plants' response to drought

Seventeen peaks were influential in characterising the response of the barley leaf metabolome to drought treatment but the majority (twelve) of these remain unannotated. In NM plants, a number of fatty acid derivatives, various sesquiterpenoids and phenylpropanoids were found to accumulate under DS treatment. The two peaks that were associated with DS in both AM and NM plants were a putative dipeptide (histidyl leucine) and an unannotated compound (m/z 98.9808). Twelve of the most influential peaks associated with WW treatment were consistent between the analyses of AM- and NM-response to drought, including a number of chlorophyll precursors, a lignan, flavonoids and triterpenoids (table 5.3).

Several previous studies have reported a strong effect of drought on the leaf metabolome of cereal species using DI-ESI-MS, LC-ESI-MS or GC-ESI-MS and tandem MS (Bowne *et al.* 2012, Marček *et al.* 2019). For example, a large study using 81 barley accessions including landraces and modern breeding lines, found that amino acids (aspartate, glutamate, and serine) were reduced by drought, as was starch, while the amino acid, proline, accumulated under both drought and heat stress for the majority of cultivars (Templer *et al.* 2017). In the results presented here, there was no evidence of proline or its precursors being associated with drought-stressed metabolomes.

Other untargeted and targeted metabolomics studies have highlighted the important role of phenolic compounds in response to drought stress. Flavonoids, hydroxycinnamic acids, other phenolics in the phenylpropanoid pathway, hordatines (polyamine derivatives) and their glycosides, and blumenol C derivatives (terpenoids) have previously been shown to be important in the barley leaf metabolomic response to drought (Chmielewska *et al.* 2016, Swarcewicz *et al.* 2017, Piasecka *et al.* 2017, Kowalczewski *et al.* 2020, Piasecka *et al.* 2020). Blumenol C derivatives are of particular interest here since they have also been implicated in the leaf metabolomic response to AM colonisation (Wang *et al.* 2018, Mahood *et al.* 2022). However, no evidence of blumenol C derivatives was observed in this study.

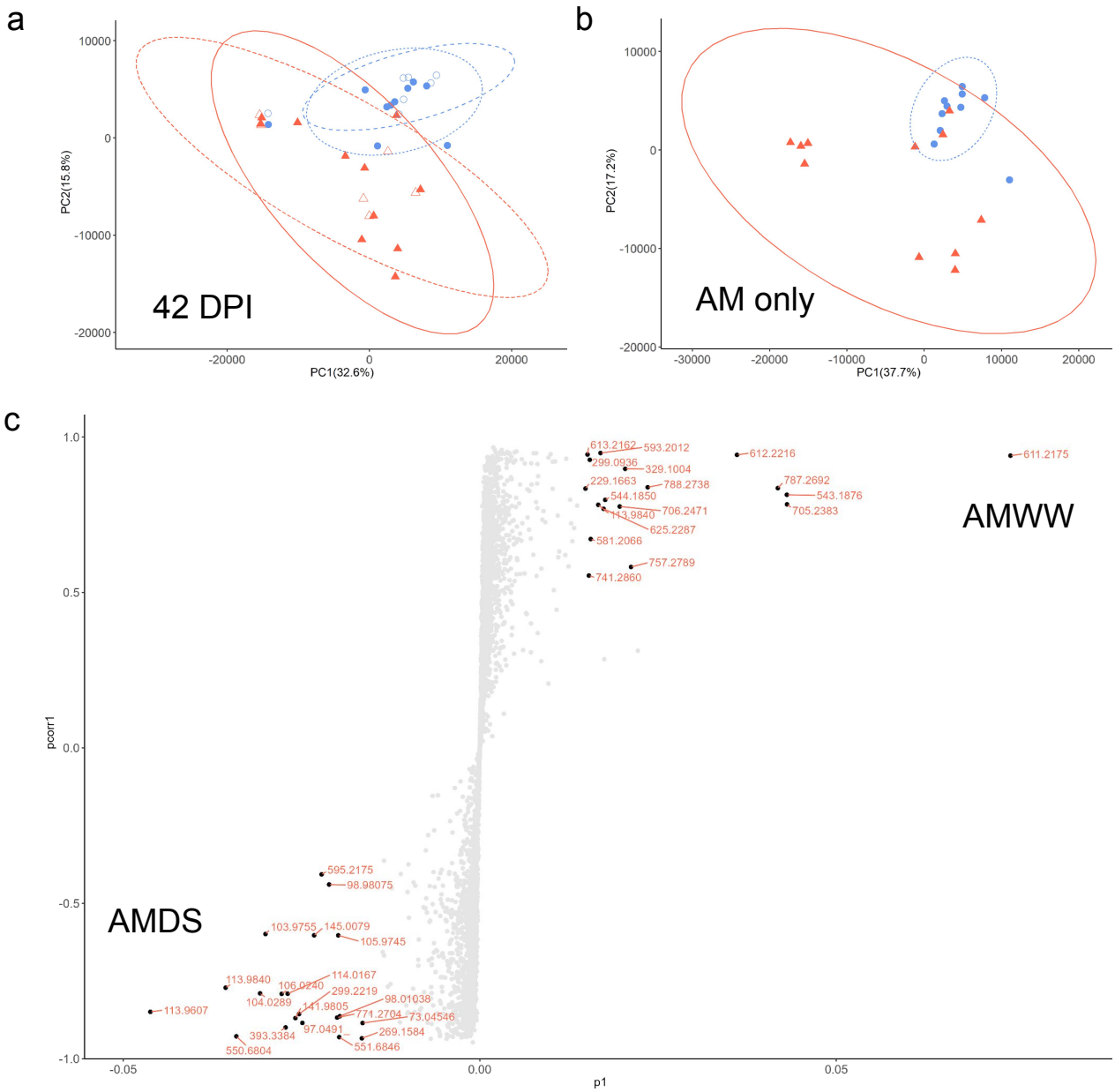

Fig. 5. 3. 1. Samples from well-watered plants clustered, as did those of drought-stressed plants and there was a tendency towards separation based on drought treatment (a). In AM plants, leaf metabolomes of droughted and well-watered did not clearly separate, though well-watered samples clustered together (b) at stem elongation stage. OPLS-DA was used to interrogate the m/z responsible for differences in the polar metabolomes of AMDS and AMWW plants. m/z considered for identification are highlighted in (c). Principal component analysis (PCA) scores plots for separate analyses of aqueous leaf extracts in positive mode with LC/MS of (a) all plants, and (b) NM plants only at 42DPI (18th day of drought period). PC1 and PC2 are shown for each. NMWW = blue open circles; AMWW = blue filled circles; NMDS = red open triangles; AMDS = red filled triangles. Ellipses represent the 95% confidence interval for a given treatment group. S plot (c) of p1 from OPLS-DA giving a measure of magnitude of the effect of a variable (m/z) on the model and pcorr1 giving a measure of confidence (extremes of x and y axis represent highest magnitude of effect with most confidence in that effect).

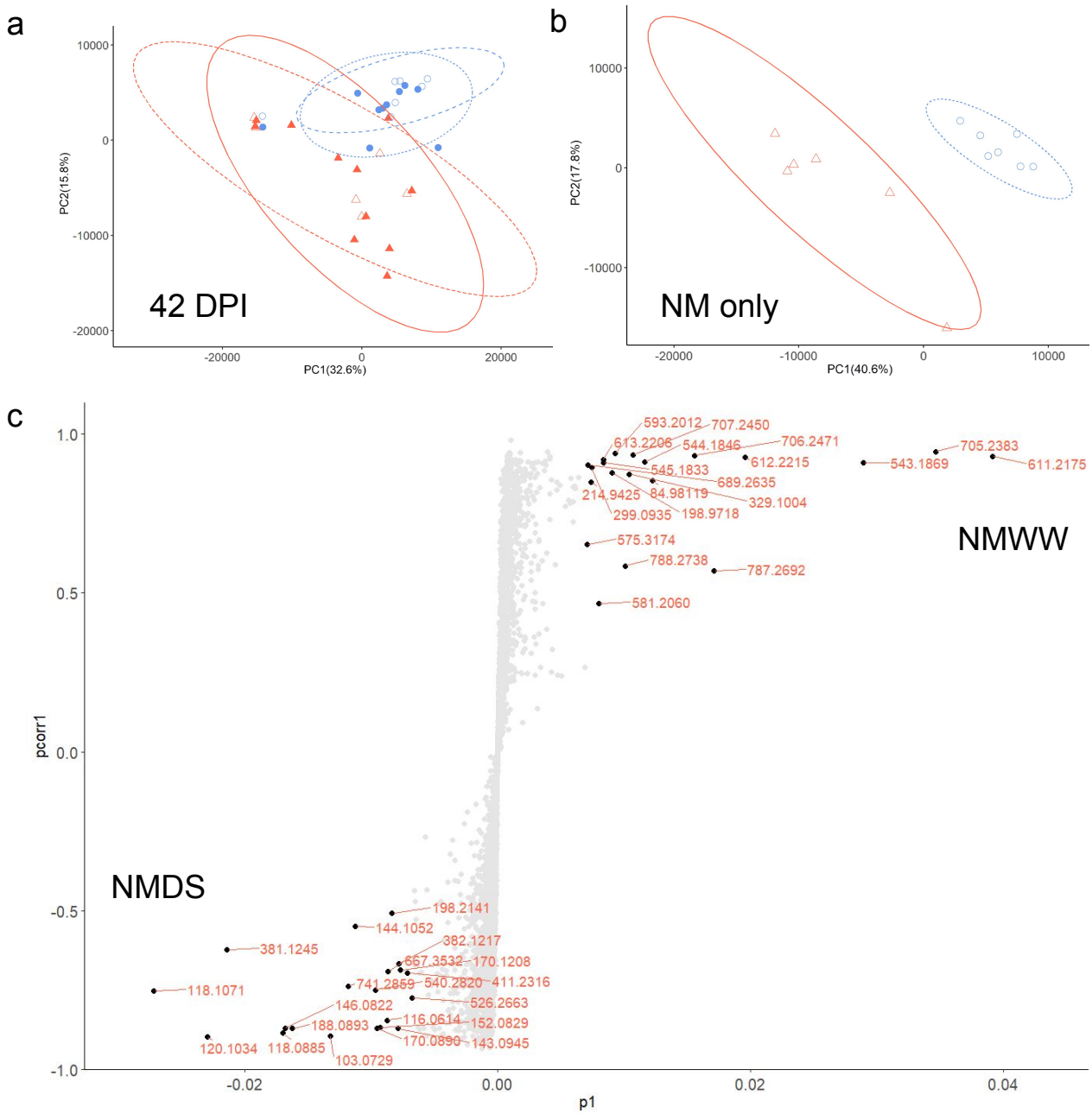

Fig. 5. 3. 2. Samples from well-watered plants clustered, as did those of drought-stressed plants and there was a tendency towards separation based on drought treatment (a). In NM plants, samples from droughted and well-watered plants could be clearly distinguished (b) at stem elongation stage. OPLS-DA was used to interrogate the m/z responsible for differences in the polar metabolomes of NMDS and NMWW plants. m/z considered for identification are highlighted in (c). Principal component analysis (PCA) scores plots for separate analyses of aqueous leaf extracts in positive mode with LC/MS of (a) all plants, and (b) NM plants only at 42DPI (18th day of drought period). PC1 and PC2 are shown for each. NMWW = blue open circles; AMWW = blue filled circles; NMDS = red open triangles; AMDS = red filled triangles. Ellipses represent the 95% confidence interval for a given treatment group. S plot (c) of p1 from OPLS-DA giving a measure of magnitude of the effect of a variable (m/z) on the model and pcorr1 giving a measure of confidence (extremes of x and y axis represent highest magnitude of effect with most confidence in that effect).

Table 5. 3. Metabolomic features implicated in the drought response of non-mycorrhizal (NM) and mycorrhizal (AM) barley plants (H*ordeum vulgare* cv. Concerto), and chemical class annotations based on comparison with METLIN, MassBank, KEGG and BarleyCyc databases. Mass error is given for annotations in ppm (parts per million).

				Database and literature m/z matches									
Also discriminated between AMDS and AMWW	**RT (mins)**	**Detected m/z**	**Putative class annotation**	**Database ID (METLIN or *MassBank)**	**KEGG ID**	**Compound**	**Common synonym**	**Chemical class**	**Formula**	**ppm**	**Ion**	**Reference m/z**	**Accurate mass**
	Associated with non-mycorrhizal well-watered treatment (NM WW) at 42 DPI												
x	18	84.9812	-										
x	20	198.9718	-										
x	19	214.9425	-										
✔	88	299.0935	Flavonoid	47617	C10199	Afrormosin		Isoflavonoid	C17H14O5	7	[M+H]+	299.0914	298.0841
				49456	C10019	Apigenin 7,4'-dimethyl ether		Flavone	C17H14O5	7	[M+H]+	299.0914	298.0841
				47574	C10527	Sayanedine		Isoflavonoid	C17H14O5	7	[M+H]+	299.0914	298.0841
				BML80380*		3-Hydroxy-3',4'-Dimethoxyflavone		Flavonoid	C17H14O5	7		299.0914	298.0841
				BML80350*		3',7-Dimethoxy-3-hydroxyflavone		Flavonoid	C17H14O5	7		299.0914	298.0841
				BML80400*		4',7-Dimethoxy-3-hydroxyflavone		Flavonoid	C17H14O5	7		299.0914	298.0841
✔	88	329.1004	Chlorophyll precursor	63924	C02880	Protochlorophyllide		Chlorophyll precursor	C35H32MgN4O5	0	[M+2Na]2+	329.1004	612.2223
✔	23	543.1869	Lignan	71761	C17529	(-)-Pinoresinol glucoside		Lignan glycoside	C26H32O11	6	[M+Na]+	543.1837	520.1945
✔	23	544.1846	-										
x	23	545.1833	Triterpenoid	65618	C01593	Limonoate		Triterpenoid	C26H34O10	9	[M+K]+	545.1784	506.2152
✔	92	581.2060	-										
✔	88	593.2012	Chlorophyll precursor	63923	C11831	Divinyl protochlorophyllide		Chlorophyll precursor	C35H30MgN4O5	4	[M+H-H2O]+	593.2039	610.2067
✔	88	611.2175	Chlorophyll precursor	63974	C16541	Chlorophyllide B		Chlorophyll precursor	C35H32MgN4O6	4	[M+H-H2O]+	611.2145	628.2172
✔	88	612.2215	Flavonoid	PR020045*	C09806	Neohesperidin	Hesperetin 7-O-neohesperidoside	Flavonone	C28H34O15	14		612.2303	610.1898
x	88	613.2206	Flavonoid	PR020039*	C09755	Neohesperidin dihydrochalcone	Dihydrohesperetin-7-O-neohesperidoside		C28H36O15	3		613.2222	612.2054
x	24	689.2635	Triterpenoid saponin (glycoside)	67172	C08753	Bruceoside A		Triterpenoid	C32H42O16	0	[M+Li]+	689.2633	682.2473
✔	23	705.2383	Triterpenoid saponin (glycoside)	67172	C08753	Bruceoside A		Triterpenoid	C32H42O16	3	[M+Na]+	705.2365	682.2473
✔	23	706.2471	-										
x	23	707.2450	-										
✔	116	787.2692	Cinnamic acid derivative	68333	C10450	Echinacoside		Cinnamic acid derivative	C35H46O20	4	[M+H]+	787.2655	786.2582

Table 5. 3. (continued …) Metabolomic features implicated in the drought response of non-mycorrhizal (NM) and mycorrhizal (AM) barley plants (H*ordeum vulgare* cv. Concerto), and chemical class annotations based on comparison with METLIN, MassBank, KEGG and BarleyCyc databases. Mass error is given for annotations in ppm (parts per million).

				Database and literature m/z matches									
Also discriminated between AMDS and AMWW	**RT (mins)**	**Detected m/z**	**Putative class annotation**	**Database ID (METLIN or *MassBank)**	**KEGG ID**	**Compound**	**Common synonym**	**Chemical class**	**Formula**	**ppm**	**Ion**	**Reference m/z**	**Accurate mass**
				71857	C17679	Magnoloside B		Cinnamic acid derivative	C35H46O20	4	[M+H]+	787.2655	786.2582
				68359	C10488	Purpureaside C		Cinnamic acid derivative	C35H46O20	4	[M+H]+	787.2655	786.2582
✔	116	788.2738	-										
	Associated with non-mycorrhizal drought treatment (NM DS) at 42 DPI												
✔	342	98.9808	-										
x	53	103.0729	Fatty aldehyde	36528	C19249	Sorbaldehyde		Fatty aldehyde	C6H8O	6	[M+Li]+	103.0735	96.0575
x	23	116.0614	-										
x	71	118.0885	Sesquiterpenoid	73288	C19678	Germacrene A acid		Sesquiterpenoid	C15H22O20	1	[M+2H]2+	118.0883	234.1620
x	23	118.1071	-										
x	53	120.1034	Sesquiterpenoid	67853	C09632	Centarol		Sesquiterpenoid	C15H26O2	1	[M+2H]2+	120.1039	238.1933
				67860	C09652	Daucol		Sesquiterpenoid	C15H26O2	1	[M+2H]2+	120.1039	238.1933
				67861	C09653	Debneyol		Sesquiterpenoid	C15H26O2	1	[M+2H]2+	120.1039	238.1933
				67908	C09722	Seiricardine A		Sesquiterpenoid	C15H26O2	1	[M+2H]2+	120.1039	238.1933
				71683	C17427	Kanokonol		Sesquiterpenoid	C15H26O2	1	[M+2H]2+	120.1039	238.1933
x	71	144.1052	-										
x	69	146.0822	Amino fatty acid derivative	44719	C02946	4-acetamidobutanoate		Amino fatty acid derivative	C6H11NO3	7	[M+H]+	146.0812	145.0739
				63455	C04076	L-2-Aminoadipate 6-semialdehyde	L-Allysine	Amino fatty acid derivative	C6H11NO3	7	[M+H]+	146.0812	145.0739
x	32	152.0829	Phenylpropanoid	73438	C19934	Prosolanapyrone III		Pyrone	C18H22O4	1	[M+2H]2+	152.0832	302.1518
				62903	C10719	Nordihydroguaiaretic acid		Lignan	C18H22O4	1	[M+2H]2+	152.0832	302.1518
x	69	170.0890	-										
x	68	188.0893	Amino fatty acid derivative	66023	C03682	6-Acetamido-3-oxohexanoate		Amino fatty acid derivative	C8H13NO4	12	[M+H]+	188.0917	187.0845
				63444	C12988	N2-Acetyl-L-aminoadipate semialdehyde		Amino fatty acid derivative	C8H13NO4	12	[M+H]+	188.0917	187.0845
x	394	198.2141	-										
✔	381	269.1584	Dipeptide	66283	C05010	Histidylleucine	His-Leu	Dipeptide	C12H20N4O3	8	[M+H]+	269.1608	268.1535
x	23	381.1245											
x	23	382.1217	-										
x	166	411.2316	Terpenoid	53589	C09076	Forskolin	Coleonol	Diterpenoid	C22H34O7	14	[M+H]+	411.2377	410.2305
				67190	C08772	Nigakihemiacetal A		Triterpenoid	C22H34O7	14	[M+H]+	411.2377	410.2305
x	144	540.2820	-	838	C09420	Ematamine	Ipecac	Isoquinoline alkaloid	C29H36N2O4	2	[M+ACN+Na]+	540.2833	476.2675

Table 5. 3. (continued …) Metabolomic features implicated in the drought response of non-mycorrhizal (NM) and mycorrhizal (AM) barley plants (H*ordeum vulgare* cv. Concerto), and chemical class annotations based on comparison with METLIN, MassBank, KEGG and BarleyCyc databases. Mass error is given for annotations in ppm (parts per million).

				Database and literature m/z matches									
Also discriminated between AMDS and AMWW	**RT (mins)**	**Detected m/z**	**Putative class annotation**	**Database ID (METLIN or *MassBank)**	**KEGG ID**	**Compound**	**Common synonym**	**Chemical class**	**Formula**	**ppm**	**Ion**	**Reference m/z**	**Accurate mass**
✔	386	550.6804	-										
x	135	667.3533	-										
✔ **but associated with WW**	100	741.2859	Flavonone	PR020051*	C09830	Poncirin	Isosakuranetin -7-O-neohesperidoside	Flavonone	C28H34O14	0		741.2859	594.1949
	Additional features associated with mycorrhizal well-watered treatment (AM WW) at 42 DPI												
	89	757.2789	-										
	35	113.9840	-										
	88	613.2162	Flavonoid	PR020039*	C09755	Neohesperidin dihydrochalcone	Dihydrohesperetin -7-O-neohesperidoside		C28H36O15	10		613.2222	612.2054
	84	229.1663	-										
	Additional features associated with mycorrhizal drought treatment (AM DS) at 42 DPI												
	25	113.9607	-										
	10	104.0289	-										
	5	103.9755	-										
	9	106.0240	Amine	3207	C00189	Ethanolamine	Colamine, glycinol	Amine	C2H7NO	0	[M+2Na-H]+	106.0239	61.0528
	397	393.3384	-										
	17	114.0167	-										
	17	141.9805	-										
	255	299.2219	Jasmonic acid precursor	63360	C01226	12OPDA	12- oxophytodienoic acid	Fatty acyl	C18H28O3	6	[M+Li]+	299.2198	292.2038
	24	97.0491	Hydroxy carboxylic acid	35392	C01013	β-lactic acid	3-Hydroxypropanoate	Hydroxycarboxylic acid	C3H6O3	14	[M+Li]+	97.0477	90.0317
				63094	C00256	D-lactate	D-lactic acid	Hydroxycarboxylic acid	C3H6O3	14	[M+Li]+	97.0477	90.0317
				45858	C00186	L-lactate	L-lactic acid	Hydroxycarboxylic acid	C3H6O3	14	[M+Li]+	97.0477	90.0317
	5	145.0079	-										
	118	595.2175	Chlorophyll precursor	63924	C02880	Protochlorophyllide		Chlorophyll precursor	C35H32MgN4O5	3	[M+H-H2O]+	595.2196	612.2223
	147	771.2704	-										
	5	105.9745	-										
	386	551.6846	-										

Table 5. 3. (continued …) Metabolomic features implicated in the drought response of non-mycorrhizal (NM) and mycorrhizal (AM) barley plants (H*ordeum vulgare* cv. Concerto), and chemical class annotations based on comparison with METLIN, MassBank, KEGG and BarleyCyc databases. Mass error is given for annotations in ppm (parts per million).

				Database and literature m/z matches									
Also discriminated between AMDS and AMWW	**RT (mins)**	**Detected m/z**	**Putative class annotation**	**Database ID (METLIN or *MassBank)**	**KEGG ID**	**Compound**	**Common synonym**	**Chemical class**	**Formula**	**ppm**	**Ion**	**Reference m/z**	**Accurate mass**
	19	98.0104	-										
	23	73.0455	-										

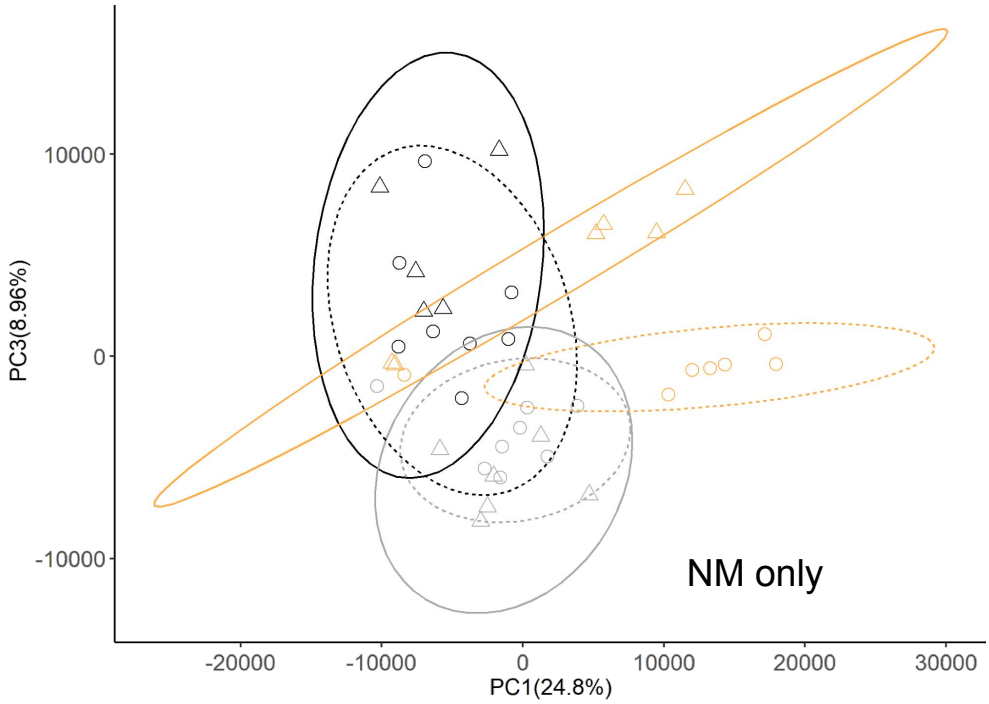

Fig. 5. 4. Separation between leaf metabolomic fingerprints of DS (drought stressed) and WW (well-watered) plants was observed at 18th day of drought period in NM plants along PC1 and PC3 but not before that. Principal component analysis (PCA) scores plot for LC/MS of aqueous extracts from NM plants in positive mode showing PC1 and PC3. Black = 29DPI/ 5th day of drought period; grey = 35DPI/ 11th day of drought period; gold = 42DPI/ 18th day of drought. NMWW = open circles; NMDS = open triangles. Ellipses represent the 95% confidence interval for a given treatment group.

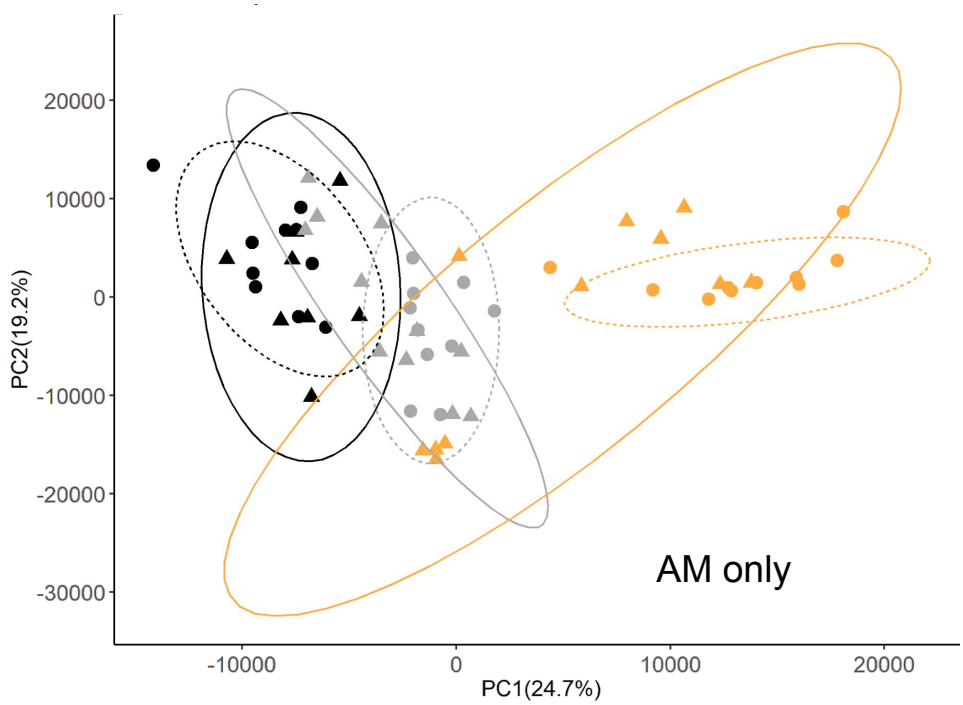

Fig. 5. 5. A tendency towards separation between leaf metabolomic fingerprints of DS (drought stressed) and WW (well-watered) plants was observed at 11th and 18th day of drought period in AM plants, with DS samples closer to earlier sampling time points than WW plants. Principal component analysis (PCA) scores plot for LC/MS of aqueous extracts from NM plants in positive mode showing PC1 and PC2. Black = 29DPI/ 5th day of drought period; grey = 35DPI/ 11th day of drought period; gold = 42DPI/ 18th day of drought. NMWW = open circles; NMDS = open triangles. Ellipses represent the 95% confidence interval for a given treatment group.

Many of these compounds act as a secondary antioxidant defence system for plants under stresses that reduce the activity of detoxifying antioxidant enzymes in the chloroplast (Agati *et al.* 2012) with glycosylation representing a dynamic and fast way for plants to translocate antioxidants within and between cells, and thus respond to oxidative stress induced by environmental conditions such as drought (Kumar & Pandey 2013). Increases in flavonoid glycosides and their acylated forms were found in both Piasecka *et al.* 2017 and 2020 to be some of the biggest changes in phenolics under drought stress.

Flavonoids and their glycosides, cinnamic acid derivatives, and triterpenoids were putative annotations for a number of peaks associated with both well-watered and drought-stressed plants. Flavonoids are a structural class that has been found to be broadly co-regulated in response to environmental stress in *Brachypodium distachyon* (Mahood *et al.* 2022) but the authors note that structural class does not seem to be a good indicator of whether metabolites are co-regulated. Reliable identification of specific flavonoids by LC-ESI-MS is challenging, particularly without the use of tandem mass spectrometry, but the results presented here support the hypothesis that some phenolic compounds such as flavonoids and their glycosylation patterns are altered by drought stress in barley.

One metabolomic feature, putatively annotated as a flavonone, was found to be associated with DS in NM plants but conversely associated with WW treatment in AM plants. However, it remains possible that the true identity of the compound responsible for this feature is different in the two different treatments - many flavonones and their glycosides share structural similarities that result in similar spectral patterns and more confident identification of these compounds requires additional MS techniques (Brauch *et al.* 2019).

Ethanolamine, 12-OPDA, lactic acid and a chlorophyll precursor additionally characterise the mycorrhizal plants' response to drought

The majority of peaks associated with DS treatment at 42DPI were different between AM and NM plants (table 5.3). This suggests that the AM plant response to drought stress is subtly different to that of NM plants, that AM plants do not sense or experience the drought stress in the same way as NM plants, or that AM colonisation delays the damage caused by drought stress.

The metabolomic drought response of AM plants differed to that of NM plants in that a putative flavonoid was associated with AM WW leaves and the putatively annotated ethanolamine, 12-oxophytodienoic acid (12-OPDA), (β-, L- or D-) lactate, and protochlorophyllide were associated with AM DS leaves.

Ethanolamine (m/z 106.0240)

Ethanolamine is an important precursor in glycerophospholipid biosynthesis and thus has a role in building biological membranes (compound **1** in fig. 5.6). Studies in hulled and hulless barley have found accumulation of glycerophospholipids in leaves in response to drought stress (Chmielewska *et al.* 2016, Yuan *et al.* 2018). Ethanolamine is also an early precursor to glycine betaine, an important compatible solute in barley (Ladyman *et al.* 1980). In the present study, an association of ethanolamine with AM DS plants could represent a reduced need to convert ethanolamine to glycerophospholipids compared to in AM WW plants, or alternatively, an increase in ethanolamine in preparation for (re)building membranes or synthesising glycine betaine (GB). Ethanolamine has been shown to be upregulated by mycorrhizal colonisation in *Medicago lupulina* leaves at the flowering initiation stage (Yurkov *et al.* 2021).

12-Oxophytodienoic acid (m/z 299.2219)

12-Oxophytodienoic acid (12-OPDA) is a precursor to jasmonic acid (JA), a phytohormone best known for its involvement in plant responses to wounding and other biotic stresses (Salvi *et al.* 2021) (compound **2** in fig. 5.6). However 12-OPDA and JA are also implicated in plant drought stress response. In *Arabidopsis*, drought has been shown to result in accumulation of 12-OPDA without accumulation of JA, while wounding results in the accumulation of both (Savchenko *et al.* 2014). *Arabidopsis* lines with higher 12-OPDA levels have reduced stomatal aperture and better tolerance to drought, and 12-OPDA is demonstrated to be a stronger antitranspirant than JA-Ile (a bioactive form of JA) (Savchenko *et al.* 2014). In experiments with grafted tomato transgenic lines, a role for JAs in long distance signalling of soil drying has been proposed (de Ollas *et al.* 2018). During periods when carbon fixation is limited due to drought, biosynthesis of 12-OPDA and JA from oxidised membrane lipids can help dissipate the excess energy harvested by chlorophyll and reduce further membrane damage (Salvi *et al.* 2021). As such there are a range of potential mechanisms by which an accumulation of 12-OPDA could ameliorate the negative effects of drought in AM plants.

The results presented in this chapter support the involvement of 12-OPDA and the oxylipin pathway in the response of mycorrhizal plants to drought in leaves. In *Medicago truncatula* the upregulation of JA and ABA biosynthesis related genes as a result of mycorrhizal colonisation, as well as *MYC2* which coordinates JA-dependent responses, are associated with accumulation of flavonoids and anthocyanins in the metabolome under non-stress conditions (Adolfsson *et al.* 2017). Other studies have found involvement of jasmonates in

drought response, for example, AM colonisation further increased sap JA in droughted plants, while no increase in sap JA under well-watered conditions was observed (Quiroga *et al.* 2018). In *Digitaria eriantha* (digitgrass), AM colonisation was found to increase the concentrations of 12-OPDA under non-stress, drought stress, and salinity stress, but not under cold stress conditions, with other jasmonic acids and derivatives showing different patterns of accumulation (Pedranzani *et al.* 2016).

Lactate (m/z 97.0491)

There is currently a lack of consensus on the effect of lactate in plant tissues but it has been found to inhibit seedling development in Arabidopsis, and is thought to cause acidification that can damage membranes (Wienstroer *et al.* 2012). Lactate is the product of methylglyoxal detoxification (compound **3** in fig. 5.7), which is itself the byproduct of many metabolic reactions and has been found to accumulate during stress causing disruption to cellular homeostasis (Jain *et al.* 2020). In the current study, lactate was associated with AM DS plants in contrast to AM WW plants, suggesting lactate may be a marker of stress for mycorrhizal plants. Alternatively the lack of this accumulation of lactic acid in NM plants could suggest that mycorrhizal colonisation alters the way that plants maintain cellular homeostasis.

Under well-watered conditions, AM colonisation only causes small alterations to the leaf metabolomic fingerprint

Combining physiological measurements with leaf metabolomics is thought to be important for understanding the interaction between plants and their arbuscular mycorrhizal symbionts, particularly as the leaf metabolome gives insight into non-nutritional effects of AM symbiosis for the plant and can be separated from the fungus' own metabolome, which is not possible when studying the root metabolome (Toussaint *et al.* 2007, Rivero *et al.* 2015).

Since, in chapter 4, the root biomass of AM plants in the WW treatment was found to be significantly lower than that of NM plants, a pairwise comparison of the leaf metabolomic fingerprints of these two sets of samples was undertaken. In an undirected PCA, there was fairly tight clustering of NM WW plants along PC3 which explained 10.3% of the variance in the PCA model (fig. 5.8). However, there was no separation between AM WW and NM WW plants in the well-watered treatment in any combination of the first 5PCs (not shown). This suggests that there is little effect of AM colonisation on the leaf metabolome of barley cv. Concerto under well-watered conditions. However, since a difference in root biomass had been observed, a supervised analysis was undertaken (using OPLS-DA) to discriminate between the two groups of metabolomic fingerprints and highlight any subtle differences.

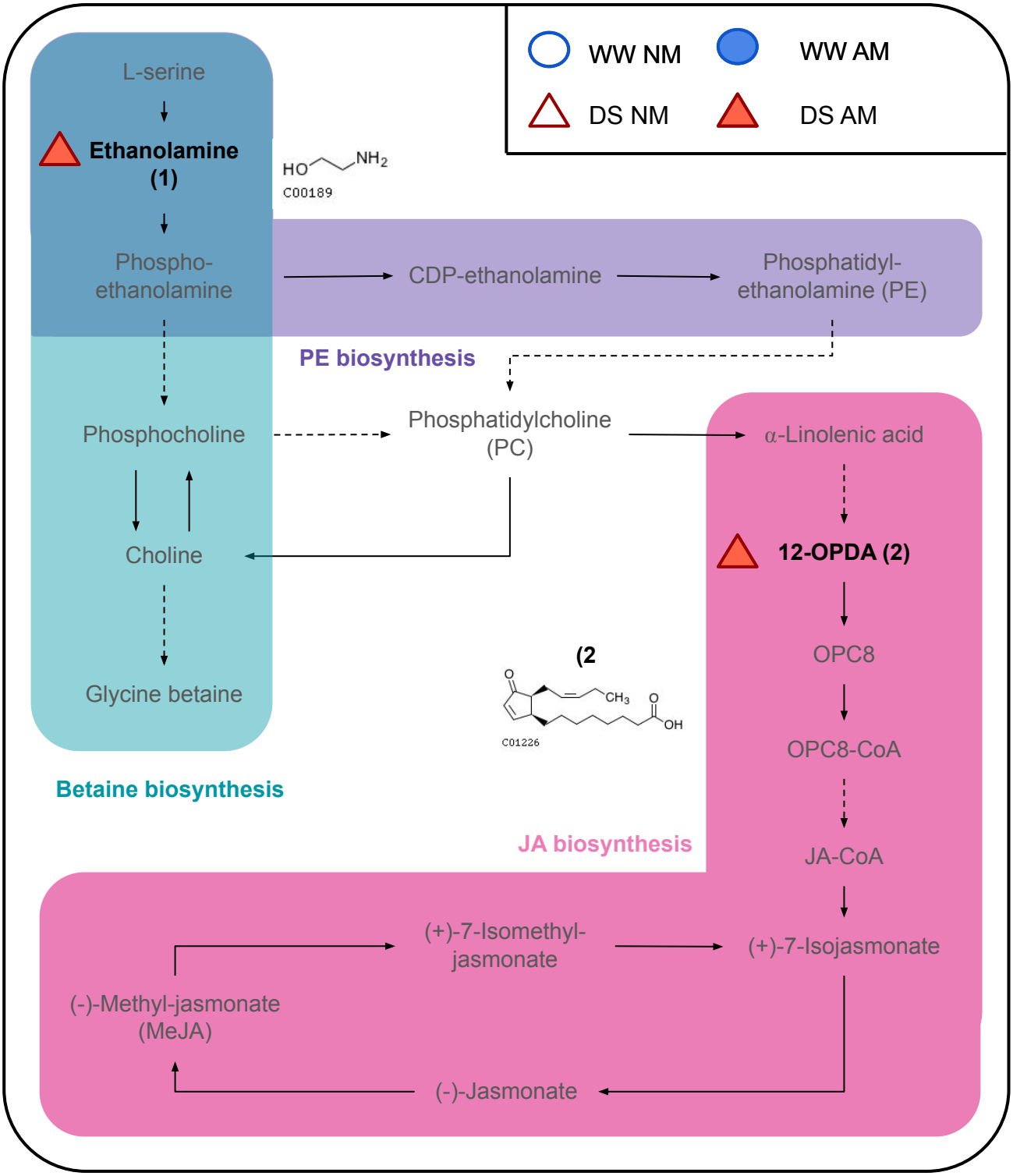

Fig. 5. 6. Ethanolamine (**1**) and 12-oxophytodienoic acid (12-OPDA) (**2**) are important in distinguishing the metabolic fingerprints of AM and NM barley plants subjected to drought stress (DS). Ethanolamine (**1**) is involved in phospholipid biosynthesis (such as PE and PC) and is also an early precursor of the compatible solute glycine betaine. 12-OPDA (**2**) is an antitranspirant and is also a precursor in the biosynthesis of jasmonic acids (JA) shown to be involved in drought- and mycorrhizal colonisation- response (Salvi *et al.* 2021, Quiroga et al. 2018).

Simplified biosynthesis pathways adapted from KEGG PATHWAY and MetaCyc. Chemical structures and KEGG IDs from KEGG COMPOUND. Solid arrows represent single step in the pathway, dashed arrows represent multiple steps in the pathway.

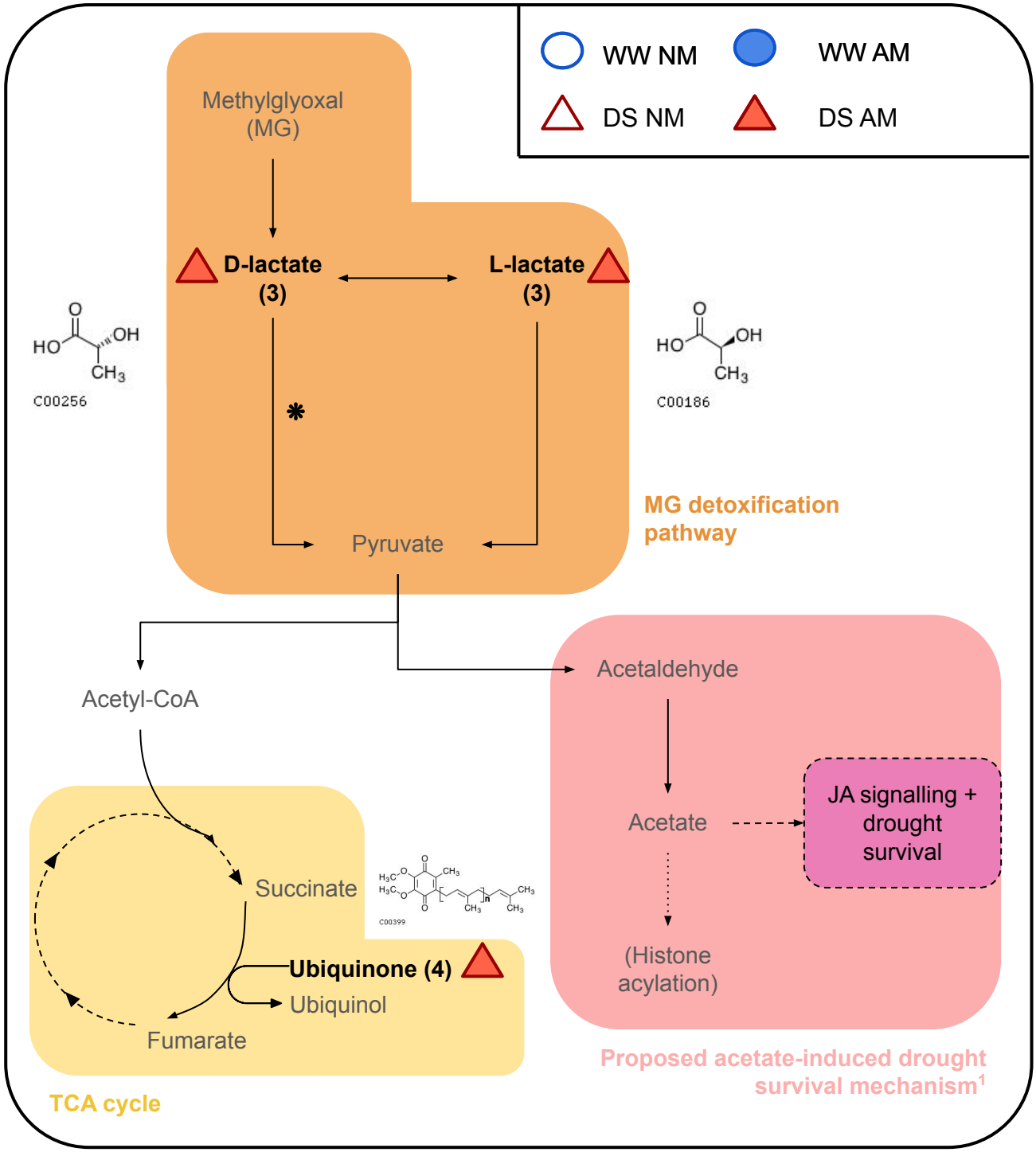

Fig. 5. 7. Methylglyoxal (MG) detoxification produces D-lactate (**3**), which (along with β-lactate and L-lactate) was a putative identity of a metabolomic feature associated with DS AM barley leaves. D-lactate dehydrogenase (D-LDH) improves tolerance to abiotic stresses by catalyzing the second stage (✱) of the MG detoxification pathway (Jain *et al.* 2020) resulting in pyruvate which has many metabolic fates. Kim *et al.* 2017 have proposed that under drought stress, a metabolic switch occurs from using pyruvate to feed into the tricarboxylic acid (TCA) cycle to acetate biosynthesis, with induction of jasmonic acid (JA) signalling improving drought survival (Rasheed *et al.* 2018).

Simplified pathways adapted from KEGG PATHWAY, MetaCyc and [1]Kim *et al.* 2017. Chemical structures and KEGG IDs from KEGG COMPOUND. Solid arrows represent single step in the pathway, dashed arrows represent multiple steps in the pathway.

Ten peaks were found to have a reliably strong association with NM WW samples in the OPLS-DA model, however seven of these remain unannotated. Three features associated with NM WW plants were all putatively annotated as neohesperidin or neohesperidin dihydrochalcone (table 5.4) and while neohesperidin, being a flavonone glycoside from citrus (metacyc DB) is not reported to be found in the leaves of barley (e.g. Piasecka *et al.* 2015, Hamany-Djande *et al*. 2021), its recurrent appearance among annotations here suggests the importance of a flavonone glycoside in differentiating AM from NM plants under well-watered conditions. A different flavonoid (dihydromyricetin) and a potential lignan were among the annotations of peaks that associated reliably with AM plants in the WW treatment (table 5.4).

Results presented in table 5.4 provide some tentative evidence that the phenylpropanoid pathway in the leaves of barley may be affected by AM colonisation under well-watered conditions. Colonisation by arbuscular mycorrhizal fungi has previously been shown to cause alterations both locally in the root metabolomic fingerprint of ragwort and sorghum (Hill *et al.* 2018, Kaur *et al*. 2022), as well as in the leaf metabolomic fingerprint of *Medicago truncatula* (Adolfsson *et al*. 2017). However, it is important in the context of the current work to note that responses vary dramatically between different plant and AMF symbiont combinations: Schweiger *et al.* 2014 found that while 18-45% of the leaf polar metabolome is conserved between five plant species (*Plantago lanceolata, P. major, Veronica chamaedrys, Medicago truncatula, Poa annua*), each species showed different patterns of metabolite modulation by AM colonisation.

Indeed, reviews in the field have cautioned repeatedly against extrapolation of metabolomic responses to AM colonisation between species and contexts (Schweiger & Müller 2015, Kaur & Suseela 2020). Moreover, in common with the results reported here, some untargeted metabolomics studies have found little to no modulation of the leaf metabolome resulting from mycorrhizal colonisation, for example, Hill *et al.* 2018 found no evidence of AM effect on shoot polar and non-polar metabolites in ragwort, using a very similar methodology to that presented here. In their research, Hill *et al.* 2018 did, however, find evidence of alterations to root metabolomic fingerprints, specifically an increase in blumenol C glycosides and hydroxyblumenol C glycosides. Wang *et al.* 2018 have since shown modulation of blumenol C derivatives in above ground tissues in a number of plant species, including barley, and have gone as far as to propose a method for using these compounds as biomarkers for arbuscular mycorrhizal colonisation (Mindt *et al.* 2019). A subsequent study has since found upregulation of blumenol C glycosides in the leaves of *Brachypodium distachyon* following AM colonisation (Mahood *et al.* 2022). No evidence of such a biomarker was found in the study presented in this chapter, which is perhaps unsurprising given the specific (targeted) protocol required to quantify these blumenol C glycosides.

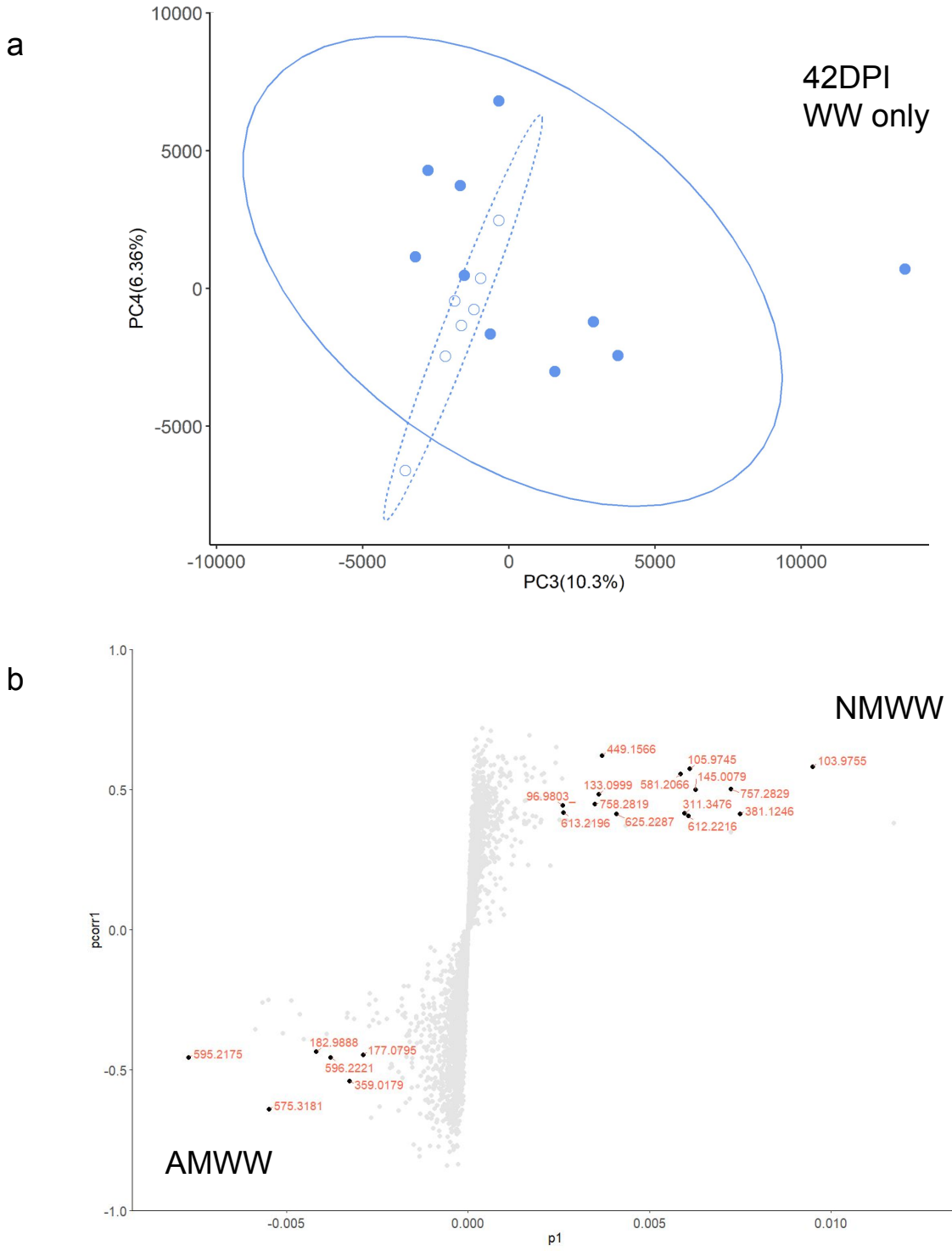

Fig. 5. 8. No strong separation between leaf metabolomic fingerprints of AM (mycorrhizal) and NM (non-mycorrhizal) plants, but some clustering, was observed under well-watered conditions at 18th day of drought period (a).These two treatment groups differed in their root biomass with AM plants having smaller root systems at 42DPI and so OPLS-DA was performed to interrogate the m/z responsible for any differences in the polar metabolomes of WWAM and WWNM plants. m/z considered for identification are highlighted in (b). Principal component analysis (PCA) scores plot for LC/MS of aqueous extracts from plants in the DS (drought stress) treatment in positive mode showing PC3 and PC4. NMWW = blue open circles; AMWW = blue filled circles. Ellipses represent the 95% confidence interval for a given treatment group. S plot (b) of p1 from OPLS-DA giving a measure of magnitude of the effect of a variable (m/z) on the model and pcorr1 giving a measure of confidence (extremes of x and y axis represent highest magnitude of effect with most confidence in that effect).

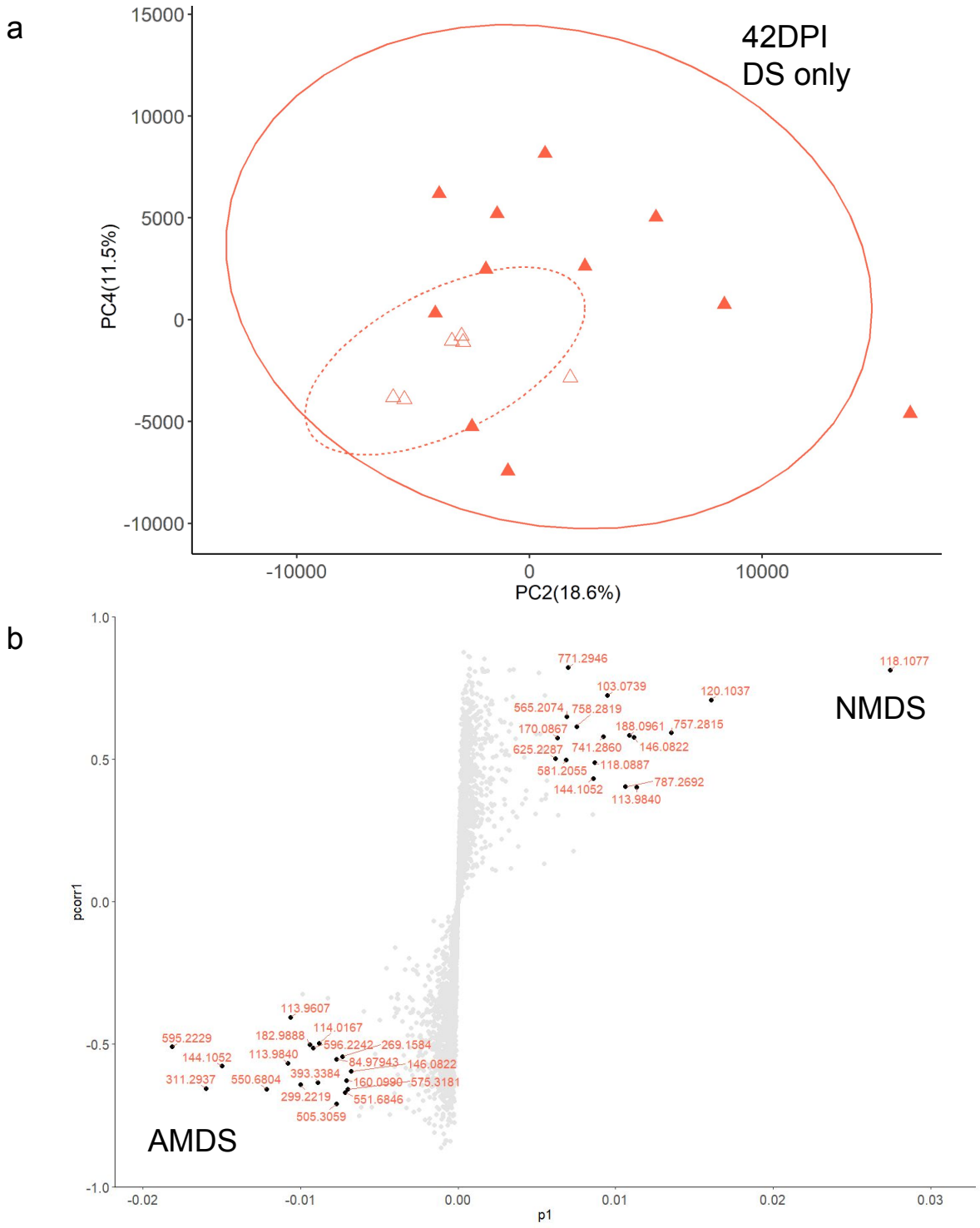

Fig. 5. 9. No strong separation between leaf metabolomic fingerprints of AM (mycorrhizal) and NM (non-mycorrhizal) plants, but some clustering, was observed under drought conditions at 18th day of drought period.These two treatment groups differed in their efficiency of photosystem II with AM plants having a significantly higher Fv'/Fm' and so OPLS-DA was performed to interrogate the m/z responsible for any differences in the polar metabolomes of DSAM and DSNM plants. m/z considered for identification are highlighted in (b). Principal component analysis (PCA) scores plot for LC/MS of aqueous extracts from plants in the DS (drought stress) treatment in positive mode showing PC2 and PC4. NMDS = red open triangles; AMDS = red filled triangles. Ellipses represent the 95% confidence interval for a given treatment group. S plot (b) of p1 from OPLS-DA giving a measure of magnitude of the effect of a variable (m/z) on the model and pcorr1 giving a measure of confidence (extremes of x and y axis represent highest magnitude of effect with most confidence in that effect).

Table 5. 4. Metabolomic features implicated in the response of well-watered (WW) and drought stressed (DS) barley plants (H*ordeum vulgare* cv. Concerto) to mycorrhizal colonisation (AM = colonised, NM = uncolonised). Chemical class annotations based on comparison with METLIN, MassBank, KEGG and BarleyCyc databases. Mass error is given for annotations in ppm (parts per million).

				Database (METLIN) m/z matches									
Also discriminated between AM WW and NM WW	**RT (secs)**	**Detected m/z**	**Putative class annotation**	**Database ID (METLIN or *MassBank)**	**KEGG ID**	**Compound**	**Common synonym**	**Chemical class**	**Formula**	**ppm**	**Ion**	**Reference m/z**	**Accurate mass**
	Associated with non-mycorrhizal drought-stressed treatment (NM DS) at 42 DPI												
x	23	118.1077	-										
x	53	120.1037	Sesquiterpenoid	67853	C09632	Centarol		Sesquiterpenoid	C15H26O2	1	[M+2H]2+	120.1039	238.1933
				67860	C09652	Daucol		Sesquiterpenoid	C15H26O2	1	[M+2H]2+	120.1039	238.1933
				67861	C09653	Debneyol		Sesquiterpenoid	C15H26O2	1	[M+2H]2+	120.1039	238.1933
				67908	C09722	Seiricardine A		Sesquiterpenoid	C15H26O2	1	[M+2H]2+	120.1039	238.1933
				71683	C17427	Kanokonol		Sesquiterpenoid	C15H26O2	1	[M+2H]2+	120.1039	238.1933
✔	89	757.2815	Flavonoid	PR020045*	C09806	Neohesperidin	Hesperetin 7-O-neohesperidoside	Flavonone	C28H34O15	3		757.2792	610.1898
x	17	113.984	-										
x	68	188.0961	-										
x	117	787.2692	Cinnamic acid derivative	68333	C10450	Echinacoside		Cinnamic acid derivative	C35H46O20	4	[M+H]+	787.2655	786.2582
				71857	C17679	Magnoloside B		Cinnamic acid derivative	C35H46O20	4	[M+H]+	787.2655	786.2582
				68359	C10488	Purpureaside C		Cinnamic acid derivative	C35H46O20	4	[M+H]+	787.2655	786.2582
x	52	103.0739	Fatty aldehyde	36528	C19249	Sorbaldehyde		Fatty aldehyde	C6H8O	3	[M+Li]+	103.0735	96.0575
x	100	741.286	Flavonone	PR020051*	C09830	Poncirin	Isosakuranetin-7-O-neohesperidoside	Flavonone	C28H34O14	0		741.2859	594.1949
x	68	118.0887	Sesquiterpenoid	73288	C19678	Germacrene A acid		Sesquiterpenoid	C15H22O20	3	[M+2H]2+	118.0883	234.1620
x	66	144.1052	-										
✔	89	758.2819	Polyamine-hydroxycinnamic acid	64189	C18073	N1,N5-Tri-di (hydroxyferuloyl)-N10-sinapoyl-spermidine		Spermidine hydroxycinnamic aicd	C38H45N3O12	10	[M+Na]+	758.2895	735.3003
x	107	771.2946	-										
x	101	565.2074	-										
✔	92	581.2055	-										
x	68	170.0867	-										
✔	106	625.2287	Alkaloid	67680	C09418	Daphnoline		Alkaloid	C35H36N2O6	0	[M+2Na-H]+	625.2285	580.2573

Table 5. 4. (continued …) Metabolomic features implicated in the response of well-watered (WW) and drought stressed (DS) barley plants (H*ordeum vulgare* cv. Concerto) to mycorrhizal colonisation (AM = colonised, NM = uncolonised). Chemical class annotations based on comparison with METLIN, MassBank, KEGG and BarleyCyc databases. Mass error is given for annotations in ppm (parts per million).

				Database (METLIN) m/z matches									
Also discriminated between AM WW and NM WW	**RT (secs)**	**Detected m/z**	**Putative class annotation**	**Database ID (METLIN or *MassBank)**	**KEGG ID**	**Compound**	**Common synonym**	**Chemical class**	**Formula**	**ppm**	**Ion**	**Reference m/z**	**Accurate mass**
	Associated with mycorrhizal drought stress treatment (AM DS) at 42 DPI												
✔	99	595.2229	Chlorophyll precursor	63924	C02880	Protochlorophyllide		Chlorophyll precursor	C35H32MgN4O5	5	[M+2Na] 2+	595.2196	612.2223
x	350	311.2937	-										
x	79	144.1052	-										
x	386	550.6804	-										
x	191	113.984	-										
x	25	113.9607	-										
x	255	299.2219	Jasmonic acid precursor	63360	C01226	12OPDA	12-oxophytodienoic acid	Fatty acyl	C18H28O3	6	[M+Li]+	299.2198	292.2038
✔	20	182.9888	-										
✔	99	596.2242	Flavonoid	PR020004*		2',6'-Dihydroxy- 4-methoxychalcone -4'-O-neohesperidoside			C28H34O14	2		596.2232	594.1949
x	397	393.3384	-										
x	17	114.0167	-										
x	19	84.97943	-										
x	350	505.3059	Quinone	3030	C05849	2,3-epoxyphylloquinone	Vitamin K1 epoxide	Quinone	C31H46O3	3	[M+K]+	505.3079	466.3447
x	368	269.1584	Dipeptide	66283	C05010	Histidylleucine	His-Leu	Dipeptide	C12H20N4O3	8	[M+H]+	269.1608	268.1535
x	386	551.6846	-										
x	54	160.099	Ubiquinone	226	C00399	Ubiquinone (Q2)	Conenzyme Q	Ubiquinone	C19H26O4	1	[M+2H]2+	160.0988	318.1831
✔	192	575.3181	Porphyrinogen	6003	C01079	Protoporphyrinogen IX		Porphyrinogen	C34H40N4O4	4	[M+Li]+	575.3210	568.3050

Table 5. 4. (continued …) Metabolomic features implicated in the response of well-watered (WW) and drought stressed (DS) barley plants (H*ordeum vulgare* cv. Concerto) to mycorrhizal colonisation (AM = colonised, NM = uncolonised). Chemical class annotations based on comparison with METLIN, MassBank, KEGG and BarleyCyc databases. Mass error is given for annotations in ppm (parts per million).

				Database (METLIN) m/z matches									
Also discriminated between AM WW and NM WW	RT (secs)	Detected m/z	Putative class annotation	Database ID (METLIN or *MassBank)	KEGG ID	Compound	Common synonym	Chemical class	Formula	ppm	Ion	Reference m/z	Accurate mass
Additional features associated with non-mycorrhizal well-watered treatment (NM WW) at 42 DPI													
	5	103.9755	-										
	23	381.1246	-										
	5	145.0079	-										
	5	105.9745	-										
	88	612.2216	Flavonoid	PR020045*	C09806	Neohesperidin	Hesperetin 7-O-neohesperidoside	Flavonone	C28H34O15	14		612.2303	610.1898
	349	311.3476	-										
	101	449.1566	Flavonoid	PR020045*	C09806	Neohesperidin	Hesperetin 7-O-neohesperidoside	Flavonone	C28H34O15	1		449.1571	610.1898
	66	133.0999	-										
	88	613.2196	Flavonoid	PR020039*	C09755	Neohesperidin dihydrochalcone	Dihydrohesperetin-7-O-neohesperidoside		C28H36O15	4		613.2222	612.2054
	199	96.9803	-										
Additional features associated with mycorrhizal well-watered treatment (AM WW) at 42 DPI													
	395	359.0179	Dihydroflavonol	3450	C02906	Dihydromyricetin		Dihydroflavonol	C15H12O8	4	[M+K]+	359.0164	320.0532
	96	177.0795	Lignan	68031	C09944	Karwinskione		Lignan	C32H32O7	3	[M+3H]3+	177.0789	528.2148

Under drought conditions, epoxyphylloquinone, ubiquinone and protoporphyrinogen IX differentiate leaves of AM plants from those of NM plants

In chapter 4, AM colonisation was found to delay the drought-induced decline in photosystem II efficiency (Fv'/Fm') by approx. one day. In order to investigate whether the leaf metabolomes of the same plants could provide any mechanistic insight into this difference, a pair-wise comparison of AM DS and NM DS leaf metabolomic fingerprints was undertaken using samples from 42DPI (at which point there was a significant difference in Fv'/Fm' - see fig. 4.2.b).

In unsupervised PCA, there was no strong separation between leaf metabolomic fingerprints of AM (mycorrhizal) and NM (non-mycorrhizal) plants following 18 days of drought (42DPI) in any combination of the first 5 PCs. However, some clustering was observed in the combination of PC2 and 4 (representing 18.6 and 11.5% of the variance in the model respectively) (fig. 5. 9). This suggests that any effect of AM colonisation on the leaf metabolome of barley cv. Concerto under drought conditions is subtle. Since a difference in photosystem II efficiency had been observed, a supervised analysis was undertaken (using OPLS-DA) to discriminate between the two groups of metabolomic fingerprints and highlight any subtle differences.

Sixteen peaks were found to reliably associate with NM DS plants in this analysis, four of which matched features distinguishing between AM and NM plants under WW conditions (table 5.4). NM DS -associated features included putative flavonoids, sesquiterpenoids, hydroxycinnamic acid derivatives, as well as eight unannotated features.

Seventeen peaks reliably associated with AM DS plants, though ten remained unannotated (table 5.4). Amongst potential annotations of these AM DS -associated features were 2,3-epoxyphylloquinone, ubiquinone and protoporphyrinogen IX (in addition to peaks that also differentiated between AM and NM plants in the WW treatment: a flavonoid and potential lignan).

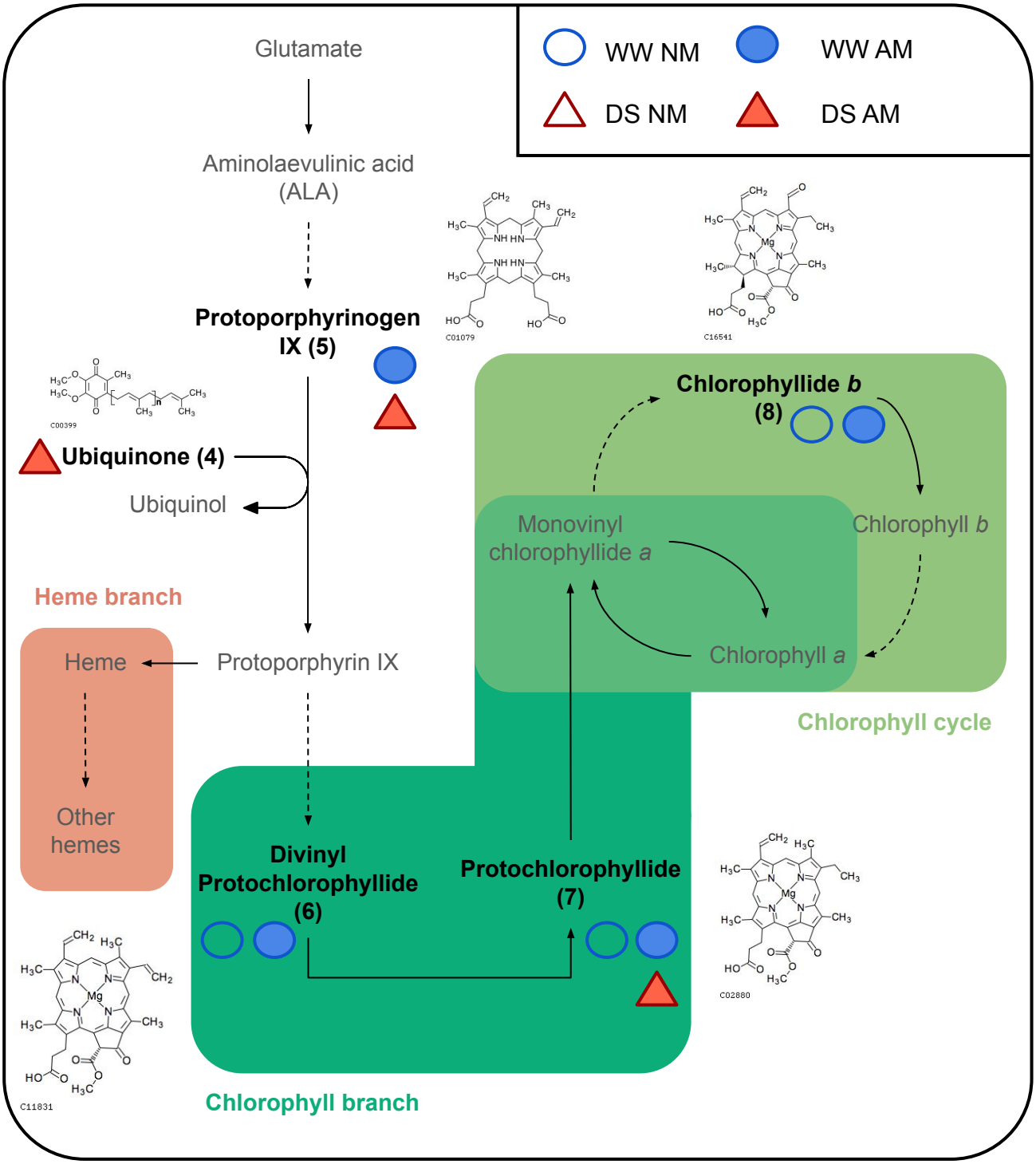

Fig. 5. 10. Chlorophylls and chlorophyll precursors are important in distinguishing the metabolic fingerprints of AM and NM barley plants subjected to drought stress (DS). Compounds in the chlorophyll branch of tetrapyrrole biosynthesis (**6** & **7**) were associated with well-watered control plants in both the AM and NM treatments, as was chlorophyllide *b* (**8**) in the chlorophyll cycle. Protochlorophyllide (**7**) was also associated with metabolomic fingerprints from AM plants in the DS treatment, making protochlorophyllide (**7**) a good candidate to explain the delayed decline in Fv'/Fm' in DS AM plants compared to DS NM plants. Protoporphyrinogen IX (**5**) was also associated with AM colonised plants (both under WW and DS). Ubiquinone (**4**) was associated with DS AM plants and is involved in conversion of protoporphyrinogen IX (**5**) to protoporphyrin IX.

Simplified tetrapyrrole biosynthesis pathways adapted from Tanaka & Tanaka 2007 and KEGG PATHWAY. Chemical structures and KEGG IDs from KEGG COMPOUND. Solid arrows represent single step in the pathway, dashed arrows represent multiple steps in the pathway.

Tetrapyrrole biosynthesis pathway is a good candidate to explain AM maintenance of photosystem II efficiency

Tetrapyrroles, including chlorophyll and heme, are important in a variety of primary metabolic processes in higher plants. A putatively identified compound in this study, protoporphyrinogen IX is a precursor to various tetrapyrroles, while divinylprotochlorophyllide and protochlorophyllide are precursors to chlorophyll *a* and *b* biosynthesis (compounds **5, 6** and **7** in fig. 5.10) (Tanaka & Tanaka 2007).

In pairwise comparisons, protochlorophyllide (**7**) was associated with metabolomic fingerprints from AM plants in both the WW and DS treatments, as well as associating with NMWW plants when compared with NMDS plants. This makes protochlorophyllide a good candidate to explain the delayed decline in Fv'/Fm' in AM DS plants compared to NM DS plants.

Accumulation of tetrapyrrole intermediatesgenerates oxidising agents in the light, resulting in high levels of ROS which are normally thought of as damaging to cellular processes. Tetrapyrrole intermediate-induced ROS accumulation has, however, been shown to improve tolerance to stresses such as viral infection, and has been proposed as a stress signalling mechanism in itself (Dilrukshi *et al.* 2015). Aminolaevulinic acid (ALA), a precursor to protoporphyrinogen IX, has been exogenously applied to various crop species, including barley, with the result of increasing chlorophyll content and ameliorating the effects of salinity, drought and high temperature stresses. Under drought stress, this has been found to occur via an increase in chlorophyll biosynthesis as well as a decrease in chlorophyll degradation, and plants were also found to have low levels of ROS (Li *et al.* 2011).

In the current study, protoporphyrinogen IX (compound **5** in fig. 5.10) was associated with AM colonised plants (both under WW and DS) as opposed to NM in pairwise comparisons. Protoporphyrinogen IX is a direct precursor to protoporphyrin IX, the "branching point" in the tetrapyrrole biosynthesis pathway, from which heme, as well as chlorophylls, can be synthesised (Tanaka & Tanaka 2007). Ubiquinone (compound **4** in fig. 5.10) was associated with DS AM plants and, among many electron transport roles within higher plants, is involved in the conversion of protoporphyrinogen IX to protoporphyrin IX.

Any increase in protoporphyrinogen IX, and thus protoporphyrin IX, could increase the availability of substrate for synthesising heme-dependent antioxidants (heme acts as a cofactor for superoxide dismutase and catalase; breakdown products of heme are ROS scavengers) and the cytochrome b6f complex, and this mechanism has been implicated in the effective dehydration response of cereals (Dilrukshi *et al.* 2015).

The use of untargeted metabolomics

Untargeted metabolomics is very useful for generating hypotheses to test with more targeted techniques (Allwood & Goodacre 2009). Since this study uses an untargeted approach, it cannot rule out the following explanations for the findings presented in this chapter:

- Compounds that are up- or down- regulated may not have been detected by the MS method employed here; may have been filtered out during the chromatography step; or may have been relatively low in intensity;
- Metabolomic effects of drought may have occurred elsewhere (e.g. in the roots) in order to maintain the "normal" biochemistry and functioning of the leaf. Different plant organs have been shown to have different metabolic responses to drought (Hein *et al.* 2016);
- The timing of leaf sampling for metabolite extraction may have missed the initial *response* to drought stress, and what was observed here may have been the *damage* resulting from drought (after 18 days). It may have been that, at 42DPI, not as much *damage* had occurred within AM leaves and this is what is observed in the PCAs. The AM plants' *response* to drought, on the other hand, may have been missed in this experiment (the magnitude of the metabolomic response peaked at approx. 8 hours in a study using hulless barley by Yuan *et al.* 2018).

Promising avenues for future research

The results presented in chapter 5 are relative and not quantitative, so, while an accumulation of putative chlorophyllide *b* (compound **8** in fig. 5.10) was observed in both WW AM and WW NM plants, it is not appropriate to draw conclusions on the effect of tetrapyrrole precursor accumulation as a mechanism by which AM colonisation may affect a host plant's perception of, or response to, drought stress. However, tetrapyrrole biosynthesis precursors and intermediates, featured in fig. 5.10, would be strong candidates for further targeted investigation. It would also be necessary to directly quantify chlorophyll and heme accumulation, associated antioxidant enzyme activity, and lipid peroxidation.

Phenylpropanoids would warrant further investigation in relation to growth stage in barley with targeted analysis such as LC-ESI-MS-MS or by using an internal phenylpropanoid standard (e.g. following the protocol of Garibay-Hernández *et al.* 2021). Phenylpropanoids were implicated in the drought and mycorrhizal responses of barley cv. Concerto leaves and targeted flavonoid LC-ESI-MS-MS protocols would permit a better characterisation of how AM colonisation alters the drought response. Phenylpropanoids also have a range of nutraceutical benefits (Dwivedi *et al.* 2016) so further information on the timing of

accumulation of specific flavonoids, and relating this to their antioxidant activity, could improve accuracy of harvesting green barley (barley grown for its seedling leaves) for maximum nutritional value (Kowalczewski *et al.* 2020).

Improved workflow for untargeted metabolomics

In preparing this chapter, a workflow was developed including R code to automate a number of steps in an existing pipeline and integrate open-source tools to replace steps that previously relied on proprietary software. The guide to the workflow is presented in Parker *et al.* 2023 (presented as chapter 6 of this thesis) and the code can be accessed at:

https://github.com/LizzyParkerPannell/Untargeted_metabolomics_workflow.git

In addition, an online guide (https://untargeted-metabolomics-workflow.netlify.app/ accessed 24 March 2023) supports researchers new to metabolomics as a discipline to analyse untargeted metabolomics data from MALDI-TOF-MS, DI-ESI-MS or LC-ESI-MS.

This effort to make the workflow findings of the untargeted LC-ESI-MS analysis presented here ensure that the study is more reproducible and complies with the FAIR (findable accessible interoperable and reproducible) for research data (Wilkinson *et al.* 2016). The documentation of the workflow will aid future analyses making them faster, cheaper and will also facilitate training and upskilling required among researchers to ensure data comply with FAIR principles.

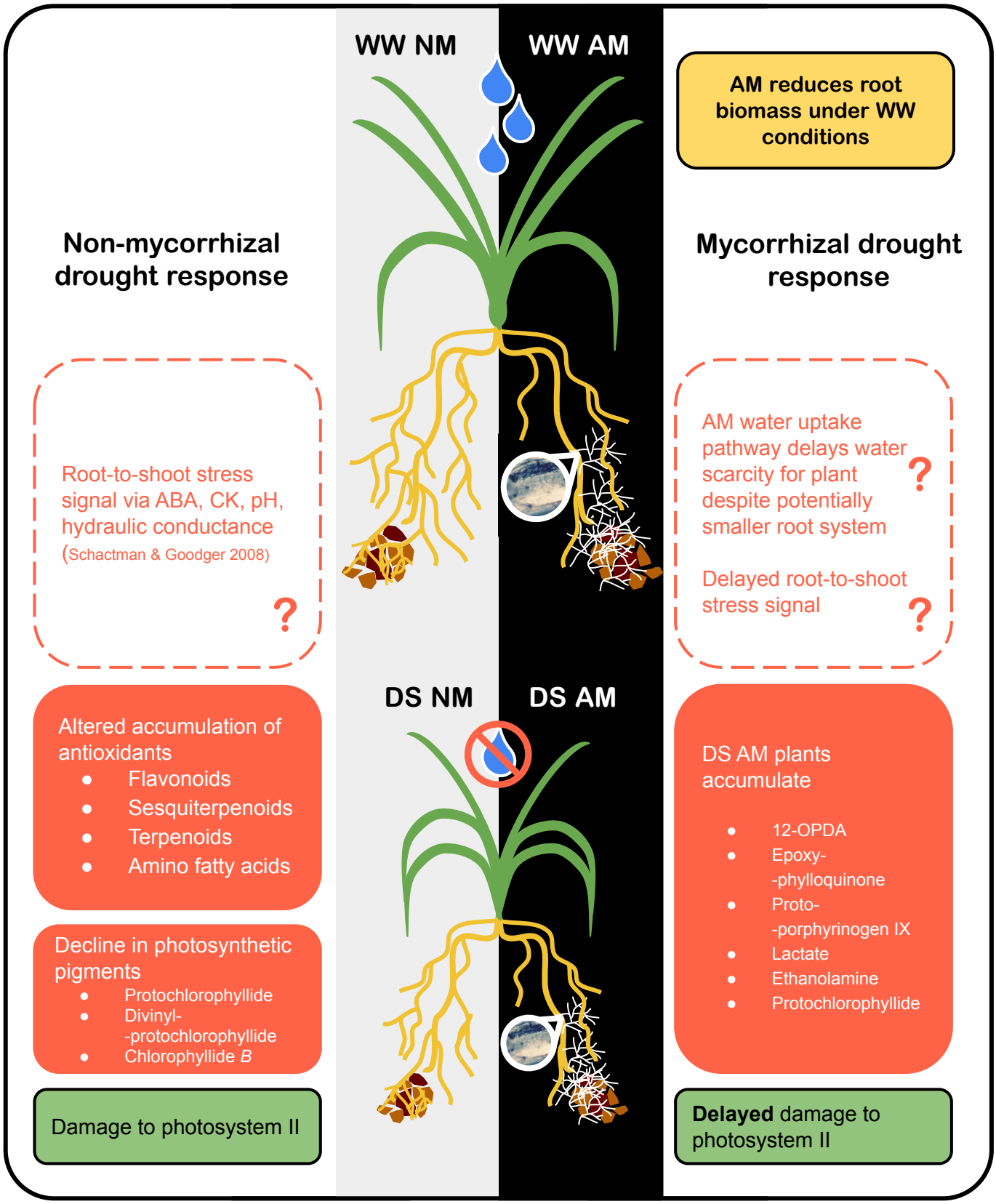

Fig. 5. 11. Summary of key findings. Evidence of accumulation of osmoprotectants and antioxidants was observed in the leaf metabolomic fingerprints of NM droughted barley. There was not as strong an effect of drought stress on AM plants' leaf metabolomic fingerprints, suggesting that AM plants entered the drought period better able to cope with drought stress and/ or provided an advantage under drought stress, though apparently not via a dramatically different metabolome. In line with a delayed decline in Fv'/Fm' observed in chapter 4, compounds involved in chlorophyll and heme biosynthesis were associated with AM DS metabolomes. Clearly defined changes in the leaf metabolomic fingerprint due to drought were not observed until the 18th day of drought in either NM or AM plants.

CONCLUSIONS

Barley cv. Concerto metabolomes at the tillering stage (29DPI) can be distinguished from those at stem elongation stage (42DPI) by accumulation of phenylpropanoids (flavonoids, lignans), alkaloids and amino acid derivatives. At stem elongation stage, cellulose and protochlorophyllide (a chlorophyll precursor), as well as different flavonoids and alkaloids were associated with the leaf metabolomes.

The effect of growth stage (age at sampling) was much greater than that of drought stress or AM colonisation on the leaf metabolomes of barley in this study.

No obvious effects of drought stress on the leaf polar metabolomic fingerprints of barley could be found at 29 and 35DPI (5th and 11th days of drought) using this methodology. A clear metabolomic effect of drought was observed at 42DPI after 18 days of drought, even though stomatal conductance and photosynthetic rate had substantially declined in droughted plants by 35DPI (11th day of drought). This highlights the importance of tracking metabolomic changes over time, of relating metabolomic changes to physiological responses and suggests that drought-induced physiological damage to the leaf can be observed before a strong metabolomic signal is detected with an untargeted metabolomics approach.

The effect of AM colonisation on barley leaf metabolomic fingerprints was weak, and could only be observed in pairwise, supervised analyses. However, this study found evidence of differences in the leaf metabolomic response to drought between AM and NM plants.

Using LC-ESI-MS and an untargeted metabolomics workflow, in combination with information from physiological assessment of drought stress and AM colonisation, it was possible to identify potential compounds and compound classes involved in drought and AM colonisation response. The most promising pathways revealed by this study which would be recommended for investigation with targeted metabolomics are:

- The phenylpropanoid pathway (particularly flavonoids) in relation to growth stage, drought and AM colonisation
- Jasmonic acid (JA) biosynthesis and glycerophospholipid biosynthesis/ glycine betaine biosynthesis in relation to the AM plants' drought response
- Tetrapyrrole biosynthesis in relation to both AM and NM plants' drought responses

Chapter 6 - Untangling the Complexities of Processing and Analysis for Untargeted LC-MS Data Using Open-Source Tools

metabolites

MDPI

Article

Untangling the Complexities of Processing and Analysis for Untargeted LC-MS Data Using Open-Source Tools

Elizabeth J. Parker [1,*], Kathryn C. Billane [1,*], Nichola Austen [2], Anne Cotton [1], Rachel M. George [3], David Hopkins [4], Janice A. Lake [4], James K. Pitman [1], James N. Prout [1], Heather J. Walker [3], Alex Williams [1] and Duncan D. Cameron [4]

[1] School of Biosciences, University of Sheffield, Sheffield S10 2TN, UK
[2] Department of Biology, University of Oxford, Oxford OX1 3RB, UK
[3] biOMICS Mass Spectrometry Centre, University of Sheffield, Sheffield S10 2TN, UK
[4] Department of Earth and Environmental Sciences, University of Manchester, Manchester M13 9PL, UK
* Correspondence: lizzyparkerpannell@gmail.com (E.J.P.); kcbillane1@sheffield.ac.uk (K.C.B.)

Abstract: Untargeted metabolomics is a powerful tool for measuring and understanding complex biological chemistries. However, employment, bioinformatics and downstream analysis of mass spectrometry (MS) data can be daunting for inexperienced users. Numerous open-source and free-to-use data processing and analysis tools exist for various untargeted MS approaches, including liquid chromatography (LC), but choosing the 'correct' pipeline isn't straight-forward. This tutorial, in conjunction with a user-friendly online guide presents a workflow for connecting these tools to process, analyse and annotate various untargeted MS datasets. The workflow is intended to guide exploratory analysis in order to inform decision-making regarding costly and time-consuming downstream targeted MS approaches. We provide practical advice concerning experimental design, organisation of data and downstream analysis, and offer details on sharing and storing valuable MS data for posterity. The workflow is editable and modular, allowing flexibility for updated/changing methodologies and increased clarity and detail as user participation becomes more common. Hence, the authors welcome contributions and improvements to the workflow via the online repository. We believe that this workflow will streamline and condense complex mass-spectrometry approaches into easier, more manageable, analyses thereby generating opportunities for researchers previously discouraged by inaccessible and overly complicated software.

Keywords: metabolomics; untargeted; mass-spectrometry; open-source; bioinformatics

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1. Introduction

Untargeted metabolomics is an increasingly popular tool for identifying perturbations within a metabolome and revealing phenotypic complexity in systems [1–4]. It is commonly the first part of a two-step research pipeline, where untargeted studies are used to gather information, identify the metabolome, and generate hypotheses. This is followed by targeted metabolomics which measures specific compounds and requires a priori knowledge of the whole metabolome [1,4,5]. Key to a metabolomics workflow are the data processing and handling steps, which take raw mass spectrometry data and convert them for use in a wide array of multivariate and statistical methods. Currently there is no one standardised pipeline for this step due to variation from sampling methods, instrumentation used, analytical methods employed and the deficit of standardised guidelines [6–11].

After over a decade of experience with proprietary software, the challenge was to address a number of issues with current common practices and embrace an open-source approach to metabolomics data processing and analysis that can have a future legacy. As well as navigating the plethora of analysis options available, with the advent of remote working, it became apparent that researchers conducting untargeted metabolomics analysis required resources to learn how to process mass spectrometry data remotely.

The objective of this work was to develop a guide focussed on processing and analysis of mass spectrometry data, collected to address untargeted metabolomics questions, primarily in the fields of environmental metabolomics and the study of complex plant stress responses. However, the tutorial and workflow have been applied in a range of experimental systems including *E. coli*, potato, barley, organic fertilisers, field soil samples, human cervical mucus, and *Chlorella.* The aim is that the guide will help to move towards standardised methodology and comparable research across the field of metabolomics.

The newly developed workflow presented here is designed to address the question:

Which compounds might be responsible for the difference in metabolomic fingerprint between the classes (groups) of samples?

The workflow converts mass spectrometry data to open formats for experiments in which a wide array of compounds are compared between two or more classes of samples. The steps may not result in a definitive difference or unquestionable compound identification, rather the workflow will direct further research and highlight potential compounds to focus on for targeted analysis. This resource is aimed at non-experts, and early career researchers who may not have extensive coding or analytical knowledge. Users are introduced and guided through pre-processing options and data formatting steps which result in a peak table data frame. This peak table forms the basis of the next steps in the workflow, multivariate analysis and putative metabolite ID to give a list of potential compounds that are differentially expressed between groups of samples which can inform the hypothesis for downstream targeted analyses. Alongside some command-line interface, GUI software has also been utilised in the workflow, which can be simpler to learn and easier to operate for new and non-expert users of metabolomics data analysis software [12]. Notably, all software approaches discussed here are free, as the authors believe it is important that the discussed pipelines are accessible.

This collaborative and open-source workflow guide for untargeted metabolomics addresses the need for data-handling tutorials [1] with the key aims of widespread use and continuous improvement, ultimately encouraging integration with multi-omic workflows.

2. Materials and Methods

2.1. Overview and Workflow Diagram

This tutorial guides the user through the untargeted metabolomics workflow that has been developed with some explanation of what each stage achieves. Further details are available in step-by-step guides on the associated website (https://untargeted-metabolomics-workflow.netlify.app/ accessed on (27 January 2023)), which includes links to relevant open-source tools, and our own interoperable code where appropriate. This tutorial covers the steps required to process LC-ESI-MS data, however detailed instructions for processing MALDI-ToF-MS and DI-ESI-MS using similar open-source tools are also available on the associated website.

An index of openly-available datasets is provided at https://untargeted-metabolomics-workflow.netlify.app/00_overview/06_demo-data/ (accessed on 9 March 2023). These example datasets can be used to demonstrate the workflow presented here.

The workflow has been divided into stages. The following number codes are used in the online guide as well as in the R [13] code and workflow diagram (for an abridged version of this diagram see Figure 1).

00. Overviews, workflow diagram & useful information
01. Metabolite extraction
02. Data acquisition (Mass Spectrometry)
03. Converting data to open format
04. Data pre-processing
05. Extracting & formatting peak table & metadata
06. Multivariate analysis (PCA) & further analysis (if applicable)
07. Putative metabolite identification
08. Archiving data & citing resources

Stages 01 and 02 are not covered in great detail in this documentation which focuses primarily on data processing and analysis.

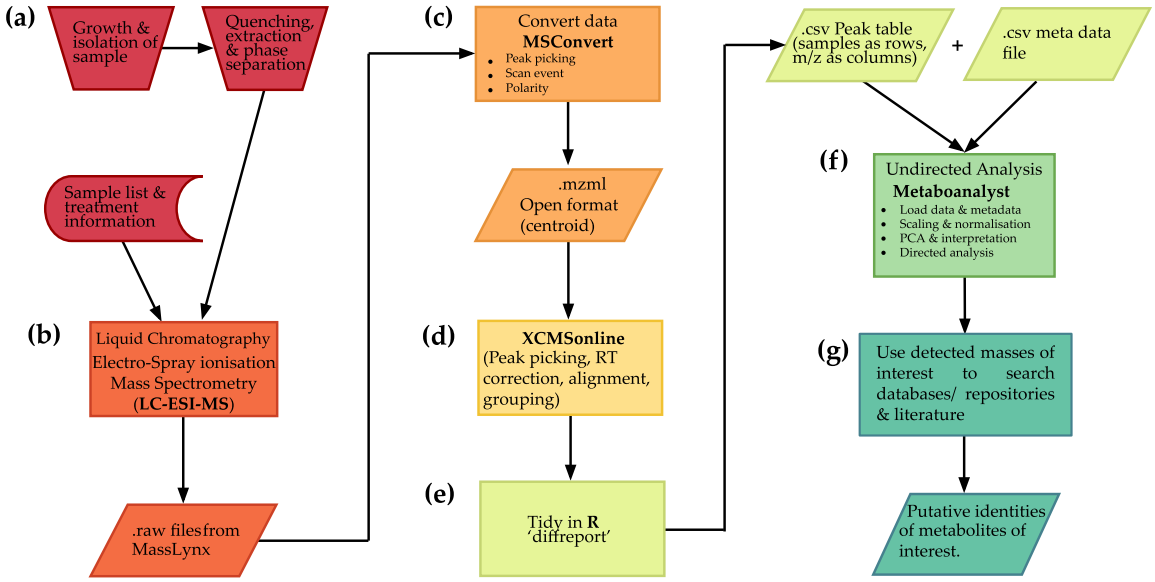

Figure 1. Workflow diagram for processing and analysis of untargeted LC-MS metabolomics data. (**a**) sample selection and preparation. (**b**) Mass spectrometry analysis of samples. (**c**) Conversion of data to open format. (**d**) Data pre-processing and (**e**) production of a feature matrix with experimental information included. (**f**) Statistical analysis for selection of features of interest and (**g**) identification of features of interest by comparison with literature and existing metabolite databases.

2.2. Experimental Design and Quality Control

Difficulties in analysis and/or workflows can arise from complexities in experimental structure. Many terms are used interchangeably in different contexts. Most tools for untargeted metabolomics are set up for one factor analysis with two or three levels e.g.,

- Case vs. control
- Wild-type vs. transgenic line
- Strain 1 vs. strain 2 vs. strain 3

However, more complex experimental designs are quite often implemented e.g.,

- Two factors with two or more levels in each such as +/− treatment for two strains
- Time course for one or two factors such as +/− treatment for two strains over three time points

To begin, the expectations of which groups of metabolite fingerprints may differ from one another must be considered, and to what extent.

- What are the biological replicates being analysed and are they independent of each other (or has the same organism/population been sampled multiple times)?
- Are there technical replicates (i.e., repeated runs of the same sample)?
- Are Quality Control (QC) samples required? Are analytical standards needed?
- What groupings are required to answer the research questions outlined?

Quality control (QC) can mean different things to researchers from different fields. There are a few simple quality control options for checking that there has not been subtle (or not so subtle) variation accumulating during the run. Decisions must be made on which one (or more) of these are necessary depending on the type of sample to be analysed and the MS techniques employed:

- Spike all prepared samples with a compound for which the m/z (and RT) is known and which is unlikely to be otherwise present in the experimental samples;

- Prepare a pooled QC sample from an aliquot of each of the samples and include this at regular intervals in the MS run;
- Include blanks and/or extraction blanks at regular intervals in the MS run;
- Use lock mass calibration (for Waters instruments).

There are some basic data quality control steps you can take to limit errors during processing and analysis:

- Check file sizes of .raw files across the MS run;
- Check file sizes of converted .mzML files—reconvert any that are unexpected;
- Compare spectra between technical replicates

2.3. Metabolite Extraction and Data Acquistion

Details of quenching, metabolite extraction or choice of mass spectrometry platform are not covered here, as they will likely be specific to the organism and/or tissue involved and the questions being addressed. Figure 2 provides a conceptual overview of metabolite extraction and data acquisition from plant tissues. See [14,15] for introductory guidance and [16] for a specific metabolite extraction method appropriate to plant tissues for this workflow.

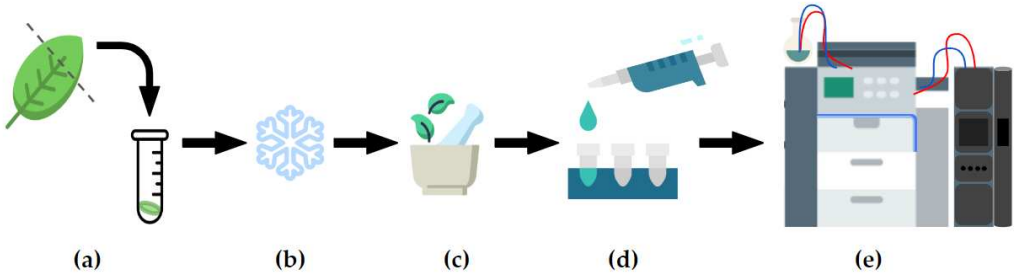

Figure 2. Conceptual diagram of an untargeted metabolomics workflow, from leaf to mass spectrometry analysis. After sample harvest (**a**), metabolic reactions in a sample tissue must be first quenched (**b**); i.e., via liquid nitrogen immersion), cell walls lysed and the sample homogenised (**c**) to permit extraction of compounds within the cells using a range of solvents (**d**). Extracts may then be diluted and submitted to mass spectrometry analysis (**e**); e.g., UPLC-ESI-MS).

2.4. Preparing Metadata for Analysis

To process and analyse data using our workflow, two .csv files are required (these can be created in excel, R, google sheets etc. depending on preference) as long as the order and headings of the columns follow the pattern detailed below.

For samplelist.csv the following columns are required:

- "Filename": this is a list of the filenames of the .mzml files (the part before the .mzml)
- "Filetext": this is the name that has been manually added to the metadata of that sample
- "MSFile" or an equivalent column that contains either "pos" or "neg" within it. Any other columns will be ignored in this file.

For treatments.csv at least two columns are required:

- "Filetext": this must contain all the distinct values of "Filetext" from samplelist.csv
- "Variable1": the naming of this column is left to the user. For example, in an MS run comparing a wild-type to a control, this column could be named "treatment" and filled with "WT" and "C" as appropriate
- "Variable2" etc: further variables. This may include batch identifiers (for example if many samples were run over multiple days), treatments or environmental variables

These are kept in a folder with the .mzml data files. Examples can be found on the website at https://untargeted-metabolomics-workflow.netlify.app/03_conversion-to-open-format/05_samples-treatments/ (accessed on 27 January 2023).

3. Results

3.1. Converting Data to Open Format Using Proteowizard

Converting proprietary data files (which contain a large amount of data and metadata about the run in separate files) to a more manageable format, such as .mzML (the standard open-data format for mass spectrometry [17]) is essential. We have developed this workflow using .RAW files, which are specific to Waters software and are not compatible with many open-source tools. To convert .RAW to .mzML, Proteowizard software [18] is used. Proteowizard is capable of converting many other proprietary file formats and guidance is available through their extensive documentation at https://proteowizard.sourceforge.io/doc_users.html accessed on (20 February 2023). Proteowizard comprises two applications: SeeMS and MSConvert.

SeeMS is useful for viewing chromatograms and spectra without access to proprietary software like MassLynx. MSConvert performs conversion of the MS data but depending on the type of MS used, different settings/parameters in MSConvert may be required, detailed in the online step-by-step instructions to complete stage 03 (https://untargeted-metabolomics-workflow.netlify.app/03_conversion-to-open-format/03_msconvert-lcms/ accessed on 27 January 2023).

It is critically important to check the size of .mzML files once converted. They should all be similar. SeeMS can be used to check any that seem unusual and reconvert any with an incongruous file size (problems in conversion can arise, for instance from intermittent internet connection when converting files from a remote drive).

3.2. Preprocessing Data

Untargeted metabolomics datasets can be several GB in size! To get from compressed .mzML files to a tractable peak table that can be interrogated with multivariate statistics, it is necessary to "tidy" the data.

A peak table is a data-frame consisting of aligned spectra with concentration or intensity values against a set of features—mass to charge ratio (m/z) or m/z with retention time (RT). The file size will be dependent on sample number but will be smaller than the .mzML files.

Different downstream tools for multivariate statistics will require the peak table in slightly different formats, so the code included in this guide will help with formatting for some common uses (e.g., MetaboAnalyst one factor and two factor peak tables) as well as helping format treatment information as metadata so that peak tables can be interrogated.

- Depending on the MS approach, different stages are involved but they broadly fall into:
- Baseline correction and/or noise reduction (estimating what part of the detected intensity is the sample and "cleaning" or adjusting the spectra to show only the signal believed to be associated with the sample);
- Normalisation and/or standardisation (these can mean a range of different things to different people but broadly cover accounting for differences in sample volume or concentration or total intensity of the signal);
- Grouping and peak picking (wave-form algorithms are used to determine which parts of the spectra constitute separate peaks utilising their m/z value);
- Alignment or peak matching (assessing across samples to determine whether peaks with slightly different m/z values are the same peak so that samples can be compared more reliably).
- The above criteria are very important when processing data as they can have a big impact on data quality however the parameters may vary with different datasets and different analysis methods. The importance of these factors have been discussed previously by [19].

By the end of this stage, data will be processed into a single table containing all the m/z and intensity values required for down-stream analysis. This stage relies on the use of open-source software (XCMS online [20] for LC-ESI-MS and MassUp [21] for MALDI-ToF-

MS and DI-ESI-MS) to process the data. These provide user interfaces for well-documented R packages (XCMS [22] and MALDIquant [23] respectively) and provide the advantage of coping well with large datasets and, in the case of XCMS online, being run remotely.

For detailed instructions on pre-processing, consult stage 04 of our online guide (https://untargeted-metabolomics-workflow.netlify.app/04_data-preprocessing/ accessed on (27 January 2023)).

R code to extract a peak table from pre-processed data is available in stage 05 of our online guide (https://untargeted-metabolomics-workflow.netlify.app/05_extracting-formatting-peak-table/ accessed on (27 January 2023)).

3.3. Multivariate Analysis

There are often two key questions when analysing a new untargeted metabolomics dataset:

- Are the metabolomic fingerprints distinct classes (treatment groups) different from each other?
- Which features of the metabolomic fingerprint are causing them to be different from each other?

To answer the first question, data ordination is required to provide a global overview of the variability and patterns within the data. Principal Component Analysis (PCA) is a commonly applied ordination tool that reduces the dimensionality of multivariate data to display complex relationships between samples in 2 or 3 dimensions [15]. As it is unsupervised the model is unaware of the classes to which the samples belong, so patterns are unbiased by a priori knowledge of the experimental design. PERMANOVA can be used to provide statistical corroboration of patterns observed in the PCA by statistically evaluating if significant trends exist at the higher levels of the experimental design within multivariate data i.e., if significant treatment and interaction effects are present. Finally, where clear differences between classes in the PCA are apparent, pairwise comparisons between classes (treatment groups) can be investigated via exploring the loadings or using a pairwise analysis such as t-tests or volcano plots. These will provide the user with features of interest that are most important at defining the statistical output [15].

Where patterns are less clear, supervised analysis, such as OPLS-DA (orthogonal projections of latent structures) may be employed to mine for differences between any two classes. The output of supervised analyses will highlight particularly highly abundant features that differ between two randomly assigned classes that may be obscured in global overview if the majority of the metabolome is conserved or unchanging (this can occur in tissues where only small numbers of metabolites respond to a stimulus, but the majority of the metabolome is unaffected). To limit false positives it is important to consider the native separation in the data (i.e., through an unsupervised ordination, like PCA) to provide a robust biological justification for comparing two particular classes. The analyses exemplified here are by no means the only option, and it is highly recommended that tools such as MetaboAnalyst [24] are employed by the researcher to explore all analytical avenues available.

In the online guide, demonstration is given on how to perform these analyses using a free online platform and how to run some alternative code in R. MetaboAnalyst is an online platform on which untargeted metabolomics data can be loaded, normalised, analysed and visualised. However, there is a strong emphasis on detailed statistics that may be more appropriate for targeted analyses, so the user must have a clear understanding of their objectives in choosing amongst the options.

MetaboAnalyst is interoperable with R and the underlying code can be accessed using the button at the top left of the "Results" page. The advantage of running the code is that the user can integrate it with other analyses (and formatting for figures). Examples of figures produced with this approach can be found in Figure 3. In contrast, the advantage of the MetaboAnalyst GUI is that it guides the user through the process and has some useful sense-checks and vignettes available.

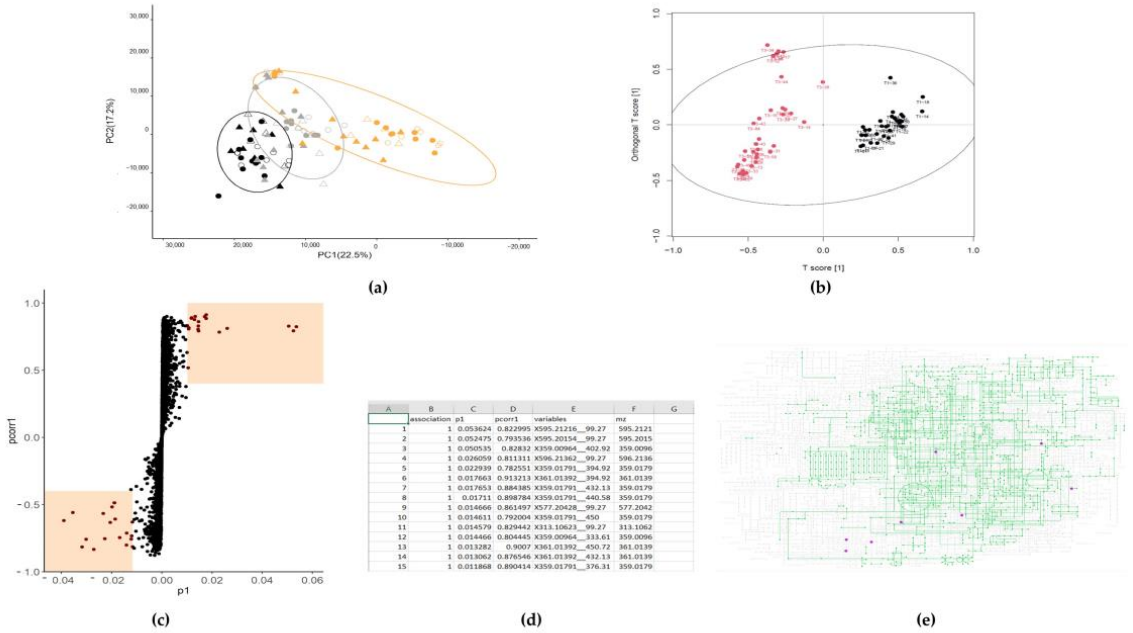

Figure 3. Conceptual diagram of examples of multivariate analysis outputs of untargeted metabolomics analysis, all produced using open-source or freely available software. (**a**) Principal component analysis (PCA) 2-D scores plot produced with *pcaMethods* and *ggplot2* packages in R; (**b**) OPLS-DA scores plot produced using the *muma* package in R; (**c**) scores plot created using *ggplot2* package and data produced by the *muma* package in R; (**d**) example list of features of interest highlighted by an OPLS-DA using *muma* in R; (**e**) example of metabolites highlighted within a KEGG pathways global *Esterichia coli* metabolism map.

Details can be found via the excellent tutorials and documentation provided by MetaboAnalyst [25].

It is also possible to analyse the same peak tables using SIMCA (Umetrics) or other proprietary softwares. However, it is much harder (and more costly) to use these remotely, and it is harder to document any analysis for sharing with other researchers. Other software worth considering includes MSDial, MetaboKit and MeV [26–28].

3.4. What Are My Metabolites?

It is very important to consider that this stage of the metabolomic process is not automated and can be incredibly time-consuming and challenging to do, so it is advisable that the preceding analysis has been adequately assessed for its effectiveness before committing time at this stage.

Annotating metabolomic features is challenging—there are some automated annotations included with e.g., XCMS that rely on the CAMERA package [29] amongst others. However, these often struggle with unusual experimental structures and/or large datasets, or "unusual" (i.e., non-human) metabolites. Thus, reducing the number of metabolomic features to those that are causing a significant (in terms of reliability and magnitude) difference between two classes of samples is advisable.

To ascertain the identity of these features, comparing the m/z (or m/z at specific RT) values highlighted by multivariate analysis with databases of reference m/z and with experimental data from the literature (usually available in a publication or in repositories like MetaboLights [30] and Metlin [31]) is key.

Stage 07 of the online guide provides guidance on using a range of databases to help annotate "metabolites of interest" (https://untargeted-metabolomics-workflow.netlify.app/07_putative-metabolite-id/ accessed on (27 January 2023)). These include:

- METLIN to search by m/z;

- KEGG PATHWAY and KEGG COMPOUND [32] to corroborate likelihood of detecting certain compounds in the study organism/sample and to gain insight on biological function;
- Data repositories such as MetaboLights;
- Details of how to find other relevant databases (MassBank, PubChem, MetaCyc, Metabolomics Workbench [33–36]);
- Reporting Metabolomics Standards Initiative (MSI) identification levels (see also [37]).

3.5. Sharing Metabolomics Data

Metabolomics data from even a small study can be very large. It can also be very complex. But there are ways of sharing it with the wider scientific community (and indeed the public) without too much trouble. It is insufficient to only prepare a data availability statement or simply share graphs or peak tables.

Metabolomics data can be analysed in lots of different ways, so it is important to comply with the FAIR principles [38]:

- Findable
- Accessible
- Interoperable
- Reusable

Institution-based data repositories are an option, but they often require extra levels of support to submit large datasets and there is no guarantee that access to other researchers is feasible.

More useful is a field-specific repository where data will be made available together with other relevant data sets. Furthermore, these repositories provide guidance on appropriate data formatting, allowing it to be compatible with other published data to form part of potential future meta-analyses. Some journals will have specific guidelines on which repository to use [39].

Time should be set aside from the outset of any project for submitting data to a repository. It is not optional!

MetaboLights is a data repository specific to metabolomics studies [30]. Data from NMR, GC-MS, LC-MS, and MALDI amongst others, may be submitted.

The repository is maintained and curated by the European Bioinformatics Institute (EMBL-EBI) meaning that the data it holds is well-formatted and integrated with several other standardised databases and ontologies (ways of describing methods, data and metadata). This "future-proofs" the data stored, making it not only open-access but also more findable and reusable, as well as facilitating integration with other -omics data, if required.

MetaboLights has various stages of submission, validation and then curation by experts to make sure each submission has all the relevant metadata needed to recreate the analysis undertaken. Following curation, there is a review process and finally data can be added to the repository and made available.

Because of the curation process, there can be a significant lag between submission and data being available so early submission is advisable. However, once submitted, there is a reference that can be linked to any publication [30].

Account creation is required, after which, a video tutorial guide on using the submission portal is available. Additional hints and tips on this can be found on the associated website (https://untargeted-metabolomics-workflow.netlify.app/08_data-archiving-citation/02_metabolights/ accessed on (27 January 2023)).

3.6. Citation of the Tools Used in the Workflow

Links to cite the following tools involved in the workflow can be found at https://untargeted-metabolomics-workflow.netlify.app/08_data-archiving-citation/03_citing-tools/ accessed on (21 February 2023). These tools are regularly updated so it is important to cite the version used and/or the date accessed:

- All R packages used;
- R and RStudio versions;
- Proteowizard (SeeMS and MSConvert);
- Metaboanalyst;
- XCMS online and METLIN;
- MassUp;
- MassBank (including access date);
- ECMDB and any other organism specific metabolite databases used;
- KEGG (including BRITE, COMPOUND and PATHWAY);
- PubChem;
- A data availability statement that links to your archived data (e.g., in MetaboLights).

4. Conclusions

At this point the choice in preparing and analysing metabolomics data is at the discretion of the research group. This guide is a useful starting point that leads the reader through an openly available, best-practice, pipeline. Complex data and analytical processes can be overwhelming, but by engaging in discussion forums, sharing ideas, troubleshooting, and having access to a community of like-minded researchers these processes can become more accessible and facilitate exploration of exciting biological questions.

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General Discussion

Research questions and hypotheses

The aim of this thesis was to characterise the effects of arbuscular mycorrhizal (AM) fungi on the physiological and metabolomic responses of spring barley to drought. *Hordeum vulgare* cv. Concerto was used throughout this thesis. Concerto (Limagrain) is a cultivar approved for malting and distilling and the market leader in the UK at the time of the experimental work (2016-18).

Research questions

The main research questions of this thesis were:

- Is it possible to simultaneously assess physiological responses to drought and obtain metabolite samples from the same plants over a time course in barley?
- Does the arbuscular mycorrhizal (AM) symbiosis alleviate drought symptoms in spring barley?
- Which biochemical pathways are highlighted by untargeted metabolomics approaches as affected by drought and by the AM symbiosis? Which compounds would be candidates for further targeted analysis?

Experimental approach

To address these questions, three experiments were carried out. To avoid repetition, methods used in more than one experiment were described in chapter 1.

In chapter 2, half the plants had been inoculated at the time of transplant with an axenic liquid inoculum of *Rhizophagus irregularis*, but showed no evidence of AM colonisation upon assessment by microscopy and also showed no evidence of physiological or growth effects of AM inoculation. As such, data from mycorrhizal (AM) and non-mycorrhizal (NM) treated plants were pooled for separate analyses in chapter 3. Physiological drought-responses were characterised and an untargeted DI-ESI-MS metabolomics approach was used to conduct a “first-pass” analysis of any distinguishing features in the metabolite fingerprints of this cultivar under drought stress.

In order to determine appropriate conditions for ensuring colonisation by arbuscular mycorrhiza, experiment 2 was carried out to compare different substrate compositions and to

confirm suitable levels of root colonisation and arbuscule formation in barley cv. Concerto using a commercially available AM fungal inoculum. In chapter 2, a primarily sand substrate, including compost and commercial AM inoculum was found to result in sufficient AM colonisation of treated plants, with no significant differences in biomass between AM plants and those treated with a control (carrier) substrate. This substrate was then used for the subsequent experiment.

Experiment 3 of this thesis used the primarily sand substrate and commercial AM inoculum in a fully factorial glass-house set up in which mycorrhizal (AM) and non-mycorrhizal (NM) barley plants were either subjected to drought stress treatment for 18 days (DS) or continued to receive adequate irrigation (WW). In chapter 4, physiological drought response of AM and NM plants was assessed by measuring photosynthetic rate (*A*), stomatal conductance (g_{sw}) and photosystem II efficiency (Fv'/Fm') across the drought period .

For chapter 5, an untargeted metabolomics workflow, detailed in chapter 6, was developed to analyse LC-ESI-MS data of polar leaf metabolite extracts with freely-available, open source software and online tools. The effects of both AM fungi and drought on the leaf metabolite fingerprint were investigated using this workflow. In initial analyses, the effects of age of plant at the time of sampling masked any differences between treatment groups when all data were included for analysis. Subsequently, therefore, subsets of the data were resubmitted to the workflow to elucidate any differences between the AM and NM plants' responses to drought.

Hypotheses

An 18 day drought treatment was expected to reduce stomatal conductance and photosynthetic rate of plants (DS) compared to those that continued to receive regular irrigation (WW). Leaf relative water content was used to assess the efficacy of the applied drought, and was expected to be significantly reduced in DS plants by the 13th day of drought as compared to WW control plants. Photosystem II efficiency as assessed by Fv'/Fm' with a handheld device was used to determine the onset of stress damaging to the photosynthetic machinery, which was expected to occur earlier in non-mycorrhizal (NM) plants, with mycorrhizal (AM) plants in the drought treatment maintaining an Fv'/Fm' closer to that of well-watered controls for longer.

It was hypothesised that leaf metabolomic fingerprints of spring barley would be distinguishable through multivariate analysis based on whether they had been colonised by AM fungi (AM) or not (NM), and based on whether they had been experiencing drought stress (DS) or not (WW). Furthermore it was expected that differences in metabolomic

fingerprints would be observed at sampling time points in advance of damage to photosynthetic machinery (i.e. before Fv'/Fm' was significantly reduced in DS plants compared to WW). Predictions of the biochemical pathways expected to be involved in barley response to drought stress, and detectable with an untargeted metabolomics approach, were:

- The **phenylpropanoid pathway**, including the glycosylation patterns of **flavonoids**, **hydroxycinnamic acid derivatives** (such as hordatines) and **terpenoids** (such as blumenol C derivatives) as well as **antioxidant systems in general**;
- **Amino acid biosynthesis pathways**, particularly those related to biosynthesis of **proline**;
- Accumulation of other **compatible solutes** and/or osmolytes was expected under drought conditions (e.g. **fructose, glucose, galactinol** and Krebs' citric acid cycle intermediates).

Furthermore, compounds and pathways expected to be involved in the response to AM colonisation, and detectable in the leaves, were:

- **Glycosides of blumenol C and hydroxyblumenol C**;
- Krebs' citric acid cycle intermediates, accumulation of **sugars and starches**;
- **Chlorophylls** and **carotenoids**/ chlorophyll precursors;
- Compounds involved in **biosynthesis of plant phytohormones**, such as ABA, SLs, JAs and SAs (although the phytohormones themselves were unlikely to be detected with the untargeted methods employed).

Since many compounds and pathways hypothesised to be affected by AM colonisation in barley are also involved in drought response (e.g. blumenol C derivatives, Krebs' citric acid cycle sugars) it was anticipated that metabolomes of drought-stressed AM plants would be more similar to those of well-watered plants than drought-stressed NM plants, and that AM colonisation would somewhat alleviate the physiological symptoms of drought.

Key findings and interpretations

A suitable experimental system was developed

With the experimental set-up developed in chapter 2, namely using an artificial mix of primarily sand with compost and commercially available AM inoculum, successful

mycorrhizal colonisation of 49% of spring barley cv. Concerto roots was achieved within 6 weeks. Plants treated with an equivalent non-mycorrhizal (NM) inoculum lacked evidence of AM colonisation. These mycorrhizal treatments were achieved again in chapter 4, though with a slightly lower root colonisation of 33.5% (AM WW treatment). Drought stress was not found to significantly reduce AM colonisation, though it was slightly lower in AM DS plants (28%).

An 18 day drought period in half the plants treated with each inoculum from 24 days post inoculation and transplant (DPI) until destructive harvest at 42DPI successfully achieved a drought-stress (DS) treatment which significantly reduced leaf relative water content (RWC), photosynthetic rate (*A*), photosystem II efficiency (Fv'/Fm') and stomatal conductance (g_{sw}) compared to well-watered (WW) control plants that continued to receive irrigation, as demonstrated in experiment 3 (chapters 4 and 5). The developed set-up permitted collection of both leaf metabolite samples and assessment of the aforementioned physiological parameters over a time course during the vegetative growth period of spring barley.

Barley morphophysiological response to AM fungal colonisation was subtle

Commercially-available AM inoculum resulted in approximately 40% root colonisation by AM fungal hyphae and presence of arbuscules in 10-16% of roots (chapter 4). However, this AM colonisation had no effect on above-ground biomass of barley seedlings at 42DPI compared to non-mycorrhizal (NM-treated) plants. The results presented here suggest that barley cv. Concerto is not responsive to mycorrhizal colonisation in the context of this experimental set-up. While this thesis did not directly assess the effect of inoculation on grain yield, the most agronomically-relevant measure of mycorrhizal responsiveness, above-ground biomass at this vegetative growth stage is strongly correlated with yield in spring barley (Křen *et al.* 2014).

While the finding that AM colonisation did not benefit barley growth cannot be extrapolated to a field situation, the evidence that glass-house studies, such as those presented in this thesis, tend to show greater beneficial effects of AM inoculation for the plant than field studies (Zhang *et al.* 2019) intimates that there would be little-to-no benefit of AM inoculation of barley cv. Concerto in the field.

A lack of growth response to AM inoculation may have been linked to the use of a multi-species AM inoculum. Sendek *et al.* (2019) found evidence of antagonism between barley and AM fungal partners at higher AM fungal species richness, particularly under drought conditions. However, recently published work has shown a similar trend to that observed in this thesis: a commercially-available AM inoculum containing only one strain of

AMF had no effect on above-ground biomass in three cultivars of wheat despite significantly increased AM root colonisation over-and-above that observed in the non-sterile field soil plants were grown in (Elliott *et al.* 2020). While that same study found that AM inoculation increased P uptake by the plant, this was not attributable to the mycorrhizal P uptake pathway, and was more likely a consequence of AM fungi altering the soil environment or rhizosphere microbiome (Elliott *et al.* 2020). In this thesis, shoot N and P concentrations were unaffected by AM colonisation (chapter 4). Furthermore, different strains of AM fungus can act as parasitic rather than mutualistic with particular plant species, as demonstrated in *Sorghum bicolor* by Kaur *et al.* 2022, who also found distinct root metabolomic responses to colonisation by different AM fungal strains with contrasting effects on plant growth.

There was a very subtle effect of AM inoculation on barley response to drought in experiment 3 (chapters 4 and 5). No difference in leaf RWC, g_{sw} *or A* was observed between AM and NM plants under either well-watered or droughted conditions. However, AM inoculation did delay the decline in photosystem II efficiency caused by drought for approximately 1 day compared to NM plants. While this very slight delay in damage to photosynthetic machinery did not translate to amelioration of drought effects on physiological parameters or biomass during the 42 day long experiment presented in chapter 4, it implies that, over the lifetime of a plant, AM colonisation may help ameliorate the damaging effects of drought in barley. It may also be that AM plants would be in a better state to recover from drought stress were irrigation to be resumed at any point after 11 days of drought when the divergence in Fv'/Fm' began. Indeed, Khalvati and colleagues (2005) found that beneficial effects of AM colonisation on barley growth were only observed following multiple cycles of drying and re-wetting.

AM inoculation did, however, reduce below-ground biomass under well-watered conditions compared to NM control plants in experiment 3 and in the primarily compost substrate in experiment 2. Under drought conditions in experiment 3, AM inoculation had no effect on root biomass. Despite having a smaller root system compared to non-mycorrhizal plants, the mycorrhizal well-watered plants maintained a similar aboveground biomass, photosynthetic rate, stomatal conductance and photosystem II efficiency (Fv'/Fm'), as well as similar shoot N and P concentrations (chapter 4). Elliott *et al.* 2020 also found AM inoculation to reduce root biomass for some cultivars of wheat, concurrent with a lack of differences in above-ground biomass.

In experiment 3, biomass was not assessed prior to initiation of the drought period (at 24DPI). However, if AM plants entered the drought treatment with smaller root systems than their NM counterparts, and a larger shoot biomass to support (see figure 7.1), the observed

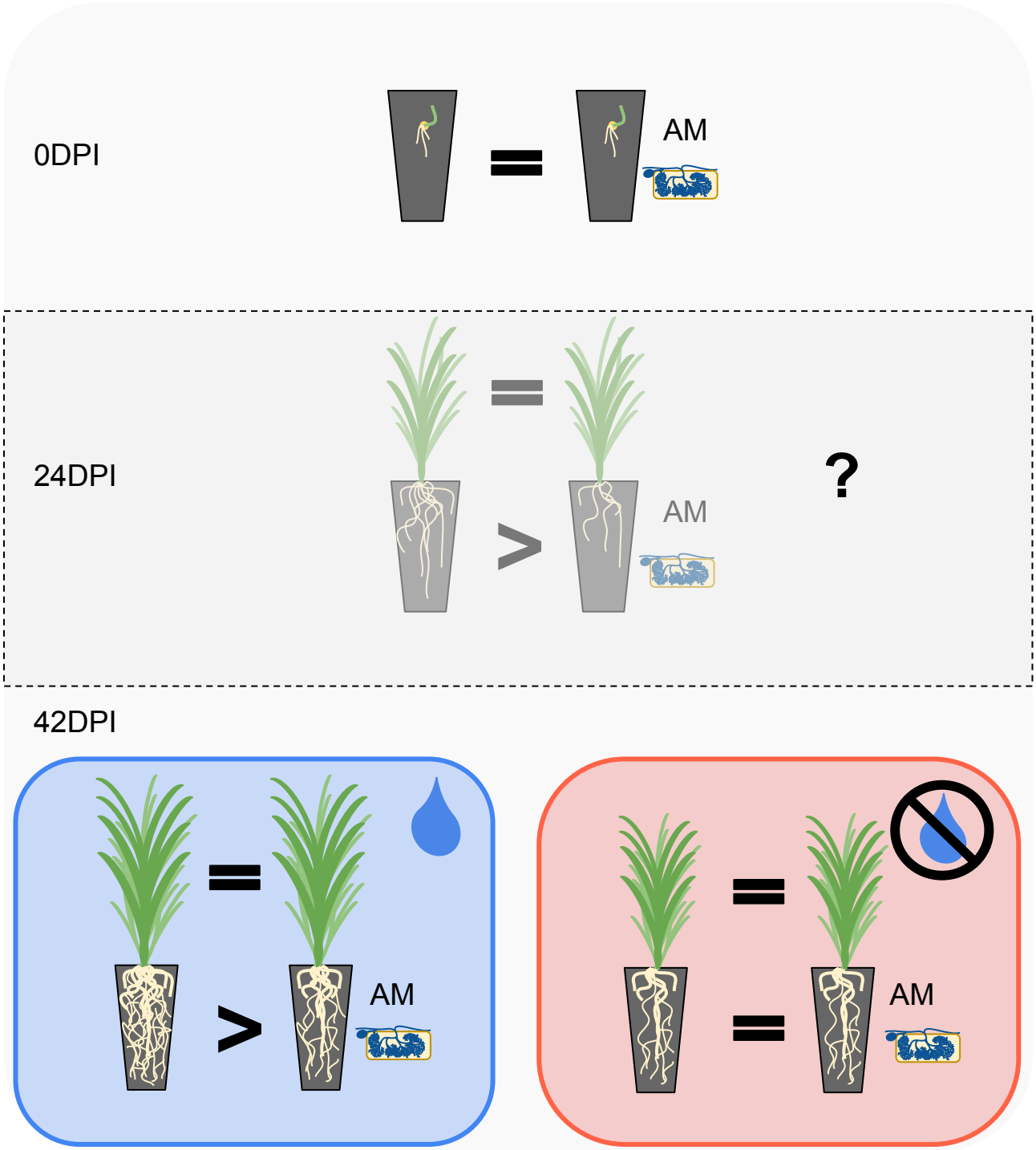

Fig. 6.1. Schematic of biomass differences between mycorrhizal (AM) and non-mycorrhizal (NM) barley plants in experiment 3 (presented in chapters 4 and 5). At 0 days post inoculation (0DPI), seedlings were transplanted into pots with an AM or NM treatment but it was not yet decided which would be subjected to WW or DS treatment. By destructive harvest (42DPI), plants in the drought treatment (red) had endured 18 days of water-withholding and both above- and below-ground biomass were smaller than for well-watered plants but there was no difference between AM and NM plants. However, under well-watered conditions (blue), AM plants had smaller below-ground biomass than NM plants but maintained a similar above-ground biomass. Biomass was not assessed prior to water-withholding (24DPI) so it is not known whether, upon entering the drought, AM plants had a smaller root system than NM plants, as they did at 42DPI in the well-watered condition.

similarities between NM and AM plants' physiological response to drought, and eventual biomasses, may represent a scenario in which AM-treated plants entered the drought period

at a disadvantage (smaller root system) but the AM symbiosis subsequently ameliorated the negative effects of drought for the host, so that, by the time biomass was assessed, there was little difference between the apparent stress tolerance of AM and NM plants.

Drought altered the barley leaf metabolomic fingerprint but age of plant at sampling was the strongest distinguishing factor

This thesis presents two separate experiments in which the polar leaf metabolite fingerprint of well-watered barley could be distinguished from that of plants subjected to drought. In chapter 3, automated direct injection electrospray ionisation mass spectrometry (DI-ESI-MS) was used while in chapter 5, a liquid chromatography step was included for improved annotation (LC-ESI-MS). Chapter 3 compared extracts from leaf samples taken at 35DPI and 42DPI (11th and 18th days of drought respectively) while chapter 5 included an extra, earlier sampling at 29DPI (5th day of drought) in addition to 35 and 42DPI.

Effects of drought stress on the leaf metabolome of non-mycorrhizal (NM) barley could be detected using an untargeted metabolomics approach (both DI-ESI-MS and LC-ESI-MS). In chapter 3, this effect was observed in both positive and negative ionisation modes, and was evident at both 35 and 42DPI (11th and 18th day of drought). In chapter 5, however, clear distinction between drought stress (DS) and well-watered leaf metabolomes was only observed at 42DPI (18th day of drought) (see fig. 7.2).

Directed (supervised) comparison of well-watered and drought-stressed non-mycorrhizal barley leaf metabolomes produced a number of metabolomic features that were causing discrimination between the treatments. Some of these were putatively annotated to MSI level 2 (tentative annotation based on comparison with reference literatureand including orthogonal datta such as LC retention times). These annotations suggested the involvement of the following compounds in the drought response of barley cv. Concerto:

- Flavonoids
- Sesquiterpenoids and terpenoids
- Amino fatty acids
- Chlorophyll precursors (protochlorophyllide, divinylprotochlorophyllide, chlorophyllide *B*)
- Hydroxycinnamic acid derivatives

Many of these compound classes contain antioxidants and may be a response to the generation of reactive oxygen species (ROS) by osmotic stress (Agati *et al.* 2012).

Flavonoids and hydroxycinnamic acids, as well as some terpenoids, and their glycosylation patterns in particular, have been found in other untargeted studies to accumulate in barley leaves under drought stress (Chmielewska *et al.* 2016, Swarcewicz *et al.* 2017, Piasecka *et al.* 2017, Kowalczewski *et al.* 2020, Piasecka *et al.* 2020).

In chapter 5, chlorophyll precursors were associated with the well-watered (WW) treatment and their importance in discriminating between WW and drought-stressed (DS) plants was concurrent with a significant decline in photosystem II efficiency in DS plants.

Contrary to expectations, the results presented in this thesis provided no evidence to support proline accumulation as distinguishing droughted and well-watered barley plants from each other, in contrast to the findings of Templer *et al.* (2017) who highlighted proline accumulation as important across cultivars in the barley response to drought.

This thesis also provided evidence of an altered metabolite profile in barley at different growth stages - in chapter 5, age at sampling had the largest effect on the leaf metabolomic fingerprint and masked the effects of drought and AM inoculation treatment. Phenylpropanoids and alkaloids were of particular note among putatively annotated metabolites that were differentially associated with tillering or stem elongation stages of development. Results from chapter 5 support the findings of other studies in observing the tillering stage to be particularly important for flavonoid production (Lee *et al.* 2016, Yan *et al.* 2022).

Drought-induced metabolome changes did not precede observed physiological responses to drought

Contrary to expectations, drought-induced metabolome changes were only clearly observed following a significant decline in photosystem II efficiency (at 18th day of drought in chapter 5 and 11th day of drought in chapter 3) (see fig. 7.2) and metabolomes of droughted and well-watered plants were not clearly distinguishable by their leaf metabolomes when sampled preceding significant differences in photosystem II efficiency or stomatal conductance. This suggests that the changes detected in the leaf metabolome in this study were symptomatic of damage experienced by the plant in response to drought stress rather than coping mechanisms for drought tolerance or avoidance. In tibetan hulless barley, PEG-induced drought responses were detected in the leaf metabolome over the first 48 hours of simulated drought exposure, and the metabolomic response peaked at 8 hours (Yuan *et al.* 2018). This suggests that the initial response of barley to drought preceded the sampling time points used in this thesis (onset of drought occurred before the 5th day of

drought treatment at which point stomatal conductance was already significantly reduced in droughted plants in both chapters 3 and 5).

AM colonisation reduced the impact of drought on the leaf metabolome

In a recent study of *Brachypodium distachyon,* the leaf metabolome was not so responsive to AM colonisation as the root metabolome, despite containing more compounds (Mahood *et al.* 2022) but it was possible to distinguish between AM and NM leaf metabolomes in an unsupervised PCA. However in the present study, effects of AM colonisation on the leaf metabolome of barley in chapter 5 were more subtle. Only through specific pairwise comparisons based on observed differences in physiology and biomass was it possible to observe differences between AM and NM plants' metabolomic fingerprints, and to form hypotheses of the differences between the AM and NM "response" to drought.

The compounds and pathways putatively annotated as important distinguishing features of the AM leaf metabolome and AM response to drought in barley were:

- Ethanolamine, a glycerophospholipid precursor, important in biological membranes, as well as an early precursor in the glycine betaine biosynthesis pathway. Glycine betaine is a compatible solute involved in drought response (Ladyman *et al.* 1980);
- 12-oxophytodienoic acid (12-OPDA), an anti-transpirant and precursor of the phytohormone jasmonic acid (JA) which has a role in drought signalling (Salvi *et al.* 2021) and has also been found to be upregulated by AM colonisation (Adolfsson *et al.* 2017, Quiroga *et al.* 2018);
- Lactate, a product of the detoxification of methylglyoxal, which is produced during a range of stress conditions (Jain *et al.* 2020);
- Protoporphyrinogen IX, a precursor to tetrapyrrole biosynthesis including heme and chlorophylls, as well as protochlorophyllide, a chlorophyll precursor (Tanaka & Tanaka 2007);
- Ubiquinone which is involved in diverse electron transfer roles in higher plants, notably, conversion of protoporphyrinogen IX to protoporphyrin prior to tetrapyrrole biosynthesis, and conversion of succinate to fumarate in the tricarboxylic acid (TCA) cycle (Tanaka & Tanaka 2007).

Taken together these candidates for AM-mediated drought response are consistent with the observed effect of delayed damage to photosystem II efficiency in AM DS plants compared to NM DS plants (chapter 4). However, little evidence of AM effect on the leaf metabolome was observed before the 18th day of drought, by which time stomatal conductance and photosynthetic rate had dramatically declined in both AM and NM plants, suggesting that any

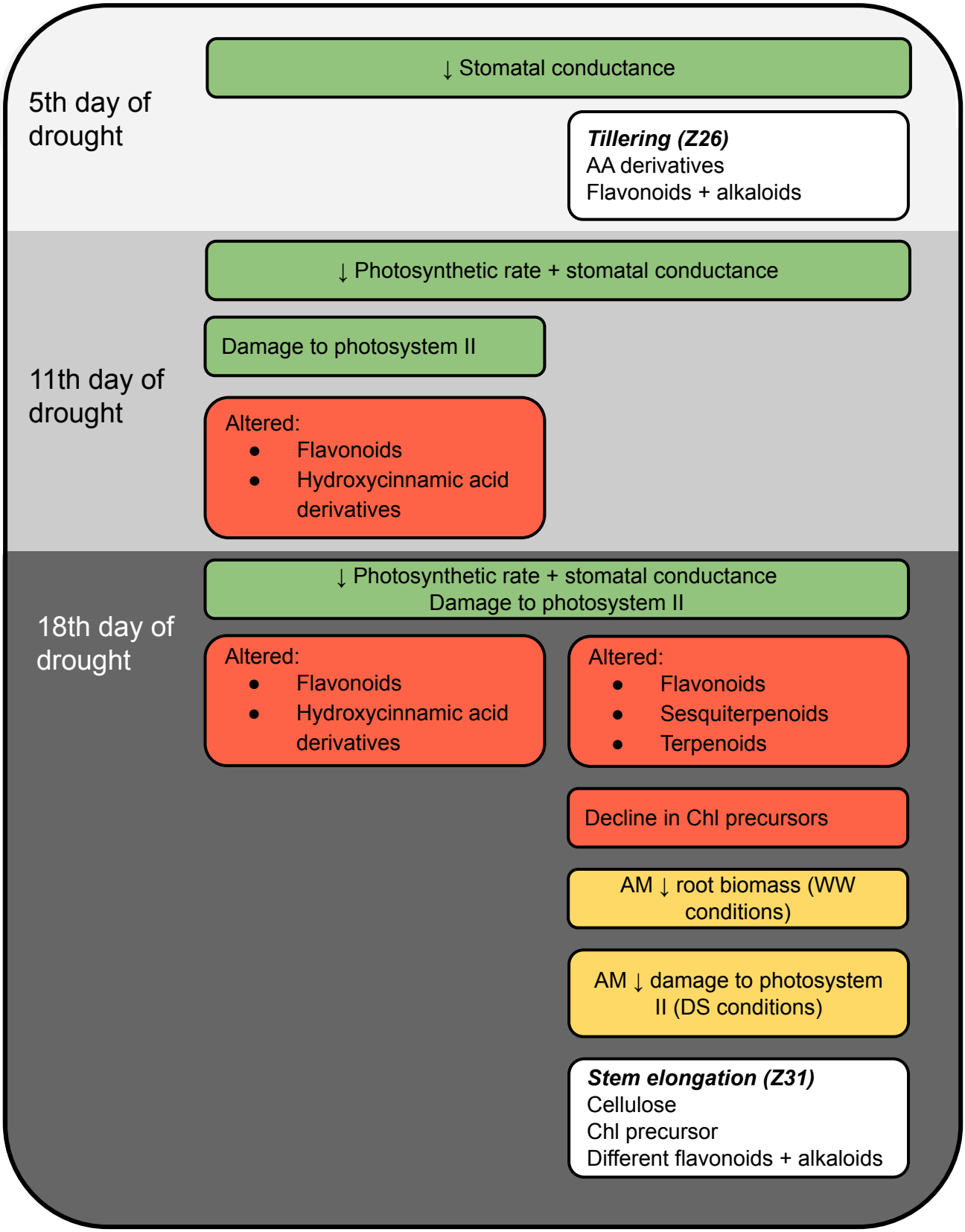

Fig. 7.2. Summary of effects of drought treatment on physiology (green boxes) and leaf metabolome (red boxes) and of AM inoculation (yellow boxes) on biomass and physiology of barley. Metabolome differences between tillering and stem elongation are shown (white boxes). On the left are results from experiment 1 (presented in chapter 3) while results on the right are from experiment 3 (presented in chapters 4 and 5).

metabolomic differences observed were more a sign of damage sustained by the leaves rather than an active drought response.

Hill *et al.* (2018) found no modulation of the leaf metabolome in response to AM colonisation when they investigated it in ragwort. Even in studies where effects of AM colonisation on the leaf metabolome have been observed, effects can vary dramatically depending on AM fungal strain: in potato *Claroideoglomus lamellosum* had an inverse effect on phenolics compared to to *Claroideoglomus claroideum* and *Funneliformis mosseae* (Fritz *et al*. 2022).

Furthermore, notable changes that have been observed in other studies tend to require semi-targeted approaches and would not be detectable by the untargeted metabolomics techniques employed here. In 2018, a group of researchers proposed a protocol for quantification of specific blumenol C derivatives as leaf biomarkers for the AM symbiosis (Mindt *et al.* 2018) and have observed modulation of these carboxy- and hydroxy-blumenol C -glucosides by AM fungal colonisation in six species including barley and wheat (Wang *et al.* 2018). No evidence of these were found in the data presented in chapter 5 of this thesis, however it is worth noting that the extraction protocol and mass-spectrometry techniques were not optimised for these compounds. Blumenols and related terpenoids are of interest in relation to AM-mediated drought responses since they have also been found to be upregulated by drought stress in barley (Piasecka *et al.* 2020).

A number of recent studies using untargeted or semi-targeted metabolomics approaches have suggested that studying the metabolomic effects of the AM symbiosis may need to consider more than just the root colonisation status of the plant. Yurkov *et al.* (2021) have proposed that AM colonisation alters the “biochemical maturation” of the plant partner, meaning that AM and NM plants at the same calendar age may actually represent different phenological ages. Developmental shifts in the metabolome have also been observed by Schtark *et al.* 2019, with leaf metabolomes of AM-inoculated pea leaves more similar to those of NM plants from earlier sampling time points. In tomato, a faster transition to reproductive growth was observed, with AM colonisation shortening the vegetative growth period and accelerating development (Fracasso *et al.* 2020).

Combining untargeted metabolomics and physiological techniques to assess plant responses to drought

The experimental set-up and timing of sampling used in this thesis made assessing both metabolomic and physiological responses to drought in the same plants possible. Despite a trade off between maintaining the number of biological replicates required for reliable metabolomic data processing and analysis with the practicalities of the frequency of

sampling, the approach used enabled detailed interrogation of the metabolomics data that would not have been possible with only treatment metadata.

An untargeted metabolomics workflow was developed to automate parts of this analysis. Incorporating open source/ freely available tools for metabolomics resulted in access to more troubleshooting help, more thorough analysis of the data, faster data processing, more reproducible methods and results.

LC-ESI-MS gave more detail for identifying compounds of interest in this thesis but DI-ESI-MS was acceptable and could be sufficient for hypothesis forming in this type of experiment. Used in conjunction with the workflow developed (rather than relying on proprietary software), particularly where both +ve and -ve mode data are available, DI-ESI-MS provides a rapid method for initial identification of compound classes of interest that can be pursued with targeted approaches. Using DI-ESI-MS would allow processing of more samples (improving speed and/ or reducing cost) and therefore could permit the use of more biological replicates or sampling time points.

Limitations of this thesis

In this thesis, only one cultivar of spring barley and one approach to metabolite sampling were employed in a fairly reductionist pot experiment, using an artificially mixed substrate. This greatly limits the possibility of extrapolating results to real-world contexts, such as field soils, but did improve the reproducibility of the experiments.

The problem of true controls in AM symbiosis research

The question of how to achieve mycorrhizal and non-mycorrhizal plants to be compared is an issue that has plagued experimental set-ups in the field of symbiosis research for decades (Gryndler *et al.* 2018). Is the non-mycorrhizal plant the control or is the true control that which occurs “in the wild”? For many plants this “true control” would be the plant engaged in mycorrhizal symbiosis. Crop plants introduce a further conundrum in that they are often capable of forming active arbuscular mycorrhizal symbiosis (with the notable exception of certain *Brassicaceae spp.*) but are bred and grown in contexts which are detrimental to the fungal partner, or result in functional redundancy of the symbiosis (e.g. inorganic fertiliser addition, fungicide use, monoculture, short growth season) (Cosme *et al.* 2018). Furthermore, the AM symbiosis has been shown to involve a diverse “mycorrhizosphere” of associated bacteria and archaea (Garbaye 1991), as well as spore endophytic bacteria (Cruz & Ishi 2012, Gulbis *et al.* 2013), all of which can have diverse effects on the host plant’s growth, nutrition and stress response.

Axenic lab-grown cultures of single strains of AM fungi represent one option for adding AM inoculum (in a liquidised form) to pot experiments. These cultures are grown on a gel media in symbiosis with Ri T-DNA transformed carrot root, due to the obligate biotrophic nature of AM fungi. This is the method of applying AM propagules to transplants used in experiment 1 of this thesis, in which inoculation failed to result in mycorrhizal colonisation of barley roots (chapter 1). While the theory is that these are axenic cultures, they do contain associated microorganisms, including endophytic bacteria, that after multiple rounds of subculturing, can sporadically take over the culture (Gulbis *et al.* 2013). Whilst the cultures used as inoculum in experiment 1 were "young" in themselves, they had been obtained from a line of cultures that had undergone many sequential subcultures. It is possible that they had become either less effective at colonising roots, could no longer acclimate to the substrate conditions or had been taken over by an unknown microorganism that interfered with the fungus' ability to colonise the barley roots.

Achieving non-mycorrhizal conditions in field soil is problematic. Most agricultural field soils contain at least some AM propagules (e.g. AM spores, AM hyphae, or roots containing AM hyphae) and therefore these have to be eradicated to achieve a non-mycorrhizal comparison treatment. To reduce the magnitude of this problem, an artificially prepared substrate was used for experiments presented in this thesis by mixing sand and compost and then autoclaving twice with a week's interval. Autoclaving substrate is known to alter the chemistry of that substrate and can be detrimental to plant health in the short term so substrate was subsequently left to air dry (Rovira & Bowen 1966) before mixing with AM/NM inocula and transplanting seedlings. However, recolonisation by air-borne spores is possible during this period.

With the liquified axenic cultured inoculum it is possible to produce comparable cultures containing media and root but lacking the AM fungus to use as NM inoculum (as in experiment 1). However, NM inocula are rarely true controls for addition of AM propagules due to the obligate biotrophic nature of AM culture. In experiment 2 and 3 in which a commercial AM fungal inoculum was used, the producer (Plantworks Ltd., Kent) was kind enough to provide an equal volume of the carrier substrate used in the inoculum production process (granular clay) which was used as an NM inoculum. The AM inoculum contained roots of the plant on which the fungi had been cultured and the NM inoculum was therefore not a complete control as these were lacking in the NM treatment group.

Where AM colonisation has been achieved in this thesis, it has been through the use of a mixed species inoculum. Where multiple cultivars are being compared, using a mixed AM species inoculum could be considered an advantage as it permits symbiont selection from a

range of options that might be more-or-less appropriate for different cultivars (van der Heijden *et al.* 2009, Wagg *et al.* 2011). However, in the experiments presented here it is not possible to determine whether differences in responses between plants has been influenced by which AM fungal species had formed a symbiosis with each plant. Furthermore there may have been variation in how many AM strains formed symbioses with each plant, which has been shown to influence the "outcome" of symbiosis for the host, particularly in controlled growth environments (van Geel *et al.* 2016) and inter-strain competition has also been shown to have impacts on the functioning of the symbiosis (Sendek *et al.* 2019). Commercial multi-strain inoculum and multi-strain field-soil derived inoculum were recently found to have a stronger effect on barley root:shoot ratio, above-ground phenolic content and phosphorus concentration than those inoculated with a single strain commercial inoculum (Frew 2020).

While inoculation methods presented in experiment 2 and 3 resulted in mycorrhizal and non-mycorrhizal plants, it is not possible to rule out effects of inter-strain competition between AM fungal strains and/ or variation due to strain-specific effects.

Drought in a pot

This thesis sought to investigate the effects of a prolonged drought stress on barley at the vegetative growth stages and the drought period was extended beyond the duration required to elicit a significant reduction in photosystem II efficiency. This was an artificial drought stress, and does not provide information on drought recovery. As in the results presented in this thesis, Khalvati and colleagues (2005) found little beneficial effect of AM colonisation on barley photosynthesis or growth after a single drought cycle. However they did demonstrate that after multiple drying-rewetting cycles, AM colonisation was of significant benefit to the drought tolerance of the host. Thus, this thesis cannot provide conclusions about the effects of AM on drought response of barley under alternative drought scenarios.

Many of the effects of AM symbiosis on the drought response of crop plants depend on their ability to explore and alter the soil and form networks of mycelia, which is disrupted by containing them within a plastic pot (Ryan & Graham 2018).

Impact

Improved understanding of the barley leaf metabolome

This thesis has contributed to the understanding of the effects of drought stress and age/ growth stage on the secondary metabolite profile of spring barley. There has recently been a significant research effort into the nutraceutical benefits of barley grass (or green barley) (reviewed in Zeng *et al.* 2018), showing that plant secondary metabolites such as flavonoids,

other polyphenols, vitamins and alkaloids from barley have antioxidant properties and that their intake could contribute to prevention of chronic diseases in humans, such as diabetes (Yu *et al.* 2002), cancers (Kubatka *et al.* 2016) and cardiovascular disease (Moussazadeh *et al.* 1992, Liu *et al.* 2017) when consumed at the vegetative stage. As such, results on the timing of accumulation of polyphenols in barley leaves during vegetative growth stages can inform decisions on optimum harvest times for barley grass intended for nutraceutical use (Lee *et al.* 2016).

In addition to direct nutraceutical benefits of barley grass polyphenols, the effect of drought stress on secondary metabolite profiles of barley leaves described in this thesis provides hypotheses that contribute to the direction of research into drought mechanisms in barley. The leaf metabolome profile of *Hordeum vulgare* cv. Concerto has been described for the first time. Furthermore, information on the effects of arbuscular mycorrhizal fungi on the secondary metabolite profile of barley, and cereal crop plants in general, remains limited (Balestrini *et al.* 2020) and so this thesis goes some way to rectifying the information gap.

Improved understanding of arbuscular mycorrhizal symbiosis in barley

The effects of AM fungi on *Horduem vulgare* cv. Concerto have been investigated for the first time in this thesis. Results suggest AM colonisation does not benefit spring barley cv. Concerto in terms of biomass or improved tolerance to drought - and may even increase susceptibility to drought through reduced root biomass.

The finding that a commercially-available AM inoculum did result in colonisation of *Horduem vulgare* within 6 weeks is an indicator that this product does indeed contain active AM fungi, however this did not translate into biomass benefits for the crop plant in this thesis. This does not discount yield benefits which were not tested here, however vegetative growth is strongly correlated with yield in barley (Křen *et al.* 2014) and the results presented here may influence decisions on whether AM inoculation of barley is worthwhile.

While extrapolation of these findings to an agronomically relevant scale is discouraged due to the reductionist nature of the experiments conducted, it still contributes important insight for research since studies detailing the effects of mycorrhizal inoculation or colonisation on barley growth and yield or response to drought are rare (Jayne & Quigley 2014, Zhang *et al.* 2019).

Improved reproducibility of untargeted metabolomics workflow

This thesis highlights the need for well-annotated metabolite libraries and openly available, detailed datasets with well-documented meta-data. It is challenging to annotate LC-ESI-MS

data with compound identities when only integer values are given for m/z in some manuscripts. In compiling this thesis, a number of correspondences were initiated to gain access to more precise LC-ESI-MS data from published studies but inadequate responses were obtained. In a number of cases, published data statements were misleading or data deposited in repositories were unavailable due to mislabelling or broken links.

The untargeted metabolomics workflow and guide included in this thesis (chapter 6 and appendix I) was developed with support from the University of Sheffield Library's "Unleash your data and software" funding. The workflow automates previously time-consuming steps of the processing and analysis of untargeted metabolomics, such as formatting peak tables for various online tools. While automatic annotation of LC-ESI-MS data-sets is possible, e.g. using XCMS online, it is still inappropriate and uninformative for many data-sets (due in part to the huge number of unannotated plant metabolites). Annotation requires a huge amount of labour for non-standard metabolites (most plant secondary metabolites). The workflow guide presented in chapter 6 somewhat reduces the time taken for this labour-intensive annotation step.

This workflow has resulted in faster analysis, an interoperable approach, and has removed the need to rely on some proprietary softwares, with their associated licence fee, thus reducing the cost of analysis. Remote access to, and analysis of, data-sets obtained by LC-ESI-MS (as well as automated DI-ESI-MS and MALDI-ToF) is now possible for researchers in the biOMICS facility and the scientific community in general thanks to the publication of an online workflow and Parker *et al.* 2023. In testing the workflow, a community of researchers from the Universities of Sheffield and Manchester have been brought together, facilitating collaborative working on metabolomics data-sets from *Escherichia coli, Hordeum vulgare, Solanum tuberosum, Pisum sativum* and *Chlorella* spp. This collaboration is ongoing and can be widened by communication and contributions to the github repository for the project (https://github.com/LizzyParkerPannell/Untargeted_metabolomics_workflow accessed 24 March 2023). The online guide to the workflow (https://untargeted-metabolomics-workflow.netlify.app/) is written in accessible language and removes barriers to metabolomics analysis normally associated with workflows that rely on academic or standard software documentation.

Further research directions

Further work would include growing barley cv. Concerto to yield with the same AMF and drought treatments to investigate:

- How does AM colonisation affect the yield of spring barley when it has been droughted?
- Do mycorrhizal plants recover quicker than non-mycorrhizal plants when a recovery period is included (as suggested by Khalvati *et al.* 2005) and is this detectable in the leaf metabolomic fingerprint?
- Do mycorrhizal plants alter the biochemical maturation of barley, as has been suggested in pea (Schtark *et al.* 2019) and *Medicago* (Yurkov *et al.* 2021), and does this aid their ability to survive drought?

LC-ESI-MS/MS and targeted metabolomic protocols would allow better annotation of leaf extracts that would permit the subsequent use of pathway/ cluster analysis tools such as those employed for *B. distachyon* by Mahood *et al.* 2022. Of particular interest would be:

- Targeted analysis of phytohormones (e.g. abscisic acid and strigolactones that were not detectable with the untargeted approach used in this thesis; further quantifiable analysis of the jasmonate biosynthesis and signalling pathways);
- Quantitative analysis of phenylpropanoid antioxidants, in particular flavonoids, as well as their antioxidant activity.
- Quantitative analysis of chlorophyll contents, and targeted analysis of tetrapyrrole biosynthesis pathways.

Concluding Summary

Studies in barley are under-represented in the research of AM-mediated drought responses and this thesis contributes an example of the very subtle effects AM colonisation can elicit, in contrast to the expectation that AM colonisation is beneficial to crop plants under drought (Jayne & Quigley 2014).

Arbuscular mycorrhizal (AM) colonisation provided a slight ameliorative effect of drought stress symptoms in barley (*Hordeum vulgare* cv. Concerto) during an 18 day drought period at the tillering and stem elongation stage of development. This was observed as an approx. 1 day delay to drought-induced reduction of photosystem II efficiency (Fv'/Fm').

Concurrent with this delayed damage to the photosynthetic machinery, untargeted LC-ESI-MS metabolomics of leaf extracts indicated the potential involvement of tetrapyrrole biosynthesis, jasmonic acid biosynthesis, glycerophospholipid biosynthesis, and glyoxal detoxification pathways in the AM plants' response to drought. No effect of AM colonisation on aboveground biomass, stomatal conductance, photosynthetic rate or leaf relative water content (RWC) was observed in this thesis in either well-watered or drought stress conditions. Age of barley seedlings at the time of harvesting metabolite samples had a larger effect on the metabolomic fingerprint than either drought treatment or AM colonisation treatment.

Phenylpropanoids (flavonoids, lignans, hydroxycinnamic acid derivatives) were highlighted by both DI-ESI-MS and LC-ESI-MS in two separate experiments as important in the drought response of barley cv. Concerto, and were also shown to be primarily associated with the tillering stage of development. Alkaloids and terpenoids were also involved in the drought and AM responses of barley leaf metabolomes. Further targeted mass spectrometry approaches could identify specific compound identities amongst these compound classes.

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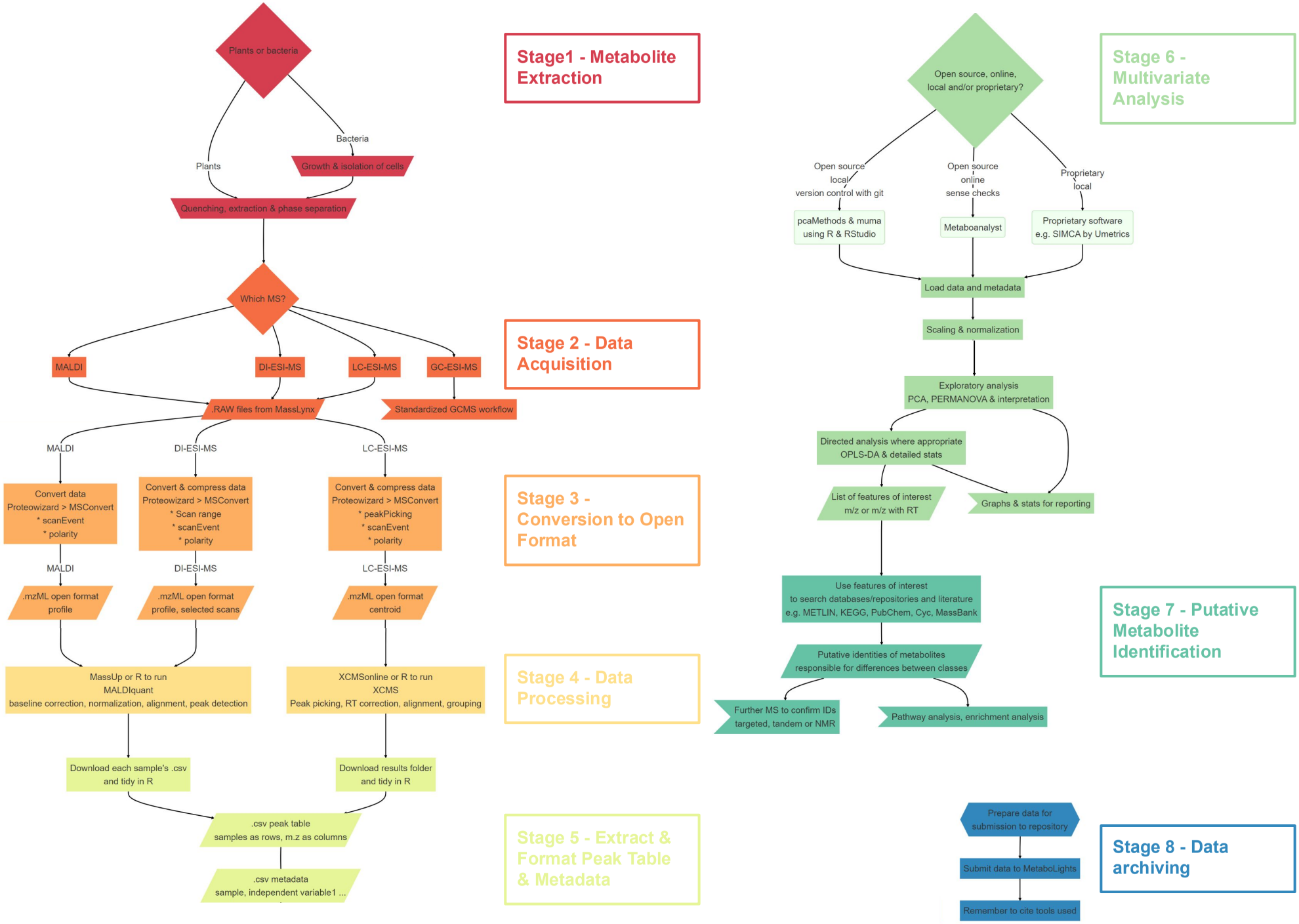
Appendices



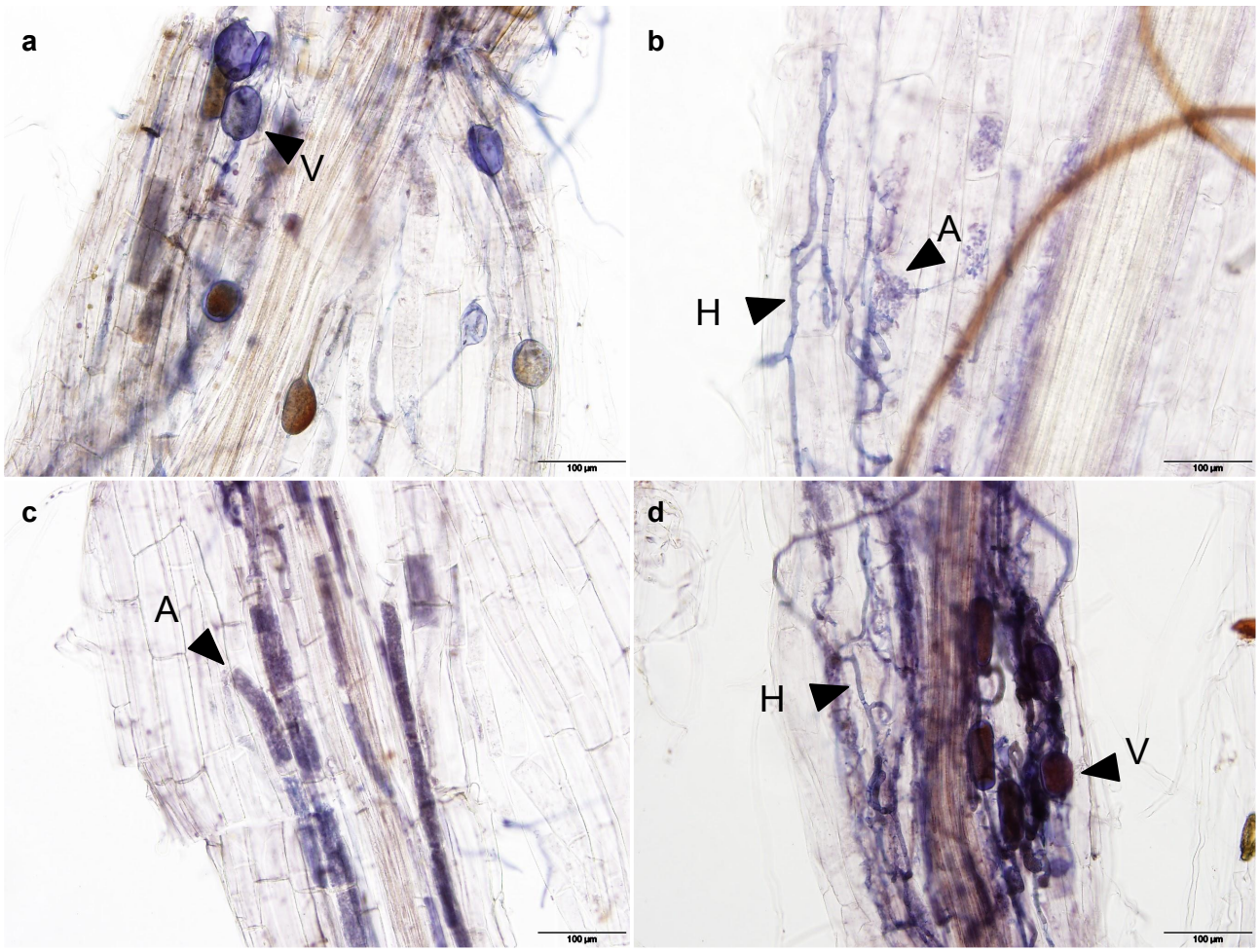
Appendix I. Conceptual diagram of stages in an untargeted metabolomics workflow (as used in chapter 5 and detailed in chapter 6)

Appendix II. Statistical summaries of the effects of Drought treatment on mass of water in pots, leaf relative water content (RWC), efficiency of photosystem II (Fv'/Fm'), stomatal conductance, photosynthetic rate, shoot and root dry weights (DW). NS = non significant, * = significant at the 90% CI, ** = significant at the 95% CI, *** = significant at the 99% CI

	Statistical test	Drought treatment	
Mass water remaining in pot (% PC)	GLMM	X2X=91.209, df=1, p<2.2e-16	***
Fv'/Fm'	repeated measures ANOVA	F=10.68, df=1, p=0.00115	***
Leaf RWC	2 sample t test	t=-6.3785, df=9.2512, p=0.0001138	**
Stomatal conductance	2 sample t test	t=-10.898, df=14.564, p=2.148e-8	***
Photosynthetic rate	2 sample t test	t=-4.741, df=11.17, p=0.0005833	***
Shoot DW	2 sample t test	t=-12.368, df=22.53, p=1.604e-11	***
Root DW	2 sample t test	t=-8.4375, df=24.595, p=9.998e-09	***

Appendix III. Summary of variance explained by PC1 and PC2 (or t and orthogonal t) and model goodness of fit for PCA and OPLS-DA analyses in chapter 3.

	Mode	N	PCA				OPLS-DA				
			R2X	Q2	PC1 %	PC2 %	R2X	R2Y	Q2	t %	to %
All samples	positive	65	0.63	0.39	19.50	9.21	-	-	-	-	-
	negative	56	0.65	0.15	15.20	10.40	-	-	-	-	-
35DPI	positive	27	0.39	0.95	19.40	16.00	0.29	0.99	0.95	15.20	6.76
	negative	21	0.61	0.20	18.80	9.43	0.72	1.00	0.92	16.60	10.40
42DPI	positive	38	0.42	0.31	25.80	8.16	0.37	0.97	0.93	10.20	5.23
	negative	35	0.41	0.23	20.40	7.56	0.49	1.00	0.93	17.20	9.20



Appendix IV. Photomicrographs showing evidence of mycorrhizal colonisation in 6 week old barley (experiment 3). Barley roots at x200 magnification show arbuscules (A), vesicles (V) and intraradical hyphae (H) in blue stained with ink and vinegar: (a) and (c) show roots from AM well-watered plants while (b) and (d) show roots from AM drought-stressed plants.

Appendix V. Statistical summaries of the effects of AM inoculation. Drought treatment and their interaction on mass of water in pots, leaf relative water content (RWC), efficiency of photosystem II (Fv'/Fm'), shoot and root dry weights (DW), root/shoot ratio, shoot and root C/N ratios.. NS = non significant, * = significant at the 90% CI, ** = significant at the 95% CI, *** = significant at the 99% CI

	Experiment 3			
	Analysis	**AM inoculation**	**Drought treatment**	**AMF * Drought**
Root colonisation by AMF	2-way ANOVA	F=77.604, df=1 p=4.58e-10	F=0.840, df=1, p=0.366	F=0.761, df=1, p=0.389
Mass water remaining in pots (% PC)	GLMM	□2□=13.625, df=2, p=0.0011	□2□=1018, df=2, p<2.2e-16	□2□=7.84, df=1, p=0.00511
Leaf RWC	2-way ANOVA	F=1.60, df=1, p=0.225	F=658, df=1, p=2e-14	F=0.233, df=1, p=0.636
Fv'/Fm'	GLMM	□2□=25.3, df=1, p=4.81e-7	□2□=6.71, df=1, p=0.00958	□2□=0.0085, df=1, p=0.927
Shoot DW	2-way ANOVA	F=2.81, df=1, p=0.103	F=136, df=1, p=4.37e-13	F=2.20, df=1, p=0.148
Root DW	2-way ANOVA	F=5.17, df=1, p=0.0298	F=85.2, df=1, p=1.55e-10	F=6.15, df=1, p=0.0186
Root/ shoot ratio	2-way ANOVA	F=2.52, df=1, p=0.122	F=25.8, df=1, p=1.56e-5	F=8.57, df=1, 0.00624
Shoot C/N ratio	2-way ANOVA	F=0.754, df=1, p=0.394	F=74.5, df=1, p=8.02e-9	F=0.112, df=1, p=0.741
Root C/N ratio	2-way ANOVA	F=27.6, df=1, p=2.18e-5	F=99, df=1, p=5.44e-10	F=16.2, df=1, p=0.000503

	Analysis	**AM inoculation**	**Drought treatment**	**AMF * Drought**
Root colonisation by AMF	2-way ANOVA	***	NS	NS
Mass water remaining in pots (% PC)	GLMM	***	***	**
Leaf RWC	2-way ANOVA	NS	***	NS
Fv'/Fm'	GLMM	***	**	NS
Shoot DW	2-way ANOVA	NS	***	NS
Root DW	2-way ANOVA	*	***	*
Root/ shoot ratio	2-way ANOVA	NS	***	**
Shoot C/N ratio	2-way ANOVA	NS	***	NS
Root C/N ratio	2-way ANOVA	***	***	***

Appendix VI. DI-ESI-MS and LC-ESI-MS instrument settings

DI-ESI-ToF-MS was performed with a Waters Synapt G2Si mass spectrometer (Waters Ltd, Manchester, UK) with a Waters ADC acquisition device. MassLynx (version 4.2) software provided instrument control, data acquisition and data pre-processing. Mass detection range was set to 50-1200 Da and scans were acquired at a rate of one spectrum s−1 (1.0 s scan time, 0.014 s interscan delay). A Lockspray™ interface was used to allow automated accurate mass measurements of plant metabolites and for daily quality control checks. Leucine enkephalin, 5 µg cm−3 in 1/1 (v/v) acetonitrile/water, was employed during Lockspray operation as an internal mass reference. Following acquisition, 30 scans covering the sample detection signal (as opposed to background signal from wash) were manually extracted from the chromatogram in MassLynx for subsequent analysis

DI-ESI-MS settings:
Polarity: **positive**
Capillary voltage: 2.5kV
Sampling cone voltage: 20V
Reference cone voltage: 30V
Source temperature: 100°c
Desolvation temperature: 280°c
Desolvation gas flow: 600 l hour-1
Nebulizer gas flow: 6.5 Bar
Solvent: 50:50 methanol to water at flow rate 5µl min-1
Injected volume: 10µl

DI-ESI-MS settings:
Polarity: **negative**
Capillary voltage: 1.8kV
Sampling cone voltage: 2V
Reference cone voltage: 30V
Source temperature: 100°c
Desolvation temperature: 280°c
Desolvation gas flow: 50 l hour-1
Nebulizer gas flow: 3 Bar
Solvent: 50:50 methanol to water at flow rate 5µl min-1
Injected volume: 10µl

UPLC-ESI-ToF-MS was performed with a Waters Synapt G2Si mass spectrometer (Waters Ltd, Manchester, UK) with a Waters ADC acquisition device. MassLynx (version 4.2) software provided instrument control, data acquisition and data pre-processing. Mass detection range was set to 50-800 Da and scans were acquired at a rate of one spectrum s−1 (1.0 s scan time, 0.014 s interscan delay) over 7.6 minutes. A Lockspray™ interface was used to allow automated accurate mass measurements of plant metabolites and for daily quality control checks. Leucine enkephalin, 5 µg cm−3 in 1/1 (v/v) acetonitrile/water, was employed during Lockspray operation as an internal mass reference.

UPLC settings:
Column: Waters Acquity BEH C18
Size: 130 Å x 2.1mm x 50mm
Solvents: Water and acetonitrile
Linear gradient system: initially at 95% water changing to 65% water at 3 mins and 0% water at 6mins at a constant flow rate of 0.4 ml min^{-1}

ESI-MS settings:
Polarity: positive
Capillary voltage: 2.5kV
Sampling cone voltage: 20V
Reference cone voltage: 30V
Source temperature: 100°c
Desolvation temperature: 280°c
Desolvation gas flow: 600 l hour-1
Nebulizer gas flow: 6.5 Bar
Solvent: 50:50 methanol to water at flow rate 5µl min^{-1}
Injected volume: 1µl

Appendix VII. XCMS online (LC-ESI-MS data processing) parameters

Polarity: Positive

Feature Detection
- Method: CentWave
- ppm: 20
- min. peak width: 2
- max peak width: 60
- mzdiff: 0.01
- Signal/ noise threshold: 6
- Integration method: 1
- Prefilter peaks: 3
- Prefilter intensity: 100
- Noise filter: 0

Retention time correction
- Method: obiwarp
- profStep: 1

Alignment
- bw: 1
- minfrac: 0.5
- mzwid: 0.025
- minsamp: 1
- max: 100

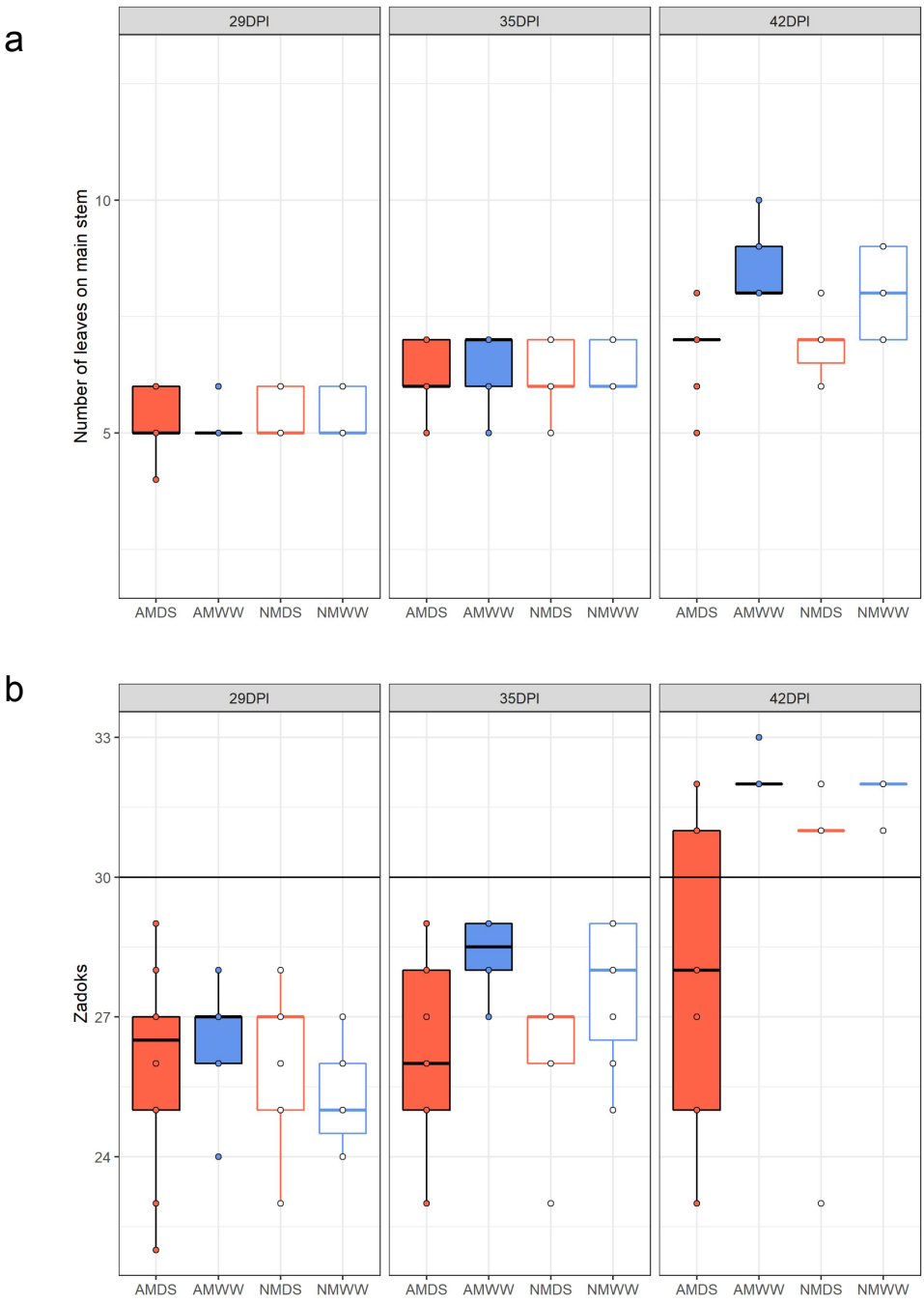
(XCMS online not used for annotation or visualisation so settings not detailed here)

Miscellaneous
- Correct mass calibration gaps (TICK)

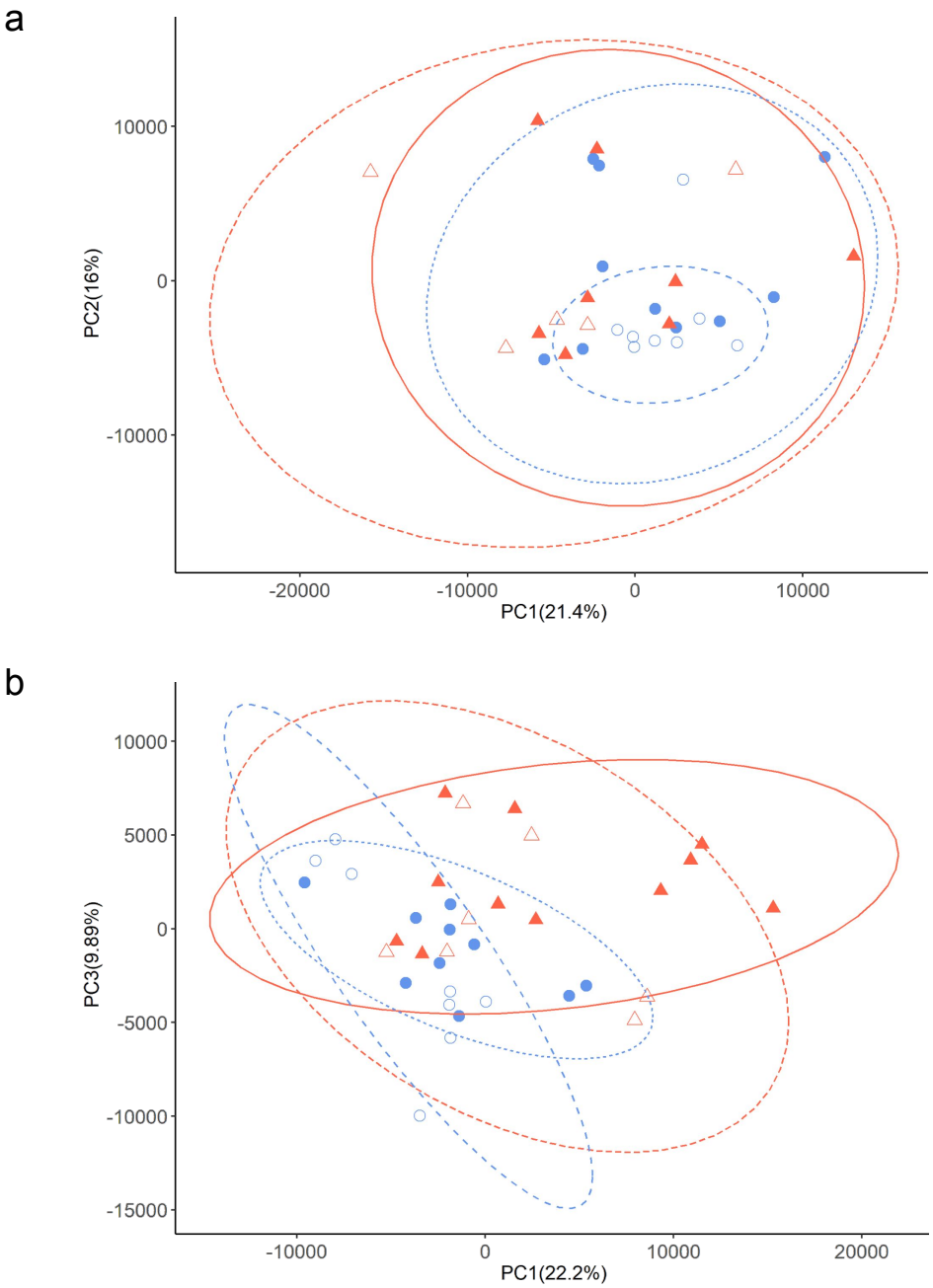
Appendix VIII. Multivariate summary statistics for chapter 5 (PERMANOVAs). NS = non significant, * = significant at the 90% CI, ** = significant at the 95% CI, *** = significant at the 99% CI

Source of variation	Df	Sum of squares	R2	F	p	
*All samples ~ Time point * Drought * AMF*						
Time	2	1.83	0.28	20.29	0.001	***
Drought	1	0.17	0.03	3.80	0.003	**
AMF	1	0.07	0.01	1.61	0.144	NS
Time:Drought	2	0.24	0.04	2.66	0.004	**
Time:AMF	2	0.08	0.01	0.91	0.493	NS
Drought:AMF	1	0.02	0.00	0.54	0.781	NS
Time:Drought:AMF	2	0.07	0.01	0.81	0.656	NS
Residual	89	4.00	0.62			
Total	100	6.49	1.00			
*NM samples only ~ Time point * Drought * AMF*						
Time	2	0.69	0.30	8.79	0.001	***
Drought	1	0.07	0.03	1.84	0.106	NS
Time:Drought	2	0.18	0.08	2.23	0.012	**
Residual	35	1.38	0.59			
Total	40	2.31	1.00			
*AM samples only ~ Time point * Drought * AMF*						
Time	2	1.30	0.32	14.16	0.001	***
Drought	1	0.13	0.03	2.84	0.018	**
Time:Drought	2	0.19	0.05	2.11	0.016	**
Residual	54	2.49	0.60			
Total	59	4.11	1.00			
*42DPI samples only ~ Drought * AMF*						
Drought	1	0.23	0.12	4.31	0.005	**
AMF	1	0.04	0.02	0.85	0.449	NS
Drought:AMF	1	0.02	0.01	0.38	0.854	NS
Residual	30	1.57	0.84			
Total	33	1.85	1.00			

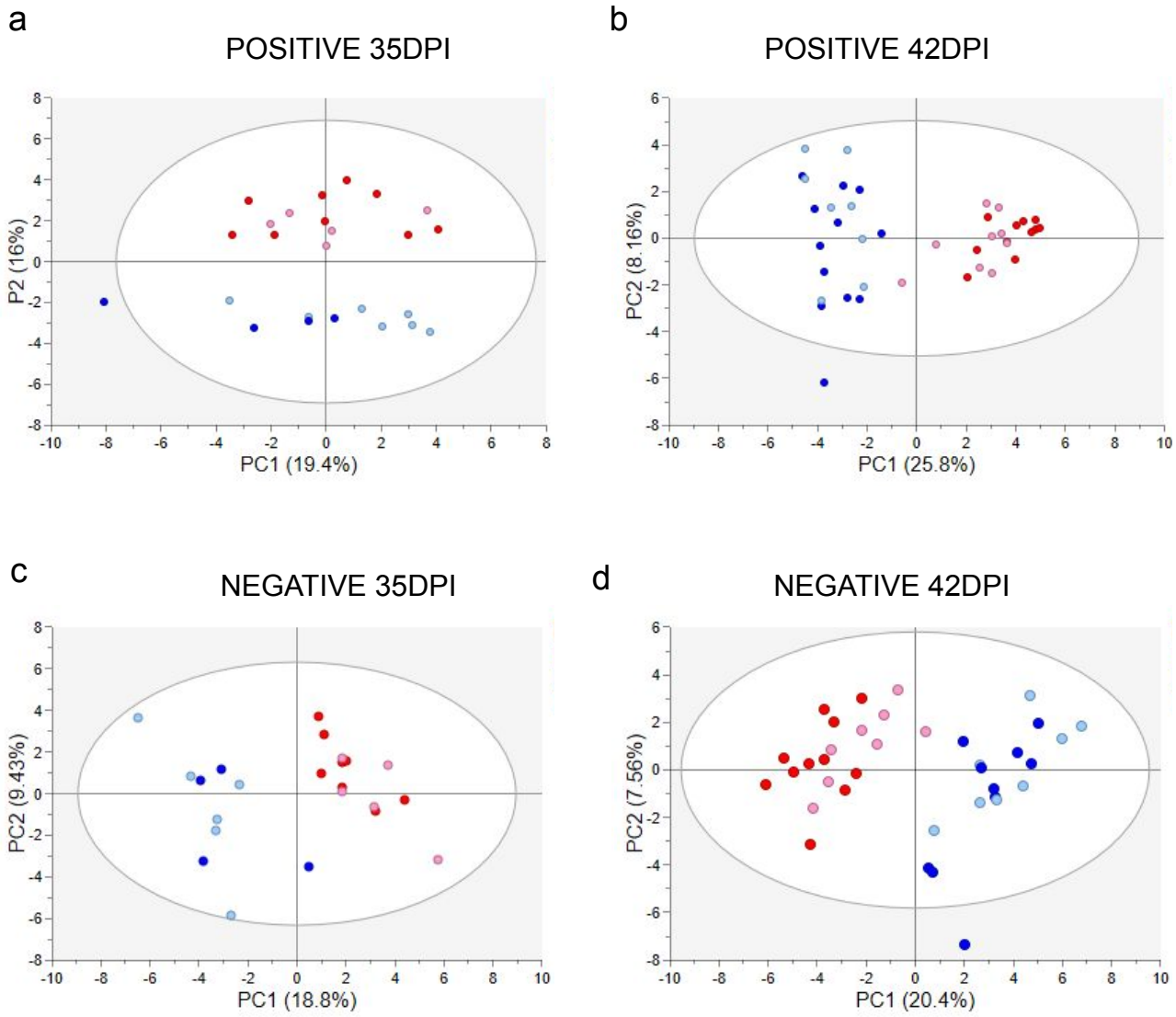
Source of variation	Df	Sum of squares	R2	F	p	
42DPI NM samples only ~ Drought						
Drought	1	0.07	0.39	7.16	0.001	***
Residual	11	0.11	0.61			
Total	12	0.19	1.00			
42DPI AM samples only ~ Drought						
Drought	1	0.24	0.19	4.43	0.01	**
Residual	19	1.03	0.81			
Total	20	1.28	1.00			
42DPI WW samples only ~ AMF						
AMF	1	0.03	0.04	0.68	0.562	NS
Residual	15	0.58	0.96			
Total	16	0.61	1.00			
42DPI DS samples only ~ AMF						
AMF	1	0.05	0.07	1.05	0.373	NS
Residual	15	0.65	0.93			
Total	16	0.70	1.00			



Appendix IX. Number of leaves on main stem of barley plants in experiment 3 at 29, 35 and 42 days post inoculation (DPI) (**a**)**.** At 29DPI, all plants were in the tillering phase (Zadoks >21) and by 42DPI, the majority of plants had reached the stem elongation stage (Zadoks >31). Horizontal line represents transition to stem elongation.AM= mycorrhizal inoculated, NM = non-mycorrhizal inoculated, DS = drought stress, WW = well-watered.



Appendix X Principal component analysis (PCA) scores plots for separate analyses of aqueous leaf extracts in positive mode with LCESI-MS from (a) plants at 29DPI (5th day of drought period) (b) plants at 35DPI (11th day of drought period). Little separation of treatment groups was observed in any combination of the first five PCs. The combination of PC1 and PC3 at 35DPI (b) shows a tendency toward separation between DS and WW plants. NMWW = blue open circles; AMWW = blue filled circles; NMDS = red open triangles; AMDS = red filled triangles. Ellipses represent the 95% confidence interval for a given treatment group.



Appendix XI. Principal component analysis (PCA) scores plots for separate analyses of aqueous leaf extracts in positive (a + c) and negative mode (b + d) with DI-ESI-MS from 35DPI (a + b) and 42DPI (c + d). Red (AM-treated) and pink (NM-treated) samples were from drought stressed plants while dark blue (AM-treated) and light blue (NM-treated) samples were from well-watered plants. Since there was no observed AM colonisation as a result of AM inoculation, samples were pooled for chapter 3. Ellipses represent hotelling at the 95% confidence interval.

Appendix XII. Nutrient analysis of experiment 3 (chapters 4 and 5)

Methods

Elemental analysis was performed on dried milled shoot material and root material for seven plants from each treatment group. Total carbon (C) and nitrogen (N) (% dry mass) were determined using a CN elemental analyser (Vario EL Cube, Langenselbold, Germany). Total phosphorus (P) and silicon (Si) were determined using a P-XRF instrument (Niton XL3t900 GOLDD analyser: Thermo Scientific, Winchester, UK) held in a test stand (SmartStand, Thermo Scientific) (Reidinger *et al.* 2012).

Results

Shoot N and P concentrations were significantly increased by drought (N: F=23.5, df=1, p=0.000112; P: F=57.2, df=1, p=3.83e-7) but unaffected by AM colonisation (N: F=0.358, df=1, p=0.557; P: F=0.059, df=1, p=0.811) while shoot Si was unaffected by either drought or AM colonisation (Drought: F=2.67, df=1, p=0.118; AMF: F=2.49, df=1, p=0.131). Shoot C was significantly reduced by drought (F=6.83, df=1, p=0.0171) but was unaffected by AM colonisation (F=0, df=1, p=0.996) (table 4.1).

Drought significantly reduced shoot C/N ratio (F=74.5, df=1, $p=8.02 \times 10^{-9}$) but AM inoculation did not (F=0.754, df=1, p=0.394) and there was no interactive effect of drought and AM inoculation (F=0.112, df=1, p=0.741) (table 4.1).

Root P concentration was significantly increased in AM plants in the well-watered treatment compared to NM plants (ANOVA AMF: F=13.9, df=1, p=0.00142; Tukey HSD p<0.05). Root C concentrations were significantly increased by AM colonisation in the well-watered treatment (ANOVA interaction: F=9.31, df=1, p=0.00656; Tukey HSD p<0.05) but not in the water restricted treatment (Tukey HSD p>0.05) and the same pattern was observed for root Si concentration (Drought: F=22.2, df=1, p=0.000151; AMF: F=2.87, df=1, p=0.107; Interaction: F=12.73, df=1, p=0.00205). Both AM colonisation and drought treatment were found to significantly affect the root N concentration of barley plants (Drought: F=50.0, df=1, p=9.98e-7; AMF: F=5.74, df=1, p=0.0271), with AM colonisation increasing root N relative to NM plants in the well-watered treatment (Tukey HSD p<0.05) and drought-stressed plants having higher root N concentrations (though there was no difference between AM and NM drought-stressed plants) (Tukey HSD p>0.05) (table 4.2).

Root C/N ratio was significantly affected by drought (F=99, df=1, $p=5.44 \times 10^{-10}$), AM inoculation (F=27.6, df=1, $p=2.18 \times 10^{-5}$) and their interaction (F=16.2, df=1, p=0.000503). Under drought stress, there was no significant difference between the C/N ratios of AM and NM plants (Tukey HSD p>0.05) while under well-watered conditions, the C/N ratio of NM plants was significantly higher than that of the AM plants (Tukey HSD p<0.05) (table 4.2).