Temporal Dynamism, Soil Processes and Niche Complementarity: Novel Approaches to Understanding Diversity-Function Relationships.

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Temporal Dynamism, Soil Processes and Niche Complementarity: Novel Approaches to Understanding Diversity-Function Relationships.

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Contents

List of Figures

List of Diagrams

List of Tables

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For Jack Schofield and Tom Morris who inspired my love of plants.

General abstract

The temporal dynamics of key processes are a poorly understood yet potentially important factor in our understanding of plant coexistence in communities. Plants occupying the same spatial but differing temporal niches can coexist through niche differentiation, allowing coexistence in complex ecosystems. This thesis used barley as a model plant to investigate the temporal dynamics of plant and soil processes associated with nutrient uptake, and whether such dynamics might promote co-existence in competing plants.

Through a series of lab-based studies I found that competition between barley cultivars can lead to a shift in the timing of peak nitrogen accumulation rate. However, estimates of peak nitrogen accumulation rate can be influenced by the experimental design, software program and statistical model used in these studies. At a molecular level, plant competition leads to temporally dynamic changes in the concentration of the plant hormone salicylic acid. There were also changes in gene expression depending on the identity of a neighbouring plant.

I also explored the temporal dynamics of soil processes associated with plant nutrient uptake at a pot and root scale. At a pot scale, plant-plant competition did not lead to a significant shift in the temporal dynamics of soil carbon, nitrogen or microbial biomass. However, at a single root level, plant-plant competition led to a shift in the timing of peak activity of soil enzymes associated with nutrient turnover, indicating that the impact of plants on the soil microbial community might be one component of the mechanisms allowing temporally dynamic responses of plants to their neighbours.

I also found that the ability to shift the timing of peak nitrogen accumulation rate in response to plant-plant competition has been conserved in modern cultivars of barley. This ability can be used in the development of greater complementarity in crop mixtures to improve crop yield stability.

I demonstrated in this thesis that shifts in the temporal dynamics of plant nitrogen uptake in response to plant-plant competition involve both plant and soil components and can be inherited. These results contribute to our understanding of plant-plant competition dynamics and are applicable to both developing approaches for sustainable agriculture and for understanding coexistence in plant communities.

Chapter 1

Temporal dynamism of resource capture: a missing factor in ecology?

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Contents

Abstract

The temporal dynamics of plant resource uptake and the impacts on plant-plant interactions have important regulatory roles in multi-species communities. By modifying resource acquisition timing, plants might reduce competition and promote coexistence. But despite the potential to advance our understanding of community processes, this aspect of plant community ecology has historically received limited attention. This is partially a consequence of an historic reliance on measures made at single points in time. However, due to current technological advances this is a golden opportunity to study within-growing season temporal dynamism of resource capture by plants. This chapter presents new technologies that can be used to study this critical aspect of temporal dynamism and help deliver a vision for future development of this research field.

1.1 - What is temporal dynamism and why is it important?

Understanding plant community composition and functioning are fundamental challenges in ecology. We have yet to fully understand why specific communities exist at certain points in space and time, why some communities are more diverse than others, and how diversity impacts ecosystem function. In plant communities many theories have been proposed to explain plant coexistence including cyclical disturbance (Grime, 1977; Bongers *et al.*, 2009), different individual responses to species interactions (Rowntree *et al.*, 2011), multiple limiting resources (Tilman, 1982; Valladares *et al.*, 2015), intraspecific trait variation (Mitchell and Bakker, 2014) and facilitative plant-plant interactions, particularly in extreme environments (Brooker *et al.*, 2007; Butterfield *et al.*, 2013).

However, short-term (i.e. within-growing season) temporal dynamism in resource acquisition might be central to addressing these fundamental questions. Temporal dynamism can be described as a form of heterochrony, controlled by intrinsic gene expression but also influenced by external environmental factors such as climatic conditions (Geuten and Coenen, 2013). However, apart from in a few cases we rarely consider within-growing season temporal dynamism in resource acquisition as a topic in its own right, in part because it has historically proven hard to measure. This is in contrast to our knowledge of plant phenology about which much is known. Phenological studies have shown the

importance of timing in the structure and functioning of plant communities (Tang *et al.*, 2016). Therefore, there can be expected to be similar important consequences for temporal dynamism in resource capture.

If different species temporally segregate uptake of common resources to avoid competition, increased complementarity can promote plant coexistence (Li *et al.*, 2014), with profound implications for biodiversity-ecosystem function relationships. Importantly, due to the wealth of analytical approaches now available, now is a good opportunity to address the historic oversight of within-growing season temporal dynamism.

Before considering these new opportunities, previous studies of temporal dynamism will be examined and why short-term temporal dynamism has been overlooked to date. New experimental approaches to address identified knowledge gaps will be presented, considering the potential influence on other areas of ecology

1.2 - Examples of temporal dynamism in plant communities

Previous research provides clear examples of the importance of temporal dynamism in the structure and functioning of plant communities. Arguably one of the most well studied examples is plant-pollinator interaction dynamics, as flowering phenology can lead to competition or facilitation for pollinators, with inter- and intra- annual dynamics (Kipling and Warren, 2014; MacLeod *et al.*, 2016). In arid environments temporal dynamism has been found in the growth response of plants to erratic inputs of water (Thompson and Gilbert, 2014), depending on the timing of the water input in the growing season, and the time since the previous water input (Schwinning *et al.*, 2004).

Other examples of temporal dynamism in plant communities involve processes linked to the temporal dynamics of nutrient uptake. One way in which non-native species can become invasive is by occupying a novel spatial or temporal niche (Wolkovich and Cleland, 2014). Occupying a novel temporal niche, left vacant by the native plant community, could allow the invasive species to capture nutrients at a time of reduced competition from the native community. The link to the temporal dynamics of nutrient uptake has not yet been proven experimentally but dynamism in resource uptake could

have a role in plant invasions. A similar example is the phenology of hemi-parasitic plants. The lifecycle of hemi-parasites occurs earlier in the growing season than their hosts, influencing nitrogen cycling with earlier leaf fall than the host community (March and Watson, 2007; Mudrák *et al.*, 2016). However, this is another case where the link to temporal dynamism of nutrient uptake has not been fully explored.

Some of the examples above clearly have a link to resource capture. A few experimental studies have sought to measure this process in more detail. One such example is that of McKane *et al.* (2002), who found in an arctic field study that coexisting species segregated the form of nitrogen, rooting depth and timing of nitrogen uptake in a tundra plant community. This is thought to lead to coexistence through niche differentiation reducing competition for key limiting factors. Another example is the Trinder *et al.* (2012) paper, which used a series of destructive harvests to examine the temporal dynamics of nitrogen uptake and biomass accumulation of *Dactylis glomerata* (Cock's foot) and *Plantago lanceolata* (Ribwort plantain). Trinder *et al.* found that in response to interspecific competition both species shifted the timing of the maximum rate of biomass accumulation and nitrogen uptake by up to 17 days (Trinder *et al.*, 2012). The species diverged the timing of these resource capture processes, presumably to limit direct competition for resources.

The presence of a range of previous studies looking at temporal dynamism but few that have been able to specifically address temporal dynamism of resource capture suggests a technological limitation that has prevented direct studies.

1.3 - Why does it matter that temporal dynamism has been overlooked?

Many of the fundamental processes and properties of many terrestrial communities are governed by the outcome of plant-plant interactions (Lortie *et al.*, 2004). Temporal segregation of nutrient uptake could support a high species diversity and have a stabilising effect on communities (Trinder *et al.*, 2013), at a species (Proulx *et al.*, 2010) and genotypic level (Fridley *et al.*, 2007), as the community uses a greater proportion of the available resources (Allan *et al.*, 2011). But despite a huge amount of work on plant-plant interactions, especially competition, there are still unanswered fundamental questions about the role of plant interactions in governing plant community composition.

For example current understanding of the defined niches available cannot explain the level of observed coexistence (Clark, 2010). However, a better understanding of short-term temporal dynamism in resource capture and plant interactions might help explain this apparent paradox. This could be due to an unmeasured trait involved in temporal dynamics of key processes such as nutrient capture (Figure 1.1).

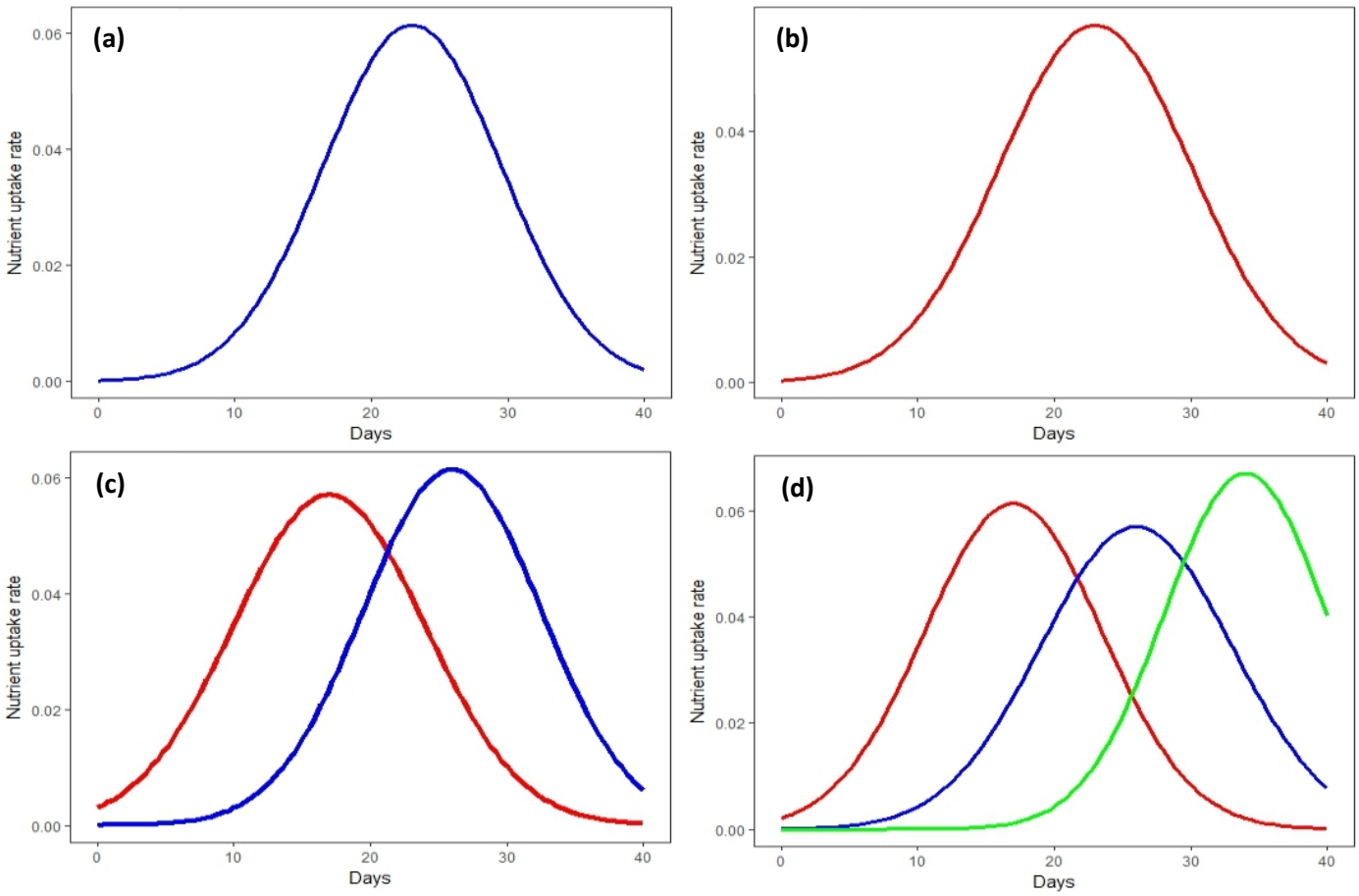

Figure 1.1 *- Theoretical role of temporal dynamism in plant coexistence. In isolation (panels a and b) plants take up nutrients in a specific profile over the growing season. But when grown together (panel c) the two plants offset the period of maximum nutrient uptake to limit competition. In a multispecies community (panel d) this may lead to species occupying distinct temporal niches, leading to coexistence.*

1.4 - Why has temporal dynamism in resource capture been overlooked?

It is clear that temporal dynamism in plant community processes and interactions can be critical for regulating community structure and function. However, there is very limited knowledge about temporal dynamism of plant-plant interactions within a growing season. Here the reasons for why this oversight might have occurred are considered below.

1.4.1 - Tradition

Plant ecology has traditionally relied on one final biomass measurement to assess the consequences of plant-plant interactions. Biomass is a relatively cheap and easy measure of plant responses, making large-scale greenhouse and field studies possible (Trinder *et al.*, 2013). However, there are some drawbacks to using single time point measurements of biomass to assess plant-plant interactions, and especially temporal dynamism. First, the accumulation of biomass is rarely solely influenced by competition alone, due to the influence of external environmental factors (Trinder *et al.*, 2012). This makes it an unreliable direct measure of the outcome of competition. Second, many studies use only single harvests to assess the outcome of plant-plant interactions, which is clearly inappropriate for measuring short-term temporal dynamism in resource capture. In addition, the precise timing of biomass harvest and measurement within a growing season can influence the perceived outcome of the plant interaction, as plants grow and develop at different times throughout the year (Trinder *et al.*, 2013). The same criticisms can also be made of other common annual, single time-point measurements, for example flower production and seed set. To understand the role of temporal dynamism of resource capture in regulating community dynamics, repeated measures of resource capture are required.

1.4.2 Traditional techniques

Comparatively traditional approaches, for example plant biomass and tissue nutrient content analysis, can be used to explore issues of temporal dynamism in plant interactions, so long as they are coupled to multiple harvesting points through time, as used by Trinder *et al.* (2012) to examine the temporal dynamics of resource capture in *Plantago lanceolata* and

Dactylis glomerata. However, although the multiple harvest approach is a valuable tool, it is destructive and requires large-scale, labour intensive studies. It also means that the subtleties of individual level temporal dynamics of resource capture and competition cannot be tracked.

1.4.3 - Difficulties in measurement

Single-harvest measurements of biomass might have become the tradition because doing anything else is difficult. The inclusion in a study of multiple harvests to track temporal dynamism of resource capture and plant interactions through time will increase the size and complexity of an experiment, and therefore reduce the complexity of the questions that can be asked (Allan *et al.*, 2011; Li *et al.*, 2014). Also, multiple harvesting means responses are averaged over many plants, potentially masking subtle individual responses in resource capture and growth. Using alternative non-destructive methods instead would allow a single plant to be studied over time.

Previous studies have looked at temporal dynamism of processes related to resource capture, with a limited look directly at temporal dynamism of resource capture directly. This is likely to be due to technological limitations to study resource uptake temporal dynamics directly such as the use of destructive harvesting. This strengthens the case for the use of innovative new technologies to give temporal dynamism of resource capture the attention it deserves.

1.5 – Research questions

A series of questions forming a research agenda is required to advance the study of temporal dynamism of resource uptake. Initially it needs to be established whether temporal dynamism in nutrient uptake really leads to a reduction in competition and promote coexistence. This is the important initial question to form the basis of future research. The mechanism by which temporal dynamism of nutrient uptake occurs is the natural follow-up area of investigation, focusing on potential signalling pathways between neighbouring plants. Also, due to the importance of soil microbes in nutrient mobilisation,

the role and influence of the soil community on the temporal dynamics of nutrient uptake merits further investigation.

This opens up a range of questions about the consequences of temporal dynamism of resource capture. The influence on the physiology and morphology of the individual is a clear starting point. However, it is the influence on the wider community that is of greater interest to a range of ecologists. There are potential impacts on any organism that interacts with plants including herbivores (vertebrate and invertebrate) and pollinators. This is likely to ultimately have cascading effects on the whole food chain, influencing the structure and function of entire ecosystems.

1.6 - How to measure short-term temporal dynamism in resource capture and competition?

In order to address the identified key research questions, new technological approaches are required to look at this complex series of processes involved in nutrient capture. This section will discuss how current technology can be used to study temporal dynamism of resource capture.

1.6.1 - Does temporal dynamism in resource capture lead to coexistence?

To address this question a method to detect the presence of temporal dynamism is needed. Destructive harvesting seems like an obvious first choice and could well form the basis of initial studies of temporal dynamism. However, to study temporal dynamism directly, non-destructive techniques are likely to be required to examine the multiple steps in the process of nutrient uptake.

To take up mineral nutrients, plants are reliant on soil biota to drive nutrient cycles that mobilise organic nutrient stocks into plant-available forms. Increasing evidence indicates that plants exert significant control over this process, changing rates of soil organic matter (SOM) mineralisation (de Vries and Caruso, 2016; Laliberté, 2016), primarily through the impacts of rhizodeposition on microbial process rates (rhizosphere priming effects, RPE (Kuzyakov, 2010; Mommer *et al.*, 2016)). As rhizodeposition varies with plant development,

species and genotype (Chaparro *et al.*, 2013; Bardgett *et al.*, 2014; Mwafulirwa *et al.*, 2016), there are likely to be important implications of these plant-microbe interactions for the temporal dynamics of resource capture in mixed plant communities that remain to be resolved.

In terms of studying components of the system that are related to plant nutrient availability and acquisition, one method is to study the timing of the rhizosphere priming effect for plants in competition vs. isolated plants. For example, recalcitrant and labile forms of nitrogen are mineralised by soil bacteria and fungi (Andrews *et al.*, 2013) and mycorrhizal fungi provide phosphate to plants (Johri *et al.*, 2015). Stable isotope labelling ($^{15}N/^{13}C$) of plants or soil provides a means of quantifying these processes, allowing plant impacts on soil nutrient cycles to be determined (McKane *et al.*, 1990). This can be done non-destructively through isotopic partitioning of soil CO_2 efflux into plant and SOM-derived components (Lloyd *et al.*, 2016) or tracing ^{15}N fluxes (derived from labelled organic matter) in soil solution (Zambrosi *et al.*, 2012; Yang *et al.*, 2013; Studer *et al.*, 2014). This allows the key processes of soil community priming and nitrogen mobilisation to be measured over time.

1.6.2 - How is temporal dynamism in nutrient uptake moderated in response to neighbours?

Traditionally plant responses to a neighbouring plant have thought to occur when the zones of nutrient depletion in the soil overlap (Ge *et al.*, 2000). However, as the complexities of plant-plant communication are revealed (Babikova *et al.*, 2013), it is becoming clear that this might not be the case. One way to look at dynamic plant responses to a neighbour is through the use of gene expression markers. The most commonly used method to study gene expression in response to an external change is RNA sequencing (RNAseq). Studies in *Arabidopsis thaliana* have identified that common stress response pathways such as jasmonate expression are activated in response to a competitor (Masclaux, Bruessow, Schweizer, Gouhier-Darimont, Keller and Reymond, 2012). However, it is unclear whether these responses can be translated to other species and more realistic experimental setups. Despite these uncertainties, the use of molecular markers represents a unique opportunity to understand competition at a molecular level and the sequence of events that take place

within a plant from competitor perception, to changes in the temporal dynamics of resource capture.

1.6.3 - How do interactions with soil organisms influence temporal dynamism in resource uptake?

The soil microbial community is known to be temporally dynamic, varying with season, plant species and plant developmental stage (Lortie *et al.*, 2004). Molecular techniques such as sequencing the 16S ribosomal RNA extracted from rhizosphere soil samples taken over a time series can provide a view of how the active microbial community changes. Shi *et al.* (2016) took this approach further and produced a network of microbial diversity over a growing season, showing how plants promote a beneficial rhizosphere, compared to the bulk soil (Shi *et al.*, 2016). This approach provides a view of dynamic interactions between plants and the soil microbial community, allowing the tracking of soil community activity and associated nutrient availability over time.

Another exciting development integrating the spatial dynamism of the soil community activity over a growing season is zymography. This approach focusses on specific functions of the soil community such as cellulase and chitinase activity (Spohn and Kuzyakov, 2014) and has already been used to identify 'hot moments' when microbial activity is higher than normal levels (Kuzyakov and Blagodatskaya, 2015). Such 'moments' can be occasional or occur periodically with events like spring growth and autumn leaf fall (Philippot *et al.*, 2009). Soil zymography can be used to identify the areas of the plant root system where temporal dynamics of nutrient acquisition is most important (Spohn and Kuzyakov, 2014). This allows not just the soil community structure but also its activity to be tracked over time and linked to plant nutrient uptake dynamics.

1.6.4 - How does temporal dynamism of resource capture influence plant physiology and morphology?

Temporal dynamism of nutrient capture is likely to influence the physiology and morphology of roots as they are directly involved in nutrient uptake. This could involve changes in root foraging behaviour and root architecture in response to a neighbouring plant.

To study root growth and foraging activity over time, one approach is the use of microrhizotrons. These are small cameras inserted into the soil to record root foraging behaviour and are particularly useful to look at fine root development (McCormack *et al.*, 2015; Warren *et al.*, 2015). However, they are limited as they do not give a view of the whole root system. Instead whole root system growth dynamics can be studied using plants grown in Perspex boxes and photographed using high definition cameras for phenomic analysis in automated root phenotyping facilities (Marshall *et al.*, 2016). This allows for a root system to be studied in-situ, as well as dynamic root architecture changes and root foraging to be tracked over time. As seen in arid environments root architecture traits can be vital for temporal dynamism studies. Therefore, techniques such as these will allow studies of temporal dynamism of nutrient uptake to include the dynamics of root growth.

For a more detailed 3D view of root architecture, X—ray CT scanning can be used to visualise plant roots grown in pots. The development of specialist root tracking software and facilities will allow much larger and more complex experiments to be carried out into dynamic competition for soil resources between the roots of multiple individuals. This approach has already been used to study root growth in response to competition between *Populus tremuloides* (quaking aspen) and *Picea mariana* (black spruce) seedlings. Both species increased rooting depth and altered root architecture in response to a competitor (Dutilleul *et al.*, 2015). Using this approach with a time series of successive scans will allow us to see a 3D view of the dynamism of root growth, and the traits of dynamic root placement to be viewed with high temporal resolution.

1.7 - What is the future strategy to study temporal dynamism?

Temporal dynamism could be a vital mechanism by which plants coexist in complex communities. There is now an ideal opportunity to understand the within-growing season temporal dynamics of resource capture as part of broader ecological system dynamics. As nutrient acquisition is a series of distinct, but interconnected processes, an integrated approach is required (Harris, 1967). A vast amount of knowledge can be gained about temporal dynamism in resource uptake from using these cutting edge technologies.

The ultimate goal in this field of research should be to integrate temporal dynamism as a factor in existing niche models, to define new niche space and aid the explanation of coexistence in complex communities. This approach can then be applied to other temporally dynamic processes, answering other fundamental questions about ecosystem functioning.

1.8 - Conclusions

Now is an ideal time to study and integrate within-growing season temporal dynamism into our understanding of coexistence. To achieve this, a clear research framework and the use of cutting-edge technology to study the individual stages of resource capture are required. This chapter has presented a clear set of questions that need to be answered in order to understand the mechanism and consequences of temporal dynamism in nutrient uptake. Although studying temporally dynamism of resource capture is not going to be straight forward, the potential benefit to our understanding of ecosystem functioning is likely to be considerable. Lessons learnt by studying the temporal dynamics of resource capture can then be applied to study other temporally dynamic ecological processes.

This thesis will use barley (*Hordeum vulgare*) as a model plant to investigate the effect of plant-plant competition on the temporal dynamics of resource capture. The use of barley allows the results of this thesis to be of relevance to both sustainable agriculture, specifically plant mixtures, as well as grassland ecology as barley is a grass species. It also allows molecular approaches to be used to study intracellular processes associated with nutrient uptake temporal dynamism, such as gene expression. Genetic approaches are often

not possible in wild grass species, as many do not have reference genomes available. Therefore, the use of barley allows these types of analyses to be carried out.

This thesis will initially take a successive harvest approach similar to Trinder *et al.* (2012), using barley as a model plant, specifically two cultivars (Proctor and Tammi) grown in isolation, inter- and intra- cultivar competition (Chapter 2). The potential influence of the statistical model design and software used to analyse the temporal datasets in these types of studies will then be assessed (Chapter 3). This will then be followed up by two studies of soil processes, the first at a pot level studying the effect of plant competition on the temporal dynamics of soil processes including soil respiration and soil nitrogen dynamics (Chapter 4). Then a second at a single root level to study the effect of plant-plant interactions on the dynamics of the soil microbial community activity using zymography (Chapter 5). This chapter will use two enzyme classes to examine the effect of plant-plant competition on soil organic matter turnover and nitrogen cycling dynamics.

The gene expression patterns of barley in inter- and intra- cultivar competition will be examined in Chapter 6. Microarrays will be used to identify genes up- and down-regulated in response to competition and differences in expression between inter- and intra- cultivar competition. The plant stress hormone production associated with plant-plant competition will be characterised in Chapter 7 using two plant hormones associated with abiotic and biotic stress, jasmonic acid and salicylic acid. The relative concentrations in roots will be used to assess the molecular response to plant-plant competition.

Descendants of the original cultivars will then be used to investigate whether the descendants of Proctor, a cultivar first introduced in 1955, have inherited a temporally dynamic response to competition for resources (Chapter 8). The same successive harvesting approach as Chapter 2 and statistical analysis from Chapter 3 will be used for this study.

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Chapter 2

Cultivar differences and impact of plant-plant competition on temporal patterns of nitrogen and biomass accumulation

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Contents

Abstract

Current niche models cannot explain multi-species plant coexistence in complex ecosystems. One explanatory factor is within-growing season temporal dynamism of resource capture by plants. However, the timing and rate of resource capture are themselves likely to be mediated by plant-plant competition. This study used barley (*Hordeum vulgare*) as a model species to examine the impacts of intra-specific competition, specifically inter- and intra-cultivar competition on the temporal dynamics of resource capture. Nitrogen and biomass accumulation of an early and late cultivar grown in isolation, inter- or intra- cultivar competition were investigated using sequential harvests. I did not find changes in the temporal dynamics of biomass accumulation in response to competition. However, peak nitrogen accumulation rate was significantly delayed for the late cultivar by 14.5 days and advanced in the early cultivar by 0.5 days when in intra-cultivar competition; there were no significant changes when in inter-cultivar competition. This may suggest a form of kin recognition as the target plants appeared to identify their neighbours and only responded temporally to intra-cultivar competition. The Relative Intensity Index found competition occurred in both the intra- and inter- cultivar mixtures, but a positive Land Equivalence Ratio value indicated complementarity in the inter-cultivar mixtures compared to intra-cultivar mixtures. The reason for this is unclear but may be due to the timing of the final harvest and may not be representative of the relationship between the competing plants. This study demonstrates neighbour-identity-specific changes in temporal dynamism in nutrient uptake. This contributes to our fundamental understanding of plant nutrient

dynamics and plant-plant competition whilst having relevance to sustainable agriculture. Improved understanding of within-growing season temporal dynamism would also improve our understanding of coexistence in complex plant communities.

2.1 - Introduction

Niche differentiation is suggested to lead to coexistence of plants by reducing competition, either for a specific form of a resource or simultaneous demand for the same resource (Silvertown, 2004). However, in complex plant communities such as rain forests and grasslands there are seemingly insufficient niches to explain coexistence of the many species present. Plants seem to occupy the same niche dimensions but without it leading to competitive exclusion (Clark, 2010).

One factor which is often not included in niche models is time, more specifically the temporal dynamism of key developmental and physiological processes such as resource capture (Schofield *et al.*, 2018). Competition can be influenced by temporally dynamic physiological processes (Poorter *et al.*, 2013), such as flowering (Kipling and Warren, 2014) and nutrient uptake (Jaeger *et al.*, 1999). Differences in the temporal dynamics of nutrient capture could reduce temporal niche overlap, reducing competition for resources. This could result in increased complementarity and promote coexistence (Ashton *et al.*, 2010).

As well as temporal dynamism influencing competition, competition can influence the temporal dynamics of resource capture, although the extent to which these processes affect each other is unclear. As there are many aspects of temporal dynamism in plant communities that are not fully understood, temporal dynamism in resource capture may be currently unsuitable as an indicator of plant-plant competition. However, a change in the temporal dynamics of resource capture may be a wider consequence of competition or a mechanism by which plants avoid direct competition for resources. Trinder *et al.* (2012) found a change in the temporal dynamics of nitrogen and biomass accumulation in response to inter-specific plant-plant competition. But the impact of competition on temporal dynamism in resource capture, and how this could influence coexistence in plant communities, remains largely unexplored (Schofield *et al.*, 2018).

There is in particular a lack of information on the relationship between temporal dynamism and intra-specific competition, and how the degree of relatedness of competitors might influence temporal dynamism. The genetic distance between competing individuals can influence the functional plasticity of an individual response to competition (G. P. Murphy et al., 2017), including biomass allocation and root morphology (Semchenko *et al.*, 2017). Differential competitive responses have been demonstrated between closely related individuals (G. P. Murphy et al., 2017), including in a number of crop species (Dudley and File, 2007). The use of two cultivars in this study allows a tight control of the relatedness of individuals, which in turn allows us to address how diversity regulates interactions and ultimately functions in a range of systems (not least for the development of sustainable agricultural practice (Schöb *et al.*, 2018)). In this sense, crop species are ideal model systems for undertaking such studies.

Here, I conducted a pot experiment with barley (*Hordeum vulgare*) as a model species, using an early and a late cultivar. Barley is a suitable model in this case as its nutrient uptake has been studied in detail to optimise the timing of fertiliser application in agriculture (Nielsen and Jensen, 1986), allowing us to address fundamental ecological questions of plant coexistence, as well as investigating a topic of relevance for agricultural practices.

It is expected that early and late cultivars of barley will have different temporal dynamics of nitrogen uptake and biomass accumulation, in a similar way to two species or genotypes in a natural system. The two cultivars in this study have been bred for different uses and therefore will have differing combinations of traits. Tammi has been bred for an early lifecycle (Nitcher *et al.*, 2013), whereas Proctor was bred for malting quality (Hornsey, 2003). The nitrogen uptake and biomass accumulation dynamics are predicted to be altered by plant-plant competition, and this will be more pronounced in intra-cultivar compared to inter-cultivar competition as the individuals will more completely occupy the same niche space.

This study aimed to understand: (1) whether early and late cultivars of barley exhibit temporal dynamics in nitrogen uptake and biomass, (2) how plant-plant competition changes the temporal dynamics of nitrogen and biomass accumulation in early and late

barley cultivars, (3) how any temporally dynamic response differs with inter- and intra-cultivar competition, and ultimately (4) how this impacts on niche complementarity.

2.2 – Materials and methods

2.2.1 - Temporal patterns of nitrogen and biomass accumulation

A pot-based competition study was used to investigate temporal dynamism in nitrogen uptake, using barley (*Hordeum vulgare*) as a model species. An early (Tammi: T) and late (Proctor: P) cultivar of barley (sourced from The James Hutton Institute, Dundee, Scotland) were chosen as they have similar height and limited tillering, enabling the study to focus on phenological rather than physiological differences. Each cultivar was grown in pots either in isolation, or with another individual of either the same or other cultivar (i.e. T, P, TT, PP, TP).

2.2.2 - Soil characteristics

Soil was sourced from an agricultural field (Balruddery Farm, Invergowrie, Scotland, 56.4837° N, 3.1314° W) that had previously contained spring barley (*Hordeum vulgare*) and had been subject to standard management for barley production (including fertiliser addition at a rate of 500 kg of 22N-4P-14K ha^{-1} yr^{-1}). The soil had an organic matter content (humus) of 6.2% ± 0.3% SEM (loss-on-ignition, n = 4) and a mean pH (in water) of 5.7 ±0.02 SEM (n = 4), a total inorganic nitrogen concentration of 1.55 ± 0.46 mg g^{-1} (n = 4) and microbial C biomass (using a chloroform extraction) of 0.06 ± 0.002 SEM mg g^{-1} (n = 4) (analysed by Konelab Aqua 20 Discrete Analyser (Thermo Scientific, Waltham, MA USA)). The soil was passed through a 6 mm sieve and then stored at room temperature until use. No fertilization of the soil occurred during the experiment.

2.2.3 - Setup and growing conditions

Seeds of both cultivars were germinated in the dark on damp paper towels and planted into cylindrical 2 L pots (diameter 152 mm, height 135 mm) with five replicate pots of each of the five treatments for each planned harvest (11 harvests in total), giving a total of 275 pots.

The pots were randomized to account for potential positional effects and grown in controlled environment rooms (Conviron, Isleham, UK) at a constant 15°C with an 8/16 (day/night) hour photoperiod (irradiance of 100 - 150 µmol m^{-2} s^{-1}) and 65% relative humidity, to mimic local spring-time conditions. The pots were watered twice weekly and the soil was kept moist to avoid competition for water. Mesh screens (45 x 16 cm, mesh size 0.08 mm (Harrod Horticulture, Lowestoft, UK)) were inserted in those pots containing two plants to separate the plants above ground, and ensure competitive interactions only occurred below ground. Foliage was relatively upright without support and the presence of a screen – although important in ensuring above-ground competition was minimised – was unlikely to have resulted in differences in shoot development in pots with two plants compared to one.

2.2.4 - Sequential harvesting

Five randomly selected pots of each treatment were harvested every five days until ear formation (when grain begins to form) was observed on the early Tammi cultivar (60 days). During this period both cultivars produced flag leaves, the stage prior to grain production, when most nitrogen has already been absorbed (Spink *et al.*, 2015). This covered the period most likely to contain the peak nitrogen and biomass accumulation rate for both cultivars, the focus of this study. The plants were then removed from the pots, the roots washed, and individual shoot and root material separated. The root and shoot material of each plant were dried at 30°C until a stable weight was reached and weighed. Milled shoot samples were analysed for carbon and nitrogen concentration (Flash EA 1112 Series, Thermo Scientific, Bremen, Germany).

2.2.5 - Data analysis

2.2.5.1 - Temporal patterns of nitrogen and biomass accumulation

To analyse temporal changes in biomass and nitrogen accumulation, the rate of each was modelled with logistic growth curves using non-linear least squares (nls) models (R Core Team, 2015). A cumulative time series data set of biomass accumulation was bootstrapped

using resampling with replacement 1000 times to estimate variability and confidence intervals. A logistic growth curve was used as the nls model and this was fitted to each of the bootstrapped data sets to produce a set of logistic instantaneous uptake rate curves for each treatment, as well as sets of modelled maximum accumulation values. This was then repeated for the nitrogen accumulation data set. A non-linear model was used as the growth dynamics of plants with determinate growth such as barley (Yin *et al.*, 2003) are mostly sigmoidal, making a linear growth model unsuitable (Robinson *et al.*, 2010). Therefore, the use of the non-linear least squares model with bootstrapping is a robust method to examine the temporal dynamism of resource capture of annual species and to properly account for uncertainty. Significant differences between the timing of peak accumulation and final maximum accumulation between treatments were determined from the difference in bootstrapped 95 % confidence intervals of the model outputs (Appendix 1, Supplementary R Code 1).

2.2.5.2 - Shoot C:N

C:N ratio at the final harvest (65 days after planting) was analysed using an ANOVA test from the MASS package *in R* (R Statistical Software, R Core Team, 2016) as the residuals were normally distributed, with treatment as the fixed factor and C:N as the response variable (Appendix 1, Supplementary R Code 2). A Tukey post-hoc test was carried out to compare the individual treatment groups.

2.2.6 - Neighbour effects

The effect of a neighbouring plant on a target plant's biomass was quantified using the Relative Intensity Index (RII; Equation 1), an index that accounts for both competitive and facilitative interactions between neighbouring plants (Díaz-Sierra *et al.*, 2017). RII was calculated using the final harvest biomass data. For each cultivar, RII was calculated separately for plants grown in intra- and inter- specific competition. The mean total biomass of each cultivar grown in isolation was used for the *Isolation* value, and the individual RII value was then calculated for each plant of that cultivar experiencing competition.

Equation 1

$$RII = \frac{(Competition - Isolation)}{(Competition + Isolation)}$$

Competition = Mean biomass of plant when in competition*, Isolation* = Mean biomass of plant in isolation.

The land equivalent ratio (LER; Equation 2) was used to determine if the inter-cultivar mixture (TP) overyielded when compared to intra-cultivar competition (TT or PP) (Mead and Willey, 1980). The mean LER value was calculated by randomly pairing inter- and intra- cultivar competition treatments using a random number generator. A LER value was calculated for each pairing, from which a mean and standard error of the mean was calculated. A mean LER value above 1 indicates that inter-cultivar pairings produced more biomass than to intra-cultivar combinations. As the residuals were normally distributed, the LER and RII values were compared between competition treatments using an ANOVA test as above, with treatment as the fixed factor and either LER or RII as the response variable (Supplementary R Code 2).

Equation 2

$$LER = \frac{Tammi\ mixture\ biomass}{Tammi\ own\ cultivar\ \ biomass} + \frac{Proctor\ mixture\ biomass}{Proctor\ own\ cultivar\ biomass}$$

Tammi mixture biomass = Tammi biomass when in competition with Proctor, *Tammi own cultivar biomass* = Tammi biomass of the focal plant when in competition with another Tammi. *Proctor mixture biomass* = Proctor yield when in competition with Tammi, *Proctor own cultivar biomass* = Proctor biomass when in competition with another Proctor.

2.3 - Results

Nitrogen (Figure 2.1a) and biomass (Figure 2.1b) accumulation were temporally distinct for both cultivars. The peak rate of nitrogen accumulation occurred between 17.5 – 19.0 days after planting for Tammi and 19.5 – 35.0 days for Proctor. The peak rate of biomass accumulation occurred between 47 – 48 days after planting for Tammi and 47.0 – 51.5 days for Proctor (Model details in Appendix 1, Table A1).

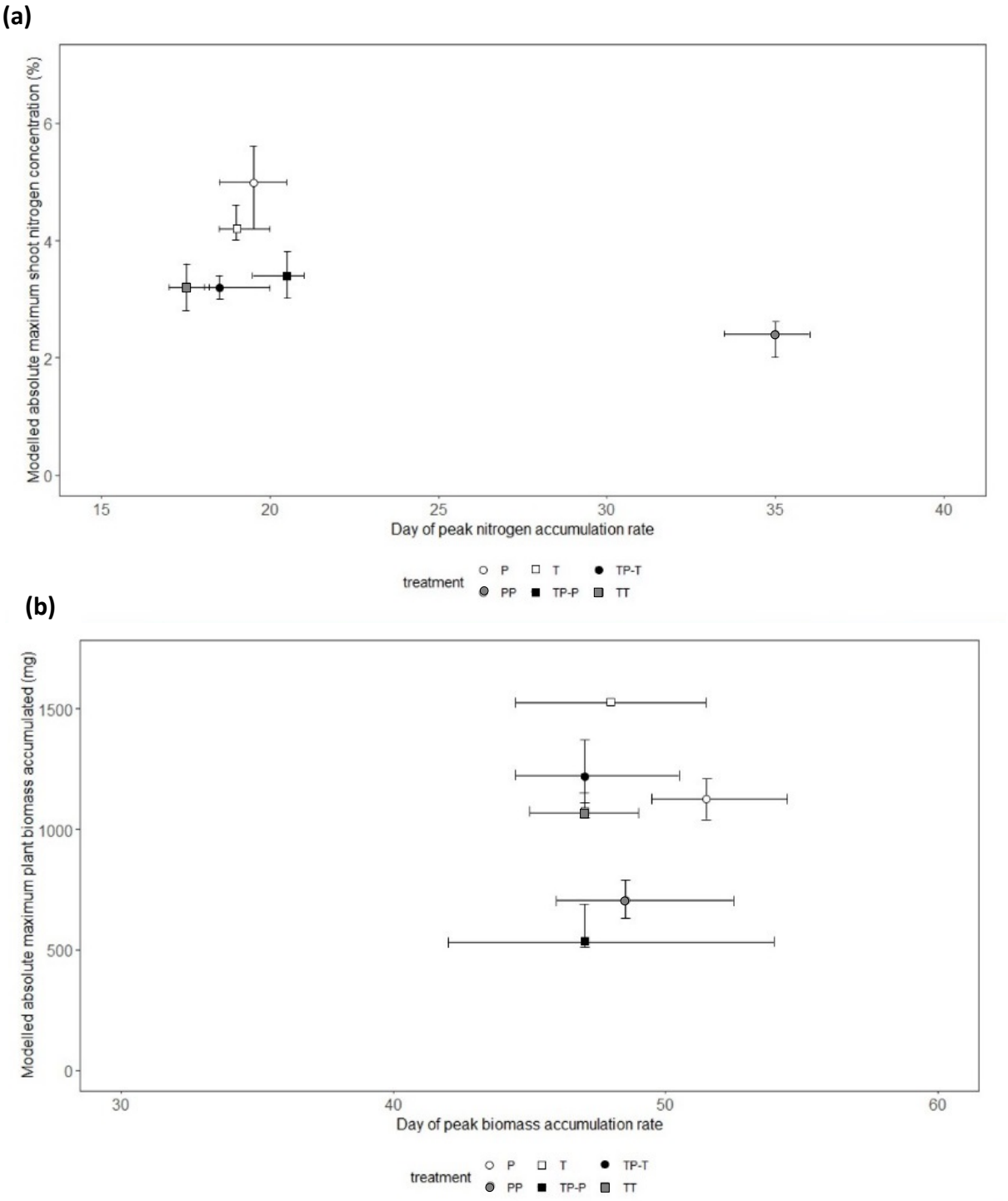

Figure 2.1 *– Timing of peak nitrogen (panel 1a) and biomass (panel 1b) accumulation rate, the shoot nitrogen concentration and absolute maximum accumulated total biomass at the end of the experiment in barley (*Hordeum vulgare*). Bootstrapped modelled accumulation derived from non-linear least squares model (T = Tammi, P = Proctor, TP-T = Tammi in competition with Proctor, TP-P = Proctor in competition with Tammi, TT = Tammi own*

cultivar competition, PP = Proctor own cultivar competition). Error bars represent the 95% confidence intervals derived from the non-linear least squares model.

2.3.1 - Temporal dynamics of nitrogen uptake

Nitrogen uptake for both cultivars followed similar temporal dynamics, increasing until 45 days after planting, then plateauing (Figure 2.2a and 2.2b). There was no significant change in the timing of peak nitrogen uptake rate in response to inter-cultivar competition for either cultivar. However, both cultivars showed a significant shift in peak accumulation rate in response to intra-cultivar competition (Figure 2.1a). Tammi demonstrated an advance in peak uptake rate by 0.5 days and Proctor a delay of 14.5 days (Appendix 1, Table A2).

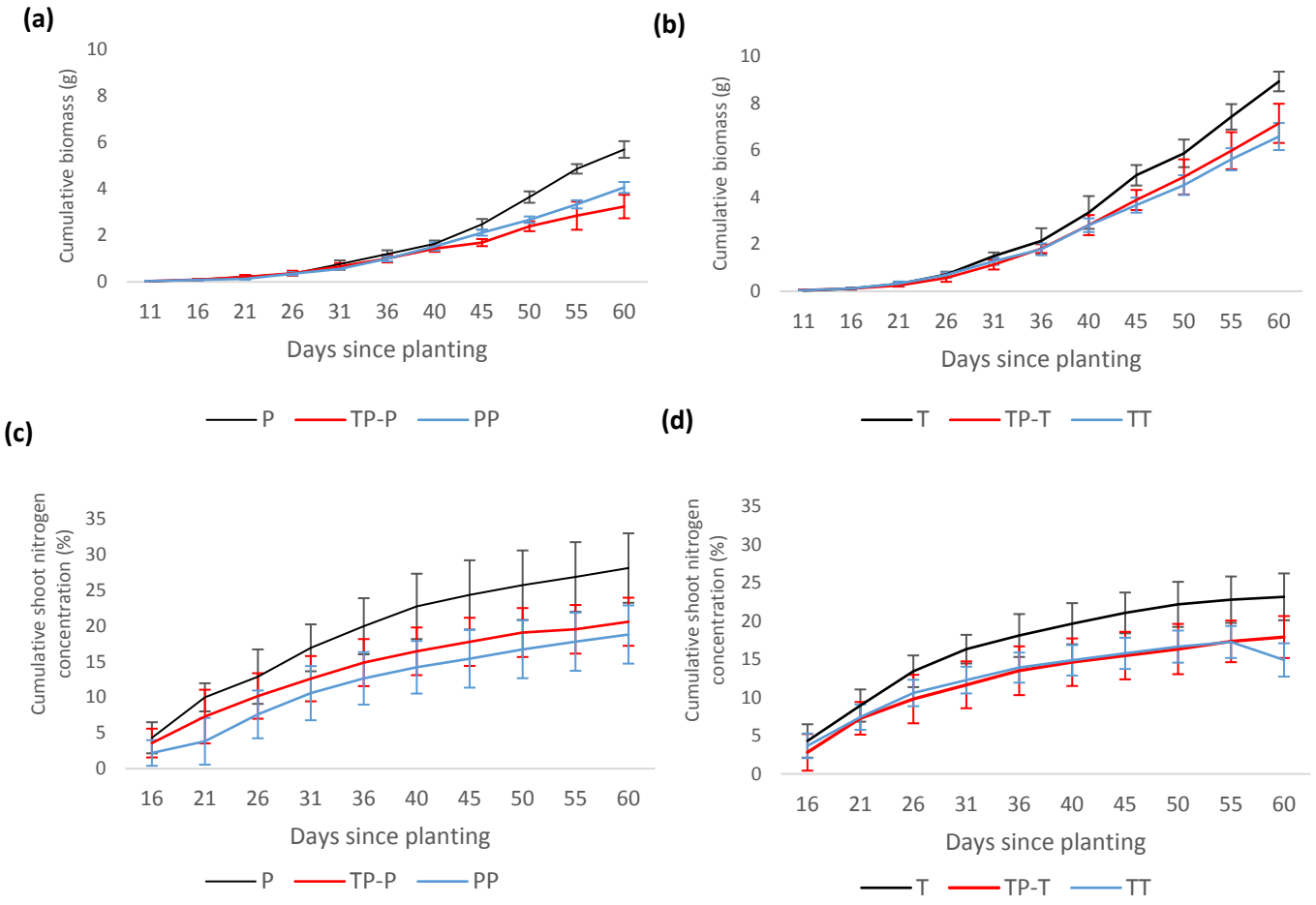

***Figure 2.2** – Mean cumulative nitrogen (panels 2a and 2b) and biomass (panels 2c and 2d) accumulation of Tammi and Proctor barley cultivars over time. Pots contained Proctor in*

isolation (P), in competition with Tammi (TP) and in competition with another Proctor (PP), Tammi in isolation (T), in competition with Proctor (TP) and another Tammi (TT). Error bars are two times the standard error of the mean.

2.3.2 - Maximum accumulated shoot nitrogen

Proctor's absolute maximum shoot nitrogen concentration was significantly lower when in competition with Tammi or Proctor compared to isolation (Figure 2.1a). Inter-cultivar competition caused a significantly lower maximum shoot nitrogen concentration compared to intra-cultivar competition for Proctor but not Tammi. Intra-cultivar competition caused a significantly lower maximum shoot nitrogen concentration for Tammi but not Proctor (Appendix 1, Table A3).

2.3.3 - Temporal dynamics of biomass accumulation

Biomass accumulation increased throughout the growing period with a lag period until 31 days after planting and then rapidly increased during the remainder of the experiment (Figure 2.2c and 2.2d). In response to competition, Tammi did not exhibit a shift in peak biomass accumulation rate, with peak accumulation rate always occurring 47 – 48 days after planting. Proctor biomass accumulation rate peaked between 48 – 51.5 days after planting (Figure 2.1b); although there was a trend towards an earlier peak in biomass accumulation when in competition there were no significant differences between treatments (Appendix 1, Table A2).

2.3.4 - Maximum accumulated total plant biomass

For both Tammi and Proctor, absolute maximum accumulated biomass was significantly lower when in competition compared to isolation (Figure 2.1b). However, neither cultivar demonstrated a significant difference between intra- and inter- cultivar competition in maximum accumulated biomass (Appendix 1, Table A3).

2.3.5 - Shoot C:N

Proctor in isolation had a C:N ratio of about half that of Tammi in isolation throughout the experiment i.e. more nitrogen relative to carbon. However, for neither cultivar were there significant differences in C:N ratio between plants in isolation compared to plants in competition at the end of the experiment (Proctor ($F_{(2,17)}$ = 1.44, P = 0.26); Tammi ($F_{(2,17)}$ = 2.74, P = 0.09) (Details in Appendix 1, Table A4).

2.3.6 - Neighbour effects

The significantly negative RII of final biomass indicated competitive interactions for both cultivars irrespective of whether they were in inter- or intra- cultivar mixtures. RII values also showed that Tammi and Proctor experienced a greater intensity of competition when in inter-cultivar compared to intra-cultivar competition (Figure 2.3). Proctor in intra-cultivar competition experienced the lowest intensity of competition; however, there was no significant difference between the competition treatments ($F_{(3,26)}$ = 2.86, P = 0.06).

The LER value for Tammi and Proctor in competition was 2.05 (± 0.35 standard error), indicating that the inter-cultivar mixture had a greater total biomass (root and shoot) than would be expected from the intra-cultivar mixtures.

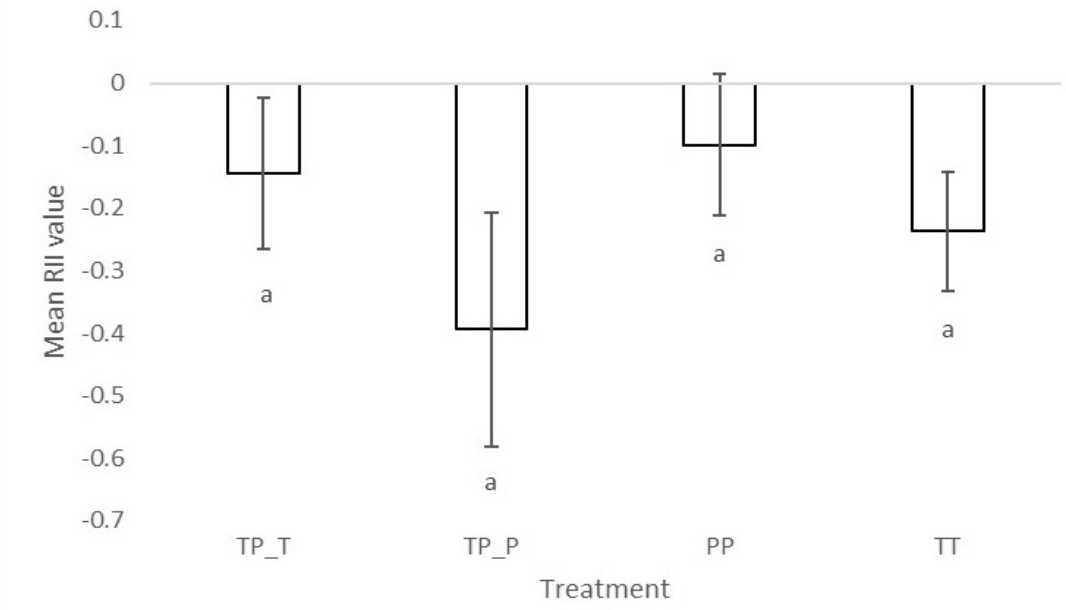

Figure 2.3 *- Mean Relative Intensity Index of barley (*Hordeum vulgare*) Tammi and Proctor cultivars in inter- and intra- cultivar competition. The more negative the result the greater*

competition the plant experienced. TP-T = Tammi in inter-cultivar competition, TP-P = Proctor in inter-cultivar competition, TT = Tammi in intra-cultivar competition, PP = Proctor in intra-cultivar competition. Error bars are two times the standard error of the mean. Letters indicate significant differences from a Tukey post-hoc test.

2.4 - Discussion

This experiment aimed to detect and quantify temporal dynamism in nitrogen uptake and biomass accumulation in two barley cultivars and determine responses to inter- and intra-cultivar competition.

I found that competition significantly reduced maximum accumulated biomass and shoot nitrogen in both cultivars. Neither intra- or inter-cultivar competition impacted the timing of peak biomass accumulation in either cultivar. However, intra-cultivar competition significantly delayed peak nitrogen accumulation rate by 14.5 days in Proctor and advanced it in Tammi by 0.5 days. Relative Intensity Index values indicated that both cultivars experienced competition, with no significant difference in intensity between intra- and inter- cultivar competition. However, a positive LER value indicated that the inter-cultivar mixture overyielded when compared to the intra-cultivar mixtures.

2.4.1 - Shifts in the timing of biomass accumulation in response to competition

Neither of the cultivars in this study significantly altered the temporal dynamics of peak biomass accumulation in response to a competitor. The mismatch between biomass and nitrogen accumulation dynamics in response to competition indicates biomass may not effectively measure the temporal dynamics of within-growing season resource capture, an issue previously raised by Trinder *et al.* (2012).

2.4.2 - Shifts in the timing of nitrogen accumulation in response to competition

Tammi and Proctor only demonstrated significant changes in temporal dynamism of nitrogen accumulation when in intra-cultivar competition. Tammi advanced peak

accumulation rate by 0.5 days and Proctor delayed it by 14.5 days. As this only occurred in intra-cultivar competition, it suggests that this is more complex than a competition avoidance response based on a source-sink (soil - plant) relationship. If this was a simple source-sink relationship, for example, based on soil nitrogen availability (Dordas, 2009), the inter- and intra-cultivar responses to competition should be identical. However, a response to only intra-cultivar competition suggests a kin recognition mechanism. Kin recognition has been suggested as a mechanism by which plants alter functional traits when in competition with closely related individuals (Sousa-Nunes and Somers, 2010). It has been found to most commonly be mediated belowground through root exudates (Bais, 2015; Biedrzycki *et al.*, 2010). This may mediate specific responses depending on the identity of a competing plant, as found in this study.

The results of this study contrast with those of a temporal dynamism study by Trinder *et al.* (2012) which examined the influence of interspecific competition on the temporal dynamics of nitrogen uptake and biomass accumulation using *Dactylis glomerata* and *Plantago lanceolata,* two perennial grassland species. *Dactylis glomerata* was the later of the two species, and *P. lanceolata* the earlier species. They found a seven day delay for *D. glomerata* and a five day advancement for *P. lanceolata* in maximum biomass accumulation rate in competition compared to plants in isolation, with a similar pattern of divergence for peak nitrogen accumulation rate. I did not find these trends between two cultivars, with no significant shifts in peak biomass accumulation rate and a significant delay in peak nitrogen accumulation rate only when Proctor was in own cultivar competition.

In this study Proctor was the less competitive of the two cultivars, as it experienced a greater decrease in nitrogen and biomass accumulation when in competition compared to Tammi. This contrasts with the Trinder *et al.* (2012) study which found that *D. glomerata* took up the most nitrogen and it could be argued was therefore the most competitive, despite being the later species for peak nitrogen and biomass accumulation rate. Therefore, it should not be assumed that the earlier species or cultivar is automatically the most competitive.

Trinder *et al.*, (2012) also found that competition reduced the period between peak nitrogen and biomass accumulation rate compared to plants in isolation, from ten days to one day for *D. glomerata*, and from fourteen to three days for *P. lanceolata*. I also found this

effect, but only when Proctor was in competition, which caused a shortening of the period between peak rate of nitrogen uptake and biomass accumulation by 18.5 days in intra-cultivar competition and 5.5 days when in inter-cultivar competition. However, the reason for this response is unclear. It could be a phenological change in response to competition, a pattern previously observed in cases of abiotic stress (Kazan and Lyons, 2016) and pathogen attack (Korves and Bergelson, 2003).

2.4.3 -Temporal segregation of nitrogen and biomass accumulation

The processes of nitrogen and biomass accumulation were temporally distinct for both cultivars. The peak rate of nitrogen accumulation was 29.0 – 29.5 days before peak biomass accumulation for Tammi and 16.5 – 27.5 days for Proctor (Figure 2.1). The gap between peak nitrogen and biomass accumulation was less variable for Tammi compared to Proctor. Tammi was specifically bred for an early phenotype (Nitcher *et al.*, 2013), whereas Proctor was bred for malting quality (Hornsey, 2003). This selection pressure for phenology in Tammi may go some way to explaining the lack of variability in the gap between peak nitrogen and biomass accumulation in response to competition. Future studies could investigate whether similar response patterns are found in the genotypes of wild species or in wild species with contrasting phenologies.

Barley has been found to have temporally distinct nitrogen and biomass accumulation, with a 23 – 24 day gap between peak nitrogen and biomass accumulation in field studies (Malhi *et al.*, 2006). The gap between the peak nitrogen and biomass accumulation rate was shortened when Proctor was in competition, indicating the impact of plant-plant competition on the temporal dynamics of nitrogen accumulation. The greatest reduction in the gap between peak nitrogen and biomass accumulation rate occurred when Proctor was in intra-cultivar competition. This was also the treatment with the lowest absolute shoot nitrogen concentration, suggesting delaying peak rate of nitrogen accumulation for this cultivar is a response to intra-cultivar competition.

2.4.4 - Impact of competition on final nitrogen and biomass accumulation

Competition significantly reduced the final maximum nitrogen concentration and biomass that both Proctor and Tammi were able to accumulate in intra- or inter-cultivar competition. A Proctor competitor caused a significant decrease in Tammi maximum biomass accumulation and nitrogen shoot concentration, despite not achieving the greatest biomass above or below ground. This suggests that another factor influenced the rate of nitrogen uptake. Signaling through root volatile compounds or root exudates has been found in a number of species including legumes and grasses (Pierik *et al.*, 2013) and may be acting here. Plant root exudates select for a specific microbial community (Shi *et al.*, 2016) and have been found to affect the rate of microbial soil organic matter turnover (Mergel *et al.*, 1998). Therefore, plants may influence the timing of soil microbial community activity in order to reduce direct competition for resources. However, as we are only starting to understand the role of short term-temporal dynamism in plant interactions (Schofield *et al.*, 2018) it is not surprising that further studies are required to determine the role of the root exudates in neighbour recognition and temporally dynamic responses, and why this response is greater for intra- compared to inter- specific competition.

2.4.5 - Shoot C:N in response to identity of a competing individual

The two cultivars differed in their C:N ratio by the end of the experiment. This is likely due to the earlier cultivar Tammi being more advanced developmentally than Proctor. By the end of the experiment, Tammi had begun grain production, whereas Proctor had produced a flag leaf, the stage before grain formation. However, there was no significant increase in C:N in either cultivar in response to competition. Due to selective breeding for a specific seed C:N (grain nitrogen content) with known mapped genes (Cai *et al.*, 2013) it is unlikely that C:N is highly plastic in barley, making it a poor measure of competition in this case.

2.4.6 - Is greater complementarity achieved?

The negative RII indicated both cultivars experienced competition when grown with a neighbouring plant, but no significant difference depending on the identity of the

competitor. This contrasts with the positive LER value which indicated overyielding of the two cultivars when grown in inter-cultivar competition compared to intra-cultivar competition. The reason for this is unclear and may be due to the timing of the final harvest, before both cultivars had set seed. This highlights the difficulty of using multiple metrics to measure the outcome of competition, especially as the measurements were only taken at the end of the experiment i.e. at a single timepoint. Therefore, single timepoint competition indices should be used with caution when examining the consequences of temporal dynamism of resource capture.

There is a need to understand the extent to which a species or genotype is temporally dynamic and the factors that lead to temporal dynamism in resource capture. This will allow temporal dynamism in resource capture to be included in models of coexistence, furthering our understanding of coexistence in complex plant communities.

2.5 - Conclusions

This study demonstrates how a previously understudied factor in plant community coexistence, within-growing season temporal dynamism of resource capture, can be measured through successive harvesting and the novel application of commonly used statistical approaches. Only peak nitrogen accumulation rate was temporally dynamic in response to competition, not biomass peak accumulation rate or shoot C:N. Therefore, I suggest that to understand the temporal dynamics of resource capture within a growing season, direct measures of mineral resources accumulated (e.g. nitrogen uptake) are important to understand the mechanisms of temporally dynamic responses to competition. By measuring shoot nitrogen accumulation rate over time, intra-cultivar competition was found to advance peak nitrogen accumulation rate in Tammi and delay it in Proctor. This suggests that temporally dynamic nitrogen uptake responses are greater in intra-cultivar competition and may be due to kin recognition. This may be mediated through root exudates and the soil microbial community, an area that requires further investigation and extension to semi-natural and natural ecosystems. Ultimately understanding the role of temporal dynamism in plant communities will lead to improved niche models of coexistence in plant communities.

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Chapter 3

Model and software choice affect analysis of temporal dynamism in plants – shorter harvesting intervals increase accuracy over replication

Contents

Abstract

Logistic growth curves have been used for over a hundred years to describe the dynamics of plant growth and resource capture. This includes studies of the temporal dynamics of resource capture by plants in competition, a potentially important factor influencing coexistence in complex plant communities. Logistic growth curves can enable us to assess

the dynamics, timing and scale of peak resource accumulation. However, both the data analysis approach and experimental design can influence the outcome of logistic growth curve modelling. This study first examined the effect of statistical model parameterisation and analytical software program choice on the estimate of peak accumulation rate timing. Two and three parameter models were compared in R, then Microsoft Excel and R were compared with the same model design. Second, a dummy dataset was constructed to investigate the effect of replicate number and sampling frequency on peak accumulation rate timing. Model parameterisation caused a shift of 3 – 15 days and software program a shift of 3 – 11 days in peak biomass accumulation rate estimate. The dummy dataset analysis found that both replicates and sampling frequency significantly affected the estimate derived from the model. With sampling intervals of six days or less there was little effect of replicate number. With greater sampling intervals estimates were larger. Therefore, this chapter recommends the use of a three parameter instead of a two parameter logistic model, as it accounts for variation in the starting value. It is also recommended sampling at a frequency of fewer than six days with 3 - 5 replicates in similar studies. It is also recommended that before logistic growth curve fitting is undertaken, the model design and the software program used to analyse the data should both be thoroughly explored to ensure they are fit for purpose and avoid confounding effects. Also, when designing experiments prior to such analyses, frequent sampling with a limited replicate number is the best use of limited resources whilst maintaining the accuracy and precision of timing estimates.

3.1 - Introduction

For the last century, logistic models have been used to visualise the growth dynamics of individuals and populations (Hunt, 1982; Yin *et al.*, 2003). In population ecology they are used to describe the growth of a population from initial colonisation, through a period of exponential growth until the carrying capacity of the environment is reached (Vandermeer, 2010). Logistic curves are also used to describe the growth of plants with determinate growth, such as annual species and crops which have a defined final biomass (Yin *et al.*, 2003). The characteristic sigmoidal accumulation curve creates a bell-shaped curve of instantaneous uptake rate (Hunt, 1982).

Many studies of plant growth explore the effect of environmental factors (abiotic and biotic) on the rate of resource or biomass accumulation (White, *et al.*, 1991; Hara, van Der Toorn, & Mook, 1993; Trinder *et al.*, 2012; Lipiec, *et al.*, 2013). Most use a series of successive harvests during the growing period, then fit logistic (or similar) models to successive measurements of, for example, dry weight, height or nitrogen content. Rates of change can then be derived from the fitted models as the instantaneous slopes of the temporal trajectories (Figure 3.1), and these derived quantities used to study temporally dynamic processes, including biomass accumulation or resource uptake (Trinder *et al.*, 2012).

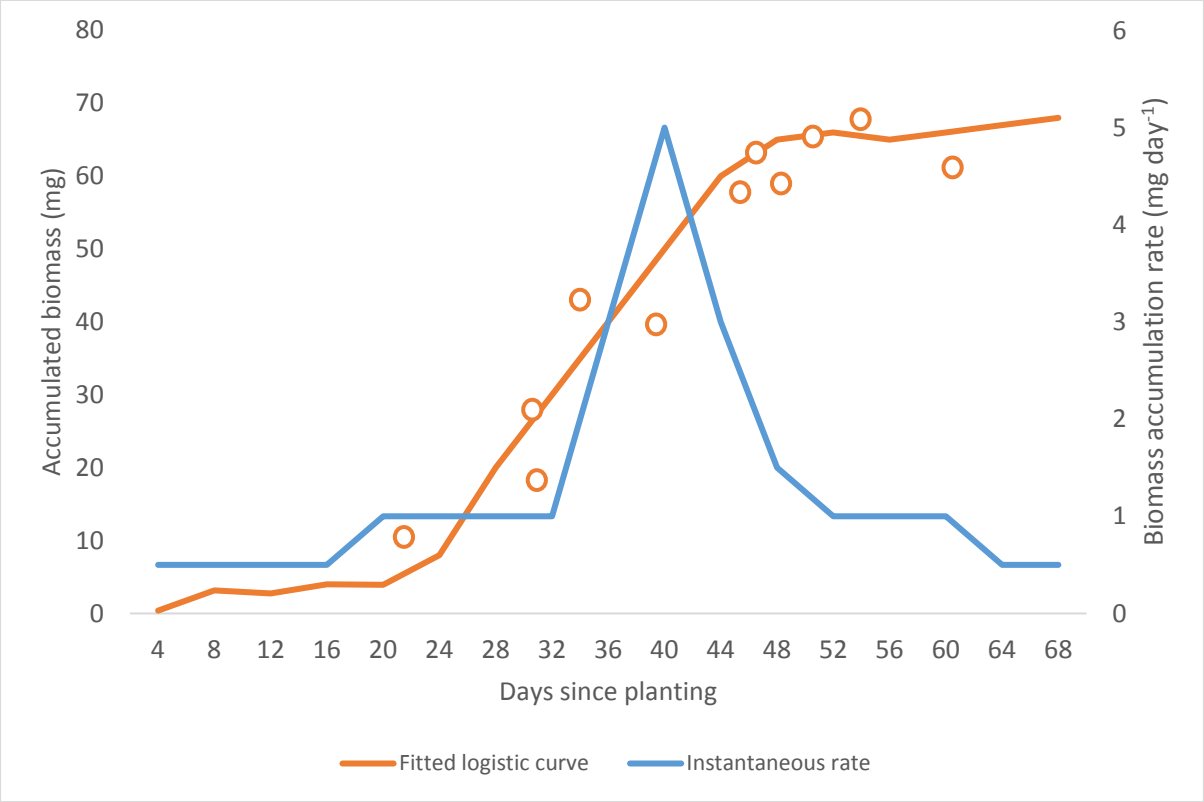

Figure 3.1 *– A hypothetical fitted logistic growth curve (orange line) of biomass from successive harvesting (orange circles) with the corresponding derived instantaneous accumulation rate curve (blue line). The number of data points and frequency of sampling influence the slope of the fitted curve. More frequent sampling and greater replicate number will lead to a more accurately fitted curve.*

Temporal dynamism is a potentially crucial factor in models of plant community coexistence (Schofield, *et al.*, 2018), and such modelling allows us to gain important information about the impact of experimental treatments (including the presence of neighbours) on the rates of processes through time. As the model-derived values are at a finer temporal resolution than the data on which they are based, they can provide greater detail about the dynamics of continuously varying processes. However, those few studies addressing directly the measurement of temporal dynamism in resource capture have employed a number of different logistic model designs and software programs to analyse their data and fit logistic (or related) models. This makes comparisons between such studies and any subsequent meta-analyses difficult to carry out.

Two studies provide the focus for this chapter: Trinder *et al.* (2012) and Schofield *et al.* (2019). Both studies focused on the temporal dynamics of resource capture using successive harvests but used different models and software programs to analyse the datasets. Trinder *et al.* (2012) measured resource capture in two competing grassland species, grown either in competition or isolation. Using successive harvesting of *Dactylis glomerata* and *Plantago lanceolata*, the study aimed to understand interspecific competition as a temporally dynamic process rather than relying on potentially misleading 'snapshot' comparisons of final yields (Gibson *et al.*, 1999). Schofield *et al.* (2019) used successive harvests to examine cultivar differences in temporal dynamics in response to intra-specific competition in barley (*Hordeum vulgare).*

When comparing these studies, the initial factor to consider is the design of the logistic model used to analyse the data. Both Trinder *et al.* (2012) and Schofield *et al.* (2019) used the logistic growth equation. Trinder *et al.* (2012) used a two-parameter form of the logistic growth curve, whereas Schofield *et al.* (2019) used a three-parameter logistic model. However, differences in the number of parameters used in fitting logistic growth curves may have influenced the modelled peak nitrogen and biomass accumulation rate values. Trinder *et al.* (2012) used a two parameter logistic model to model the growth between two time points, using the asymptote (ymax) and a scaling factor (r) from a fixed initial value, whereas the logistic curve modelling undertaken by Schofield *et al.* (2019) used the nls (non-linear least squares) function in R, taking the midpoint of the logistic curve (xmid), asymptote (ymax) and scaling factor (scal) as parameters. Understanding the extent to which such

analytical differences might impact on the results is essential in drawing informed comparisons between multiple studies, as well as improving the accuracy of peak nutrient uptake rate estimates and is the first focus for this chapter.

Another factor in the analysis of these datasets is the software program used to analyse the data. Those studies that have addressed the measurement of temporal dynamism in resource capture have employed a number of different software programs to analyse their data and fit logistic (or related) models, including SAS (Andersen *et al.*, 2007; Moreira *et al.*, 2015), SPSS (W. P. Zhang et al., 2017) and MATLAB (Neumann *et al.*, 2017). Two of the more commonly used software programs are Microsoft Excel using the SOLVER add-in (Robinson, Davidson, Trinder, & Brooker, 2010; Trinder *et al.*, 2012; Li, et al, 2014; Li *et al.*, 2016) and R Statistical Software (R Core Team, 2015) (using nls within the stats package) (Dormann & Roxburgh, 2005; Paine *et al.*, 2012; Wei *et al.*, 2018; Schofield *et al.*, 2019). As Trinder *et al.* (2012) used Microsoft Excel and Schofield *et al.* (2019) used R, a comparison of these software programs as analytical tools is the second focus for this chapter.

In addition to the influence of analytical approach, the frequency of sampling and sampling effort at each time point (number of replicates) can affect estimates (Figure 3.1) of temporally dynamic processes (Miller-Rushing *et al.*, 2008). Sampling frequency and effort are often limited by practical considerations such as the growth form of the plant being studied, time, space and funding available (Goldberg & Barton, 1992; Trinder *et al.*, 2013). But there must exist both a minimum sampling effort below which the quality of information provided is worthless, and a maximum above which further increases in effort provide only disproportionately small returns. Identifying sampling regimes that are both optimal and practical is a long-standing problem in experimental design. Temporal dynamism studies often vary in sampling frequency and replicate number. Paine *et al.* (2012) suggested that a minimal number of replicates with very frequent sampling would provide the most accurate representation of growth dynamics. However, there are few studies that test this hypothesis to provide recommendations using a specific curve fitting model. One such study by Kreyling *et al.* (2018) found that, when sampling environmental drivers along a gradient, an increase in sampling locations at the expense of replicates improved the predictive success and reduced systematic over or under estimation of the

model. However, replication improved local precision and prediction of the true value (Kreyling *et al.*, 2018). Therefore, an estimation of the optimum sampling frequency and replicate number, while accounting for practical considerations, would strengthen the experimental design of resource capture temporal dynamism studies. This approach can then also be applied to other uses of logistic and general non-linear growth curves to optimise experiment size and sampling frequency.

To summarise, here I explore the impact of analytical approach and sampling regime on the assessment of temporal dynamism of plant processes. In particular I tested two hypotheses: 1) the number of parameters in the logistic model and a different software program will alter the estimation of peak accumulation rate; 2) increasing replicate number and sampling frequency will increase the precision of the estimates of instantaneous rates of nitrogen and biomass accumulation up to a point, beyond which further replicates and more frequent sampling frequency will not improve estimates.

3.2 - Materials and Methods

The first hypothesis, concerning analytical approaches, was addressed using the Trinder *et al.* (2012) biomass accumulation dataset. The second hypothesis, concerning sampling regime, was addressed using a dummy dataset of biomass accumulation derived from the Trinder *et al.* (2012) biomass data. The use of a dummy dataset allows the effect of replicate number and sampling frequency combinations to be compared to a known value of peak accumulation rate timing.

3.2.1 - Comparison of model parametrisation

The Trinder *et al.* (2012) biomass dataset was used to compare two and three parameter logistic models. To avoid potential confounding effects from use of a different software program, a two-parameter model with a fixed initial value was constructed in R. This effectively recreated in R the model used for the Excel-based analysis of Trinder *et al.* (2012) allowing us to separate the effect of software package (R vs. Excel) from model (2- or 3-parameter). Two and three parameter models in R were then compared.

3.2.2 - Comparison of software programs

The Trinder *et al.* (2012) biomass dataset was used to compare analytical results from both the Microsoft Excel 2007 with (v12) SOLVER add-in (two parameter Excel model) (Trinder *et al.,* 2012) and R nls 2-parameter approaches to analyse temporal patterns of biomass and nitrogen accumulation. Running the same model in both software programs allowed the effect of software program to be examined.

3.2.3 - Effect of sampling frequency and replicate number

To test the effect of sampling frequency and replicate number, a dummy dataset was created using the SSlogis function in R (R Core Team, 2015), with defined parameters based on the Trinder *et al.* (2012) biomass accumulation dataset for *Dactylis glomerata* grown in isolation. This provided a dataset with a known timing of peak biomass accumulation rate to which the model outcomes under different replicate and sampling frequency conditions could be compared. The dummy dataset was subsampled to produce datasets with 3, 5, 10 and 20 replicates and sampling every 1, 3, 6 and 9 days after planting. These subsampled datasets were then run using the R nls model (Schofield *et al.,* 2019) and estimates of peak timing and confidence interval width were plotted. The effect of sampling frequency, replicate number and interaction between the two factors were tested using an ANOVA test with the MASS package in R.

3.3 - Results

3.3.1 - Comparison of model parameterisation

A comparison of the three-parameter R nls model and the two-parameter R nls model provided information about the effect of the number of parameters on model estimates with the same software. The two models produced different shaped logistic curves (Figure 3.2a). The two-parameter model produced an earlier estimate in all treatments compared to the three-parameter model. The mean difference in peak biomass accumulation rate between the two models was 3 – 15 days (Figure 3.2b).

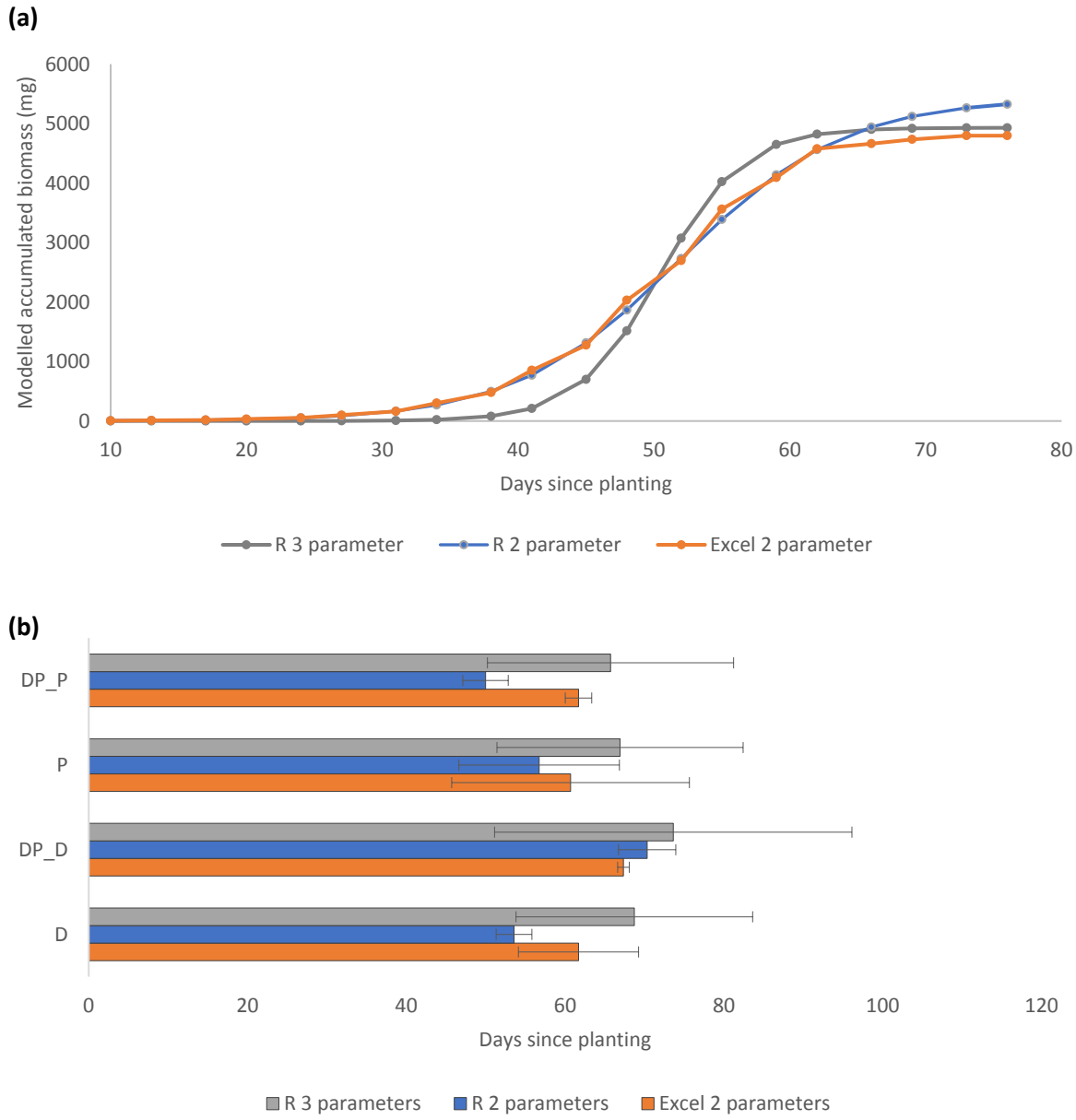

Figure 3.2 *– Panel (a) shows an example of the fitted logistic curves produced by the different models and software programs. In this case the modelled accumulation of biomass in* D glomerata *in isolation. Panel (b) shows the timing of peak biomass accumulation rate of* Dactylis glomerata *(D) and* Plantago lanceolata *(P) in isolation or interspecific competition (DP_P =* P. lanceolata *in competition, DP_D =* D. glomerata *in competition), using data from Trinder* et al. *(2012). The timing of peak biomass accumulation rate was modelled using a two parameter Excel model, R nls, and a 2 parameter model in R. Error bars are 95% confidence intervals.*

3.3.2 - Comparison of software programs

When the two-parameter model was run in both Excel (two parameter Excel model) and R (two parameter R model), the model estimates were different between software programs. However, the two programs produced similar shaped curves (Figure 3.2a). The two parameter R model produced an earlier estimate of peak biomass accumulation rate in three of the four treatments when compared to the estimate produced by the two parameter Excel model (Figure 3.2b). The mean difference in peak biomass accumulation rate was 3 to 11 days

3.3.3 - Effect of sampling frequency and replicate number

The dummy dataset peak accumulation rate timing was at 55.0 days after planting (54.6-55.5 95% CI) (Figure 3.3a). When sampling was less frequent than 3 days, the estimate became less precise, as indicated by a widening of the confidence interval widths (Figure 3.3b). With a 6-day sampling interval, the estimate was still close to the known value, i.e. within 1.6 days (52.02 - 57.66 95% CI with 5 replicates). At a 9-day sampling interval, the estimate varied by up to 3 days from the known value and had confidence interval widths of 6 - 8 days (e.g. 50.67 – 57.60 95% CI with 5 replicates). Sampling frequency had a significant effect on the estimate of peak accumulation rate timing ($F_{(3, 14985)} = 864.5$, P = < 0.01).

Less frequent sampling coupled with fewer replicates led to a less accurate estimate with larger confidence intervals. The 95% confidence interval width was decreased with an increase in replicate number, but the confidence intervals were still wider than with more frequent sampling. The greatest disparity with the known value was at a 9-day sampling frequency, when only 3 replicates were used. Beyond ten replicates there was little impact of replicate number (Figure 3.3a). Although replicate number had a minimal effect with the most frequent sampling, it had a much greater impact when sampling was less frequent. Overall, replicate number had a significant effect on the estimate of peak accumulation rate timing ($F_{(3, 14985)} = 263.1$, P = < 0.01). The combination of a low replicate number and infrequent sampling led to the estimates furthest from the known value with the largest confidence intervals. There was a significant interaction between replicate number and sampling frequency ($F_{(8, 14985)} = 411.7$, P = < 0.01).

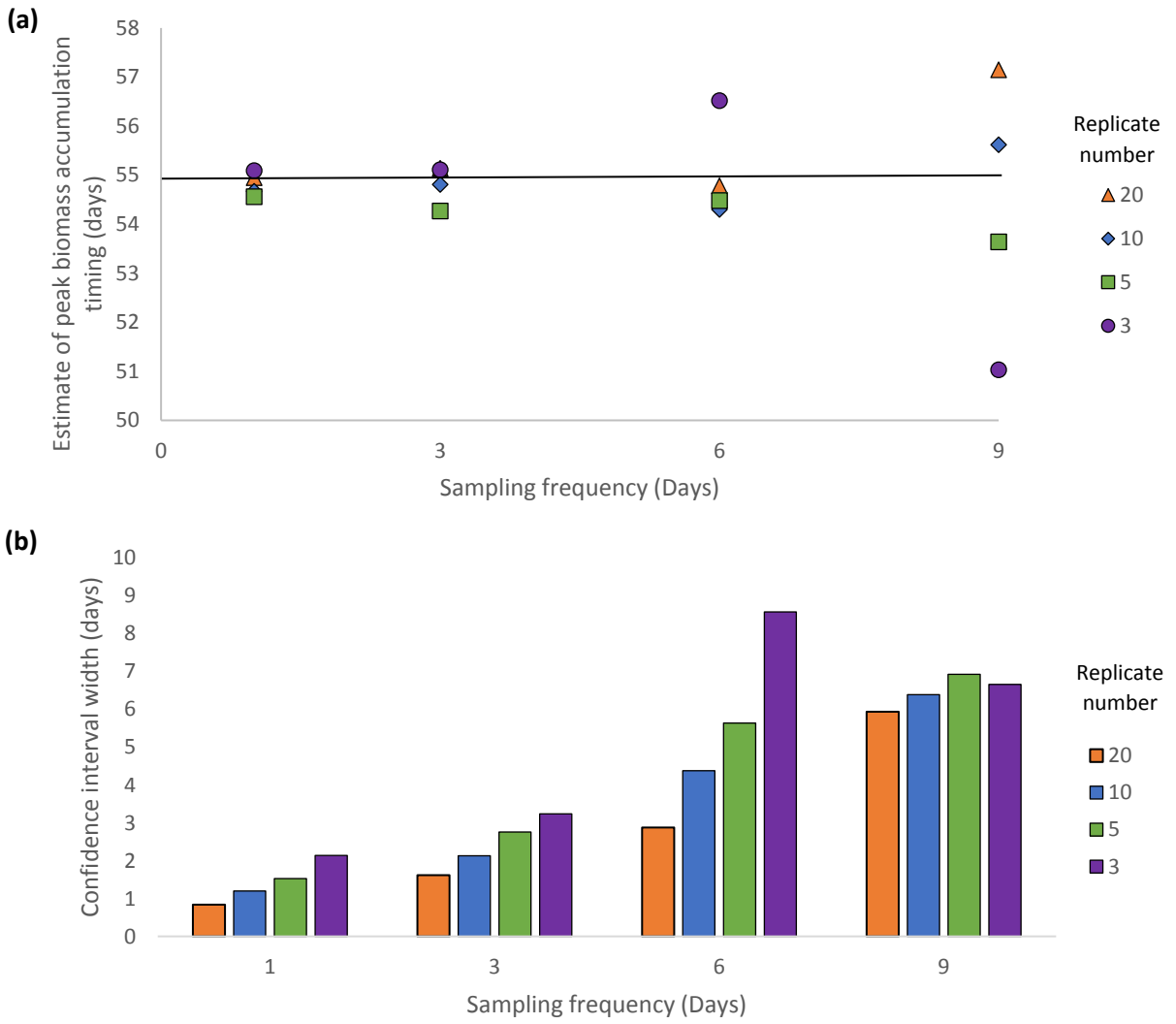

Figure 3.3 *– The effect of replicate number and sampling frequency on the estimate of peak biomass accumulation rate. Panel a shows dummy dataset estimates of timing of peak biomass accumulation with an increasing number of replicate. The black line shows the known value of peak biomass accumulation rate. The optimum estimate was found with a sampling interval of less than 6 days, with little effect of replicate number at less than this sampling frequency. Panel b shows the corresponding confidence interval width associated with each estimate. Smaller confidence interval widths were found with sampling at less than 6 day intervals.*

3.4 - Discussion

This study looked at the effect of model parameterisation and the software program used to analyse the data on the modelled timing of plant peak biomass accumulation rate. The effect of two commonly used software programs for data analysis, Microsoft Excel and R as well as two and three parameterised logistic models were examined using the Trinder *et al.* (2012) dataset of biomass accumulation. The software used and model parameterisation caused a shift in peak biomass accumulation rate of up to 15 days. A dummy dataset was also constructed with a known peak biomass accumulation rate timing to investigate the effect of sampling frequency and replicate number on the model outcome. Both sampling frequency and replicate number had a significant impact on the timing of peak biomass accumulation rate, affecting the estimate of peak accumulation rate and the associated confidence intervals.

3.4.1 - Effect of model parameterisation

The number of parameters used to fit the logistic growth curves impacted the estimate of peak biomass accumulation rate timing by 3 -15 days. The two-parameter model used a fixed initial value as one of the model parameters, giving only two available parameters with which the model could be fitted (Trinder *et al.*, 2012). The use of a model with a fixed initial value assumes this starting value had no error associated with it, which cannot be true of a measurement. This limitation in model fitting is likely to have accounted for the differences observed between the two and three parameter models in this study. This suggests that the use of a three-parameter logistic model would be more appropriate than a two-parameter model in temporal dynamism studies, as it accounts for variation in the initial starting value. Therefore, the design and parameterisation of a logistic model can have a profound effect on the estimate of peak biomass accumulation rate timing.

3.4.2 - Effect of curve fitting statistical software

There were differences in the estimate of peak accumulation rate between the two software programs even when the same basic model was used to analyse the Trinder *et al.*

(2012) dataset. The two parameter R model produced an earlier estimate of peak biomass accumulation rate compared to the two parameter Excel model. The difference between the two software programs was 3 – 11 days, a similar effect as found for differences in model parameterisation. Consequently, direct comparisons of conclusions drawn from logistic growth curves calculated using different software programs should be made with caution, as differing calculation processes appear to affect modelled estimates of peak accumulation rate. The raw data of temporal dynamism studies therefore should be made available and reanalysed with the most up to date models when comparing multiple studies in order to draw accurate comparisons between different ecosystems and species.

The version of Microsoft Excel SOLVER add-in used by Trinder *et al.* (2012) to fit the logistic growth curve has been found to have significant issues when fitting nonlinear least squares models (calculated using the SOLVER add-in). McCullough and Heiser (2008) found that SOLVER tended to state it had found a converged result when in fact it had not. The methodology of the calculation is opaque and not readily available, making it unclear if a solution has been reached or not (Mélard, 2014). These consistent errors lead many statisticians to recommend against the use of Excel to carry out statistical tests (McCullough and Wilson, 2005; Mccullough and Heiser, 2008) and this study would echo these recommendations.

When fitting logistic growth curves, the user often has to provide starting values for the program to fit the model. In a previous study, when Excel and R were compared using test datasets with starting values close to the true value, Excel was found to successfully fit logistic curves to 20 of the 27 test datasets, whilst R successfully fit logistic curves to all of the same test datasets provided (Odeh *et al.*, 2010). However, the same study (Odeh *et al.*, 2010) found both software programs performed equally well when analysing linear regressions. Therefore, the limitations of the software program used for analyses should be researched prior to use to ensure they are fit for purpose, as each have strengths and weaknesses when performing different statistical tests.

3.4.3 - Effect of sampling frequency and replicates on model estimates

The most accurate estimate (closest to the known value) of peak accumulation rate timing was found with a sampling frequency of 1 - 6 days. At this sampling frequency there was little effect of replicate number, with variation from the known value of less than 1 day between the different numbers of replicates. The dataset only covered 60 growing days, a relatively small proportion of the total lifecycle of most plants. However, many plant competition experiments are of a similar length (Trinder *et al.* 2012). This is not to say that a sampling interval less than every 6 days is ideal for all plant lifeforms or environmental conditions, as the dummy dataset used was based on a forb (*D. glomerata*) growing in greenhouse conditions. Further studies are required to find the optimum sampling frequency and replicate number for other lifeforms, timescales and environmental conditions. Therefore, running a pilot or simulation study prior to carrying out a large scale temporal dynamism study would determine the appropriate sampling frequency and replicate number under different conditions.

When designing experiments there are practical considerations including: space, time and cost (Trinder *et al.*, 2013). This dummy dataset analysis demonstrates that in temporal dynamism studies of this type, more frequent sampling led to more accurate estimates of peak accumulation rate timing. With up to a three day sampling frequency, three replicates is sufficient, whereas at six days and above five replicates are required for good temporal resolution. This echoes the findings of Kreyling *et al.* (2018) that sampling locations (or in this case sampling frequency) are of greater importance when examining trends compared to replication. However, replicates are of importance when detecting differences between treatments, as increased replication reduced the confidence interval size. Therefore, three replicates should be the minimum for statistical robustness to allow the model to run successfully. The balance between sampling frequency and replicate number should therefore be in favour of sampling frequency but not completely disregard replicate number, to ensure the balance between the detection of treatment differences and sufficient temporal resolution.

3.4.4 - Are there potential ecological consequences of the analysis approach and experimental design of temporal dynamism studies?

Both the number of model parameters and software program caused a change of up to 15 days in the estimate of peak biomass accumulation rate. This suggests that both the model and software program have a similar impact on the estimated outcome. The ecological importance of these shifts in peak accumulation rate depends on the species being studied and the length of the study. For annual or short-lived species, differences in estimates of peak accumulation rate timing may have important consequences for the ecological conclusions drawn from them. For example, the lifespan of spring barley can be as little as four months or ~120 days with ~60 days of active nutrient uptake (Spink, Blake, Bingham, Hoad, & Foulkes, 2015; Schofield *et al.*, 2019). Therefore, a difference of 15 days represents 25 % of the total nutrient uptake period and 12.5 % of the total lifecycle and so, in short lived species such as annual crops, the differences in peak accumulation rate caused by differences in model parameterisation and software program used for analyses may represent a significant proportion of the lifecycle of the plant. However, in perennial species that store resources between seasons these differences may have less impact on ecological conclusions as a result of different model and software program use.

3.5 - Conclusions

Both model parameterisation and the choice of software program caused a similar shift in the estimate of peak biomass accumulation rate, by up to 15 days. Therefore, when analysing data from temporal dynamism studies, both the number of parameters included in the model and the software used should be considerations. Although these differences are a matter of days, when studying short-lived individuals they may be important in the drawing of ecological conclusions. Microsoft Excel should be used with caution as there is evidence of errors in calculations when fitting non-linear models. Before a software program is used the parameterisation and calculation process should be researched to ensure that it is fit for purpose, as these can affect estimates of temporally dynamic processes. The number of replicates was found to have an overall smaller impact on the timing of peak accumulation rate, whilst sampling frequency had a greater effect on model

estimates. Therefore, when there are practical limitations, sampling frequency and experiment length should be prioritised. These factors are important in the design of experiments looking at the within-growing season temporal dynamics of resource capture and the factors that influence temporal dynamism of resource capture. The findings of this chapter will be used in the experimental design of Chapter 8.

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Chapter 4

Temporal patterns of soil processes: cultivar differences and the impact of plant-plant competition

Contents

Abstract

The soil microbial community has an important role in plant-plant competition by converting nutrients locked away in soil organic matter into forms suitable for plant uptake. Plants can reduce competition for resources through niche differentiation including changing the temporal dynamics of nutrient uptake to reduce direct competition and therefore promote coexistence. However, the role of soil processes in the temporal dynamics of nutrient uptake in response to plant-plant competition has yet to be explored. This study used two barley cultivars, Tammi and Proctor, grown in isolation, intra- or inter-cultivar competition. Root derived and primed soil respiration were measured using ^{13}C labelled CO_2, alongside soil solution samples taken to analyse soil nitrogen and organic carbon dynamics. Soil nitrogen concentrations and microbial biomass were analysed at the end of the experiment. Root derived and primed soil respiration peaked at 29 days after planting and then decreased but showed no change in temporal dynamics with competition. However, plants in competition had lower respiration per unit biomass. Mineral soil nitrogen decreased during the experiment and dissolved organic carbon concentration increased, indicating soil organic matter breakdown. Only organic carbon demonstrated a change in temporal dynamism in response to competition. This suggests that there is a need to look at specific soil processes as not all may be temporally dynamic in response to competition.

4.1 - Introduction

The soil microbial community has an important role in nutrient cycling, mining soil organic matter (SOM) for nutrients that are then made accessible to plants through microbial turnover (Hodge *et al.*, 2000). This background process is influenced by seasonal pulses of nutrients associated with spring warming in temperate environments (Bardgett *et al.*, 2005) or seasonal rains in semi-arid environments, providing temporally dynamic inputs of nutrients into a system (Chesson *et al.*, 2004).

Much of the activity of the soil microbial community is supported by plant roots, through the exudation of low molecular weight organic compounds into the rhizosphere (Alegria Terrazas *et al.*, 2016; de Vries and Caruso, 2016; Laliberté, 2016). This stimulates

the soil microbial community to mine SOM for nutrients (Rhizosphere Priming Effect (RPE) (Dijkstra *et al.*, 2013)). In this study priming is defined as the plant driven SOM mineralisation activity carried out by the soil microbial community (i.e. the increase in SOM mineralisation, relative to unplanted soil). The RPE is dependent on the availability of nutrients (Dijkstra *et al.*, 2013), soil carbon-nitrogen ratio (C:N) (Lloyd *et al.*, 2016) and microbial demand for resources (Hodge *et al.*, 2000). There is increasing evidence that root exudation of labile carbon can stimulate the activity of SOM decomposers and provide the plant with enhanced nutrient availability (Hamilton III and Frank, 2001). The RPE is could well be temporally dynamic, as root exudation quality and quantity varies with plant developmental stage (Chaparro *et al.* 2012), as well as between genotypes and species (Mommer *et al.* 2016). Therefore, RPE is a critical component of plant nutrient uptake and is likely to be an intrinsic component of the temporal dynamics of nutrient uptake.

The temporal dynamics (timing and rate) of nutrient uptake are important for plant development, successful flowering (Ne'eman *et al.*, 2006), viable seed set (Fenner, 1991) and long term species survival. The timing and rate of nutrient uptake varies over the course of a growing season, and in some species coincides with plant demand (Trinder *et al.*, 2012). Differences in the temporal dynamics of nutrient uptake are evident at a species (Trinder *et al.*, 2012) and genotype level (Schofield *et al.*, 2019). These dynamics may be one mechanism by which plants segregate nutrient uptake over time, reducing direct competition for resources and ultimately promoting coexistence. This may be important to understand coexistence in complex ecosystems. In current niche models there are seemingly insufficient niches to explain the coexistence of species in ecosystems such as rain forests and grasslands (Clark, 2010). However, these models often overlook time as a factor, in particular within-growing season temporal dynamism of nutrient uptake (Schofield *et al.*, 2018). Therefore, understanding the temporal dynamics of nutrient uptake would allow the integration of another currently missing factor in niche models.

Temporal dynamism in nutrient uptake has the potential to alter plant interactions but competition can also impact the temporal dynamics of nutrient uptake. Trinder *et al.* (2012) found that interspecific competition between *Dactylis glomerata* and *Plantago lanceolata* altered the temporal dynamics of nitrogen and biomass accumulation in both

species. Therefore, plant-plant competition can influence the temporal dynamics of resource uptake, which can in turn influence the extent of competition between individuals.

A previous study (Schofield *et al.*, 2019) used barley (*Hordeum vulgare*) as a model species, growing two cultivars with differing phenology, an early (Tammi) and late (Proctor), in isolation, intra-cultivar and inter-cultivar competition. Temporally dynamic shifts were not evident in the rate of biomass accumulation but were found in peak nitrogen accumulation rate. Proctor delayed peak nitrogen uptake rate by 14.5 days and Tammi advanced it by 0.5 days when in intra-cultivar competition compared to inter-cultivar competition and plants in isolation. This study will test whether, in parallel to these plant level effects, the temporal dynamics of soil processes are impacted by plant-plant competition. It is expected that peak priming of the soil microbial community will be significantly delayed when Proctor is in intra-cultivar competition but not in the other treatments, in line with the shifts observed in Chapter 2 (Schofield et al., 2019).

4.2 - Materials and Methods

4.2.1 - Soil characteristics

The soil was sampled from an agricultural field (Balruddery Farm, Invergowrie, Scotland, 56.4837° N, 3.1314° W) that had previously contained spring barley (*Hordeum vulgare*) and had been subject to standard management for barley production (including fertiliser addition at a rate of 500 kg of 22N-4P-14K ha^{-1} yr^{-1}). The soil had an organic matter content (humus) of 6.2% ± 0.3% SEM (loss-on-ignition, n = 4) and a mean pH (in water) of 5.7 ±0.02 SEM (n = 4), a total inorganic nitrogen concentration of 1.55 ± 0.46 mg g^{-1} (n = 4) and microbial C biomass (using a chloroform extraction) of 0.06 ± 0.002 SEM mg g^{-1} (n = 4) (analysed by Konelab Aqua 20 Discrete Analyser (Thermo Scientific, Waltham, MA USA)). The soil was passed through a 6 mm sieve and stored at 4°C prior to use.

4.2.2 - Experimental setup

In this experiment two cultivars of Barley (*Hordeum vulgare*), the early developing Tammi and late Proctor were grown in isolation, and in intra- or inter- cultivar competition (T, P, TT,

PP, TP). Plants were grown in a carbon-13 enriched CO_2 ($^{13}CO_2$) environment during the entire growth period. This continuous labelling ensures that the plant C is uniformly labelled above and below ground, and therefore allows partitioning of plant and soil sources of respiration. Respiration by plant roots as well as respiration of ^{13}C-labelled root exudates by the soil microbial community will result in detectable $^{13}CO_2$ in soil respiration gas samples. This is a useful measure of plant investment in roots over time. In addition, any increase (relative to the unplanted controls) in soil organic matter derived respiration ($^{12}CO_2$) found in the planted treatments provides an indication of the priming effect plant roots are having on the soil microbial community (Murphy *et al.*, 2015).

The pots were packed to a dry bulk density of 1 g cm^{-3} and watered to 54 % of the soil water holding capacity, to provide sufficient moisture to the plants whilst limiting waterlogging of the soil. This was maintained by watering twice a week, including on the day of soil respiration sampling for the duration of the experiment, to limit competition for water. Pots containing bare soil were included as controls. Seeds of Tammi and Proctor cultivars of barley were germinated before being planted into pots, with four replicates of each treatment, 24 pots in total. The pots also contained a respiration chamber, a jar with a sealed lid containing ports to allow flushing of the headspace and an open bottom inserted 2 cm into the soil in the middle of the pot (headspace volume 210 ml). Germinated barley plants were planted at the side of the respiration chamber when the pot contained one plant, and on either side of a respiration chamber in pots with two plants (Diagram 4.1). Pots with rigid sides (diameter = 102 mm, depth = 135 mm) were used to avoid shifting of the respiration chamber when the pots were moved.

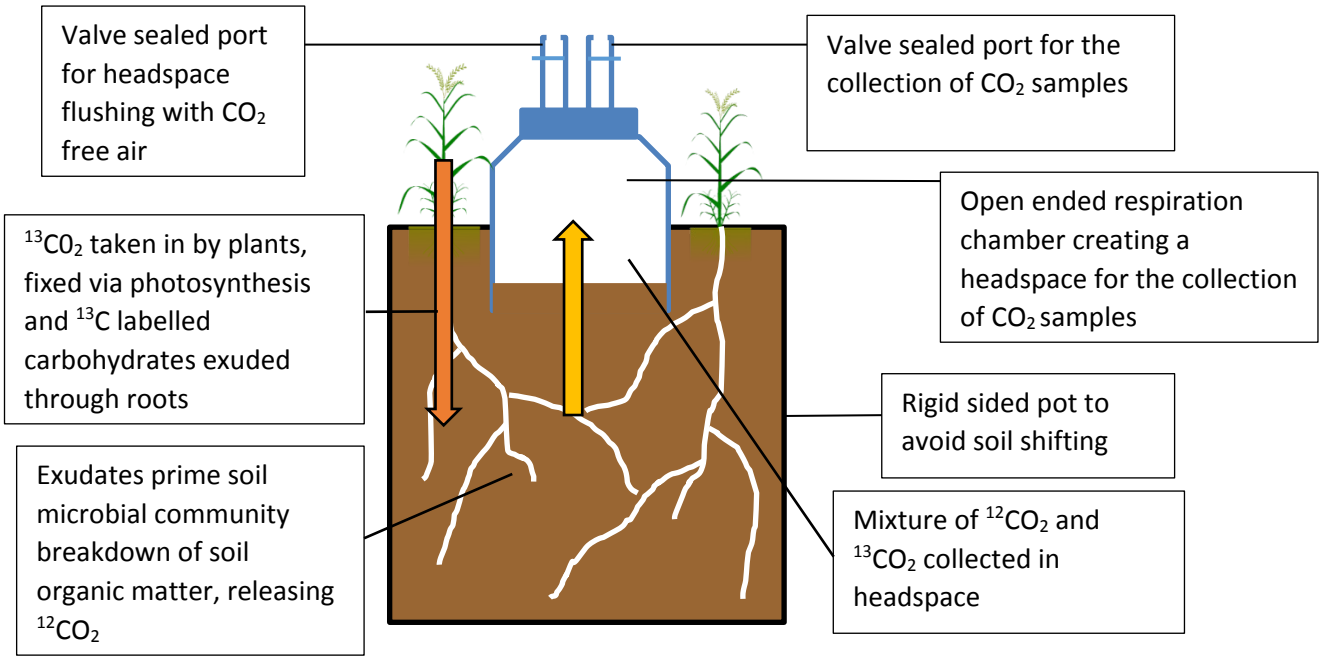

Diagram 4.1 *– Experimental setup of the soil respiration experiment, showing the positioning of the plants and respiration chamber. The seedlings were planted either side of an open-ended glass chamber, creating a headspace for the collection of CO_2 samples. Two ports with valves were attached to the top of the sealed headspace, one to allow for the flushing of the headspace with CO_2 free air prior to incubation and the other for the collection of CO_2 post incubation.*

The pots were placed into labelling chambers with an atmosphere of 400 ppm carbon dioxide, containing a mixture of ^{12}C and ^{13}C CO_2 (total flow rate 20 L min^{-1}) giving a ^{13}C isotopic enrichment of 2.60 atom percent (atm %), using mass flow controllers (Flotech Solutions, Stockport, UK). The tanks were kept at 15°C, with an 8/16 (day/night) photoperiod and 75 % relative humidity, higher than the previous experiment (Schofield *et al.*, 2019) due to the smaller size of the tanks. The ambient temperature of the controlled environment room was reduced when the lights were on, to maintain a constant temperature in the labelling chamber. The pots were arranged randomly in six rows of four pots and were repositioned within the tank containing the ^{13}C CO_2 atmosphere every few days to minimize potential positional effects.

4.2.3 - Soil respiration sampling

Soil respiration samples were taken once a week for six weeks through a valve in the respiration chamber. On sampling days, the headspace of the respiration chamber was flushed with CO_2 free air for five minutes, the CO_2 concentration recorded, and the jars sealed (C1 value in Equation 2). The pots were then replaced into the tanks and incubated in the dark for three hours. Ten ml of air from the headspace of each pot was sampled, the CO_2 concentration recorded (EGM-4, PP Systems, Amesbury, Massachusetts, USA) and samples taken for isotopic analysis. The ^{12}C to ^{13}C ratio was analysed using a gas bench (Deltaplus Advantage Thermo Scientific, Bremen, Germany) interfaced with an isotopic ratio mass spectrometer (Trace Ultra GC Thermo Scientific, Bremen, Germany). After 42 days, the pots were harvested, the shoots separated and the total root biomass for the pot washed. The shoots and roots were stored at -20°C then freeze dried and weighed.

4.2.4 - Soil solution sampling

Soil solution samples were taken weekly using a micro-rhizon soil solution sampler (Van Walt Environmental Equipment & Services, Surrey, UK) and frozen at -20°C prior to analysis. Soil solution samples were selected for analysis covering the period of 21-35 days after planting, the period of maximum soil microbial community priming based on the soil-derived CO_2 flux results. Solutions were analysed for nitrate (NO_3), ammonium (NH_4), total organic carbon (TOC), total nitrogen in soil solution (TN) and total organic nitrogen (TON) concentration directly, using a Konelab Aqua 20 Discrete Analyser (Thermo Scientific, Waltham, MA USA).

4.2.5 - Soil mineral nitrogen

The bulk soil mineral nitrogen concentration at the end of the soil respiration experiment was measured using potassium chloride (KCl) extraction (McTaggart and Smith, 1993). Fifty ml of 1 M KCl was added to the wet equivalent of 12.5 g of dry soil. This was then mixed in an end-over-end shaker for 1 hour, then filtered through Type 1 Whatman filter paper (GE Healthcare Life Sciences, Buckinghamshire, UK), which had been pre-rinsed with 1 M KCl

three times. The samples were analysed using a Konelab Aqua 20 Discrete Analyser (Thermo Scientific, Waltham, MA USA).

4.2.6 - Microbial biomass

Microbial biomass was measured by the difference in dissolved organic carbon (DOC) concentration between extracts from chloroform fumigated and non-fumigated soil samples (Vance *et al.*, 1987). Two samples of moist soil were taken from a homogenous soil sample from each pot at the end of the experiment, with the equivalent mass of 12.5 g dry soil. The samples to be fumigated were placed in a vacuum desiccator containing chloroform and a vacuum applied overnight. The fumigated and non-fumigated soils were then added to 50 ml 0.5 M potassium sulphate (K_2SO_4) and mixed using an end-over-end shaker for 30 min. The samples were filtered through Whatman 42 filter paper (GE Healthcare Life Sciences, Buckinghamshire, UK) and analysed using an OI 1010 TOC Analyser (O.I. Analytical, Texas, USA). The difference in carbon content between the fumigated and non-fumigated samples was then used to determine the microbial biomass (conversion factor 0.45 (Wu *et al.,* 1990)) per pot.

4.2.6 - Statistical analysis

The data were analysed differently, depending on whether it was a repeated measure or was sampled at the end of the experiment (Table 4.1).

Table 4.1 *– Sampling frequency of data collected in this study and the statistical test applied for analysis. NH_4 = ammonium, NO_3 = nitrate, TOC = total organic carbon, TON = total organic nitrogen, TN = total nitrogen, DOC = dissolved organic carbon.*

Measure	Time of sampling (Days since planting)					Statistical test used
	15	21	29	35	42	
Root respiration						Peak respiration timing compared using a Kruskal-Wallis test. Random factor = Pot number, Treatment = fixed factor.
Root Priming Effect						Peak priming timing compared using a Kruskal-Wallis test. Random factor = Pot number, Treatment = fixed factor.
Soil solutions: NH_4^+, NO_3^-, TOC, TON, TN						Linear model, Fixed factors = treatment and time point sampled. Effect of each factor and interaction between each factor tested.
Bulk soil nitrogen: NH_4^+, NO_3^-, TON						Kruskal-Wallis test. Random factor = pot number, Fixed factor = Treatment.
Microbial biomass (DOC)						Kruskal-Wallis test. Random factor = pot number, Fixed factor = Treatment.
Final root biomass						Students t-test comparing treatments with one and two plants per pot. Random factor = Pot number. Fixed factor = treatment.
Root respiration per unit biomass						Students t-test comparing treatments with one and two plants per pot. Random factor = Pot number. Fixed factor = treatment.

4.2.6.1 - Root derived respiration

Root derived respiration is a measure of the overall respiration derived from the root carbon. The delta value (δ) describes the ratio of $_{12}C$ to $_{13}C$ in the sample. The control $\delta^{13}C$ value measured the soil source signature (i.e. CO2 flux from unplanted soil), accounting for the diffusion of $^{13}CO_2$ from the atmosphere into the soil. The root derived respiration value was a proxy for plant and microbial mineralisation. It was analysed directly from milled dried root samples taken at the end of the experiment providing the $\delta^{13}C$ root for Equation 1 (Murphy et al., 2017). These values were then expressed as respiration per gram of soil per hour ($\mu g\ CO_{2\ g}^{-1}\ hr^{-1}$).

Equation 1

$$Root\ respiration = (\delta^{13}\text{C sample} - \frac{(\delta^{13}\text{C sample} - \delta^{13}\text{C control})}{(\delta^{13}\text{C root} - \delta^{13}\text{C control})}$$

δ^{13}C (‰) sample was taken from the planted pots, δ^{13}C (‰) control from unplanted pots

δ^{13}C root is a proxy for root derived respiration including microbial mineralisation of root derived substrates (Murphy et al. 2017).

4.2.6.2 - SOM derived respiration

This is the respiration derived from the microbial mineralisation of SOM alone. It was calculated by subtracting the root derived respiration rate from the total respiration rate over the incubation period (Equation 2).

Equation 2

$$SOM\ derived\ respiration = (C2 - C1) - root\ derived\ respiration$$

C1 = CO_2 concentration after flushing the respiration chamber with CO_2 free air, C2 = CO_2 concentration after three hours of incubation (Mwafulirwa et al., 2017).

4.2.6.3 - Root priming effect

The root priming effect is the soil respiration rate promoted above that of the bare soil controls when the plant respiration rate is subtracted (Equation 3).

Equation 3

$$Priming\ effect = SOM\ derived\ respiration\ rate - control\ soil\ respiration\ rate$$

4.3 – Results

4.3.1 - Root biomass

At the point of harvest there was no significant difference in the total root biomass between the treatments containing one plant compared to those containing two (t_9 = -0.72, P = 0.45). Therefore, there was a lower root biomass per plant in pots where plants were in competition compared to those containing plants in isolation.

4.3.2 - Root derived respiration

The rate of root-derived respiration increased until a peak at between 21 - 29 days, and then declined; this was true for all the treatments. There was no significant difference between the two cultivars, or any of the treatments in terms of the magnitude or timing of root-derived respiration. At the end of the experiment when the plants were harvested, the treatments with two plants had significantly lower root-derived respiration per unit biomass compared to those with one plant (t_9 = 2.72, P = 0.02) (Figure 4.1).

4.3.3 - Root priming effect

There was no significant difference in the timing of peak priming promoted by either cultivar grown in isolation compared to when in competition (χ^2_4 = 2.77, P = 0.59) (Figure 4.2), as the peak in root primed activity occurred at 29 days for both cultivars, regardless of whether in competition or isolation. This followed the pattern of root-derived respiration, as peak priming coincided with peak root respiration rate.

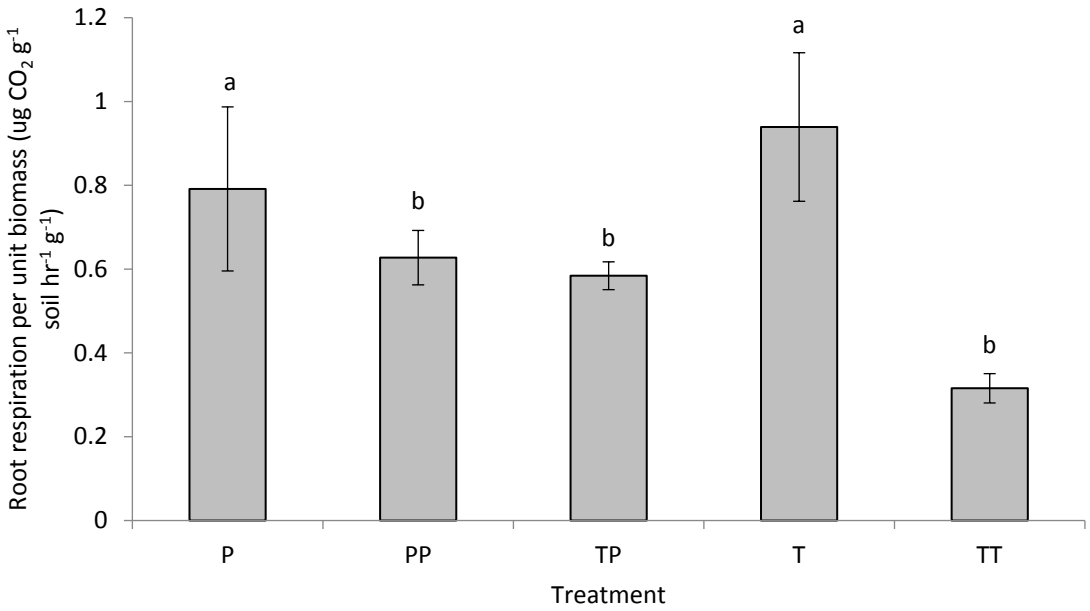

Figure 4.1 *– Root-derived rate of soil respiration per unit biomass at the end of the experiment, after 42 days of growth. Root-derived rate of soil respiration per unit biomass was derived from isotopic and respiration data of two barley cultivars grown together or in isolation. Pots contained Proctor in isolation (P), in competition with Tammi (TP) and in competition with another Proctor (PP), Tammi in isolation (T), competition with Proctor (TP) and another Tammi (TT). Error bars are two times the Standard Error of the Mean (SEM). Letters indicate significant differences.*

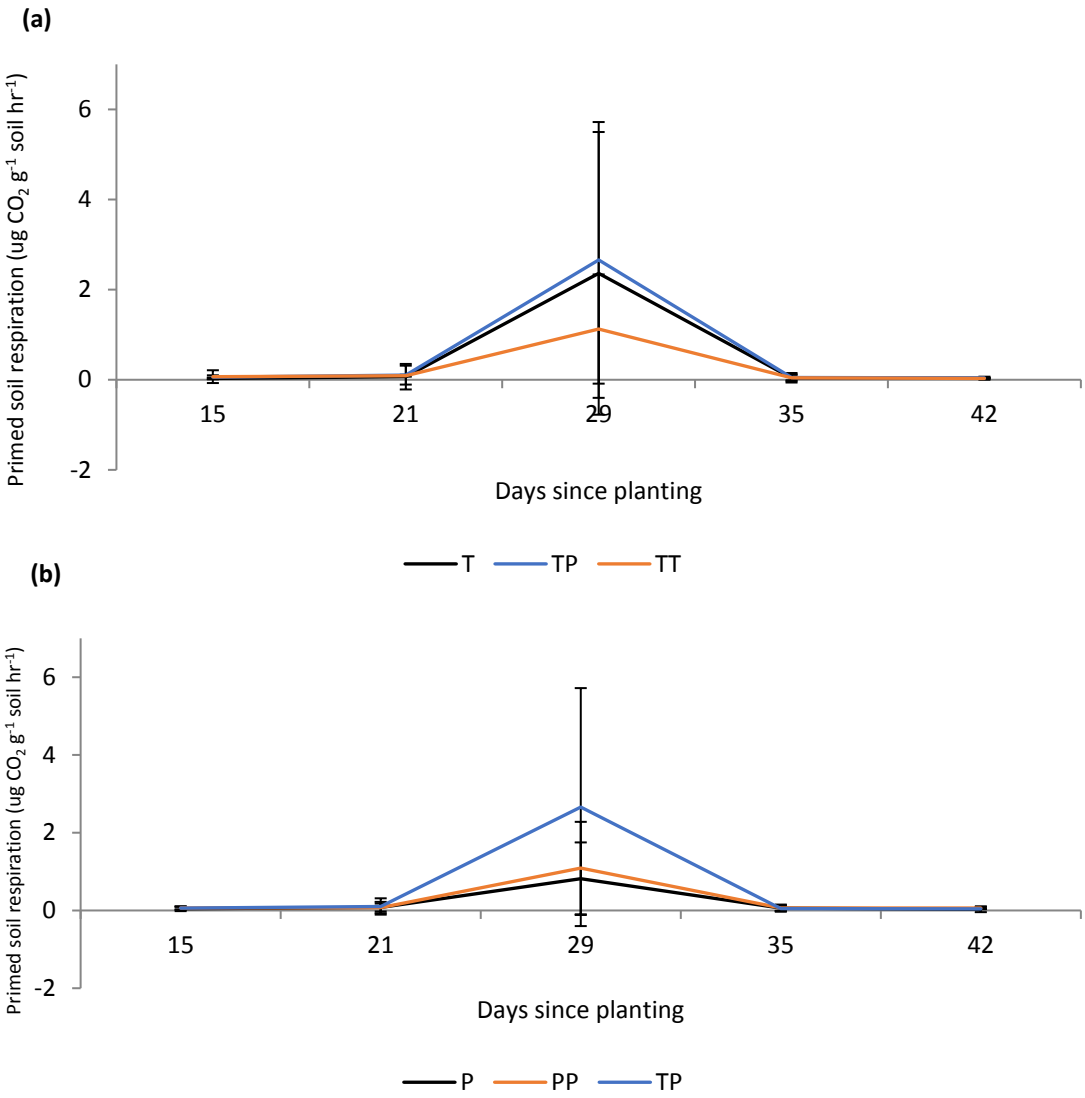

Figure 4.2 *– Root priming effect over time in soils under two barley cultivars relative to the unplanted controls. Pots contained Proctor in isolation (P), in competition with Tammi (TP) and in competition with another Proctor (PP) (panel a), Tammi in isolation (T), competition with Proctor (TP) and another Tammi (TT) (panel b). Error bars are two times the Standard Error of the Mean (SEM).*

4.3.4 - Microbial biomass

At the end of the experiment total microbial biomass was not significantly higher in the planted treatments compared to the bare soil control ($\chi_5^2 = 8.93$, P = 0.11), and there was no significant difference between any of the planted treatments ($\chi_4^2 = 1.71$, P = 0.78).

4.3.5 - Soil solution analysis

The concentration of ammonium (NH_4^+) in the soil solution samples was not significantly affected by time or treatment between the planted treatments ($F_{(12,46)} = 1.09$, $P = 0.39$). This was also found for nitrate ($F_{(12,46)} = 1.92$, $P = 0.06$), total organic nitrogen ($F_{(14, 44)} = 1.62$, $P = 0.11$) and total nitrogen ($F_{(14, 45)} = 1.55$, $P = 0.13$).

There were significant effects of both time ($F_{(2, 45)} = 9.52$, $P < 0.01$) and treatment ($F_{(4, 45)} = 4.23$, $P < 0.01$) on the concentration of TOC in the soil solutions (Figure 4.3). However, at the end of the experiment there was no significant difference between the treatments. There was also no significant interaction between time and treatment ($F_{(8, 45)} = 0.79$, $P = 0.61$).

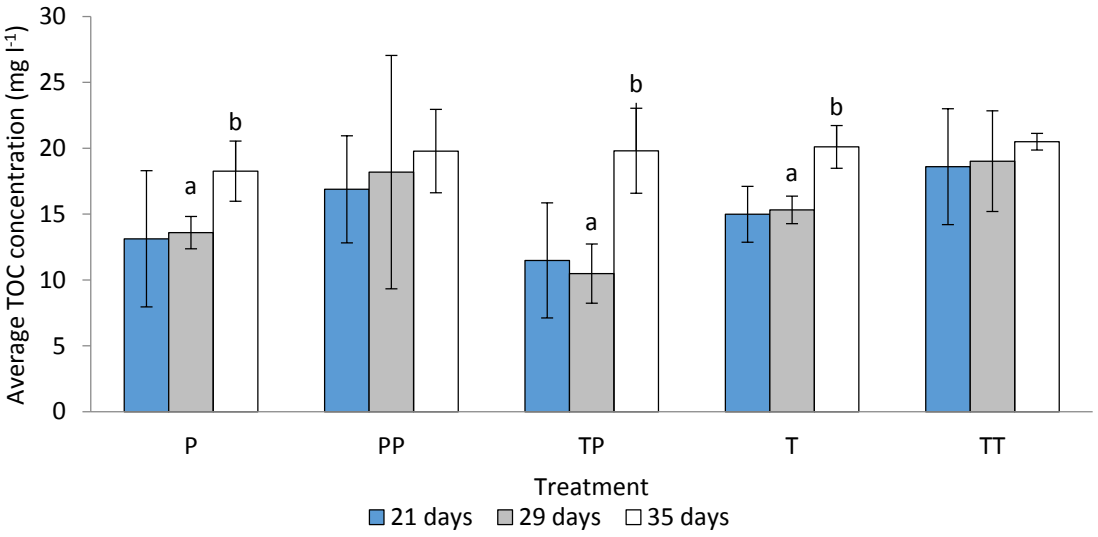

***Figure 4.3** – Mean soil solution concentration of total organic carbon from pots containing barley cultivars grown in isolation, inter- or intra- cultivar competition. Proctor = P, Proctor and Proctor = PP, Tammi and Proctor = TP, Tammi = T, Tammi and Tammi = TT. Error bars are the twice the standard error of the mean. Letters indicate significant differences.*

4.3.6 - Soil nitrogen

By the end of the experiment the concentrations of both ammonium and nitrate in bulk soil samples were low (Figure 4.4). There were no significant differences in ammonium concentration at the end of the experiment ($\chi^2_5 = 4.88$, P = 0.42). However, there was a significant difference between the treatments ($\chi^2_5 = 14.29$, P = 0.01), with nitrate concentration lower in the PP and T treatments compared to the other treatments.

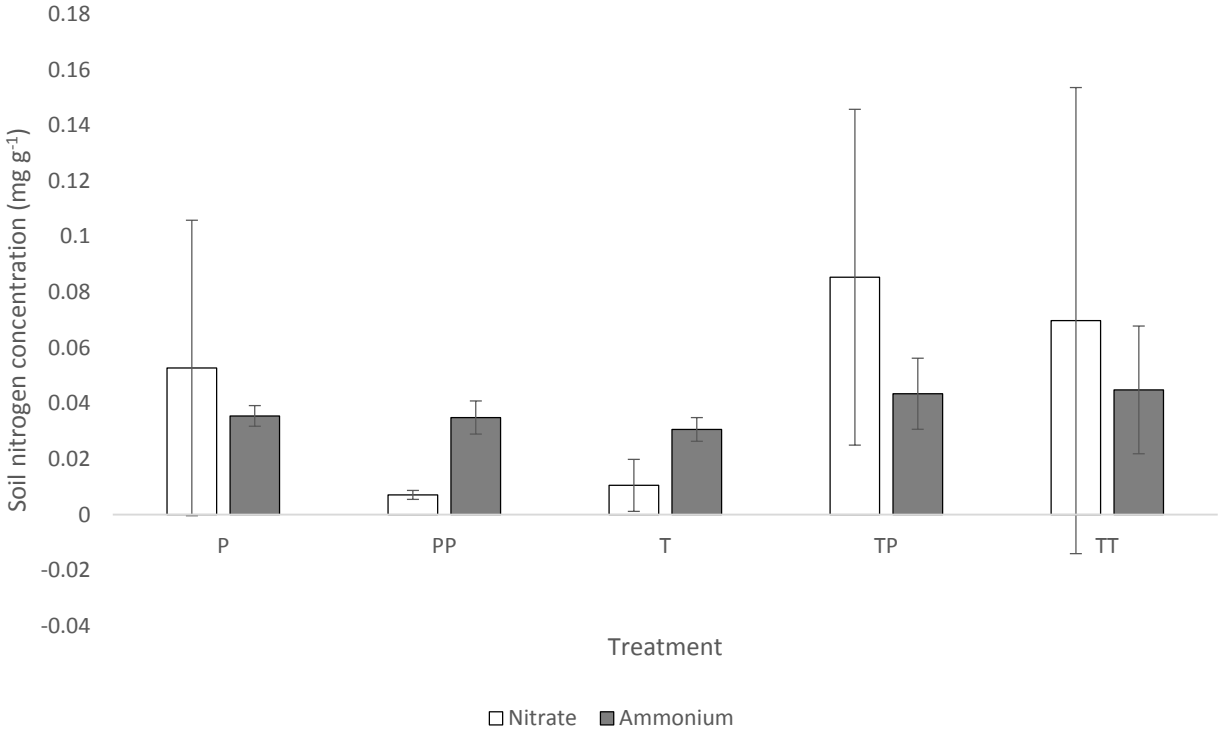

***Figure 4.4** – Mean concentration of NO_3 and NH_4 extracted from soil samples at the end of the soil respiration experiment. (T = Tammi, P = Proctor, TP = Tammi and Proctor in competition, TT = Tammi own cultivar competition, PP = Proctor own cultivar competition, bare = bare soil control). Error bars are two times the standard error of the mean.*

4.4 - Discussion

This study aimed to determine the temporal patterns of soil processes in response to barley cultivars with differing phenologies and plant-plant competition. An early (Tammi) and late (Proctor) barley cultivar were grown either in isolation, intra-cultivar competition or inter-cultivar competition. Root and soil derived respiration were measured over the early stages of plant growth. Soil solution samples were taken during the period of peak priming to

examine potential impacts of priming on nitrogen dynamics. Bulk soil samples from the end of the experiment were taken to determine if there was any cultivar or competition effect on the final soil mineral nitrogen concentration and microbial biomass.

4.4.1 - Root respiration and priming are not temporally dynamic in response to competition

Root respiration and priming were not significantly different between pots containing plants in competition compared to isolation. This indicates the impact that competition had on net root respiration and priming as the measurements taken were at the pot scale. The measurements taken in this study when plants were in competition represent the combined fluxes of both plants, instead of each individual plant separately. Competition may have caused upregulation in one individual and downregulation in the other, leading to a lack of individual response resolution. Further studies labelling an individual plant could determine if there is variation at an individual plant scale.

Root biomass and total respiration at the end of the experiment were not significantly different between treatments containing one or two plants. However, at the end of the experiment there was a lower root respiration per unit biomass in treatments with two plants. This indicates that competition limited plant growth and activity. A low respiration per unit biomass in the competition treatments may also be due to the period of low RPE at the end of the experiment (Figure 4.2). This may have been because soil nitrogen became depleted more quickly in these pots compared to those that contained only one plant.

Peak root respiration and the root priming effect both occurred between 25-29 days after planting in all treatments. The fact these events coincide supports the root priming effect theory, as greater root respiration indicates more root activity (including the exudation of organic carbon), which can lead to priming of the soil community and turnover of the soil microbial community within days (Hodge *et al.*, 2000).

The rhizosphere priming effect, mediated by impacts of root exudation of microbial communities, did not exhibit any temporally dynamic change in response to the presence of a competitor, regardless of the identity of the competitor. Soil community priming is

important when concentrations of bioavailable nutrients are insufficient to support plant requirements (Dijkstra *et al.*, 2013). This experiment used a fertilized agricultural soil, with high nitrogen content at the beginning of the experiment, which may have delayed a competition response in a neighbouring plant. Detection of a neighbouring plant can occur through overlapping of nitrogen depletion zones (Craine and Dybzinski, 2013) or through belowground signaling, for example using volatile organic compounds or root exudates (Pierik *et al.*, 2013). However, the dataset was highly variable and the frequency of sampling may have been too low to measure the timing of these soil processes, many of which occur over the timescale of hours (Hodge *et al.*, 2000). Therefore, a clear determination of fine scale temporal dynamism of RPE may require a similar study at a finer temporal scale. Also, a direct focus on specific microbial processes involved in nutrient mobilisation from organic matter rather than carbon mineralisation would lead to a more sensitive assay of soil microbial processes.

Despite the effect of RPE, at the end of the experiment there was no significant difference in the microbial biomass between the planted treatments. By the end of the experiment there was extensive root growth, and so all soil within the pots was considered to be rhizosphere. This could indicate that any increase in microbial biomass was transient, occurring during the period of peak priming and was therefore not recorded at the end of the experiment. Alternatively it could suggest that priming by plants in competition compared to those in isolation does not support a larger microbial population but a community with different functioning (Houlden *et al.*, 2008). The observed temporal dynamics of TOC indicates SOM mining during the period, with a stronger response likely to be found as soil closer to roots is sampled.

There was a significantly higher total TOC concentration in the soil solutions sampled from planted treatments compared to the unplanted control. As positive priming of the soil community occurred it indicates SOM was being broken down by the soil microbial community (Dijkstra *et al.*, 2013). There were also significant differences between some of the planted treatments and over time. However, there was no significant difference in TOC concentration at the final sampling date, suggesting that although cultivar and competition may have altered the temporal dynamics of TOC formation, it had little effect on the final TOC concentration. This contrasts with the soil respiration results, which suggest little

temporal dynamism in the soil community activity. This suggests that changes in the temporal dynamics of the soil community may be better examined by looking at individual processes instead of total activity. Further studies including measures of microbial community structure and function changes over time are needed to test this hypothesis.

The lack of difference in soil nitrogen at the end of the study suggests that plants took up all the available nitrogen, depleting the mineral nitrogen pool. The concentration of nitrogen in soil solution is a function of both production (SOM breakdown) and consumption (plant uptake) processes. Therefore, differences in the concentration of nitrogen forms may be due to an increase or decrease in either the production of nitrate and ammonium or plant consumption rates. As NO_3 and NH_4 are intermediates between SOM and plant assimilated nitrogen (Hodge *et al.*, 2000), they are unlikely to be highly abundant in the soil solution, as they are rapidly taken up by plants. Any differences between the treatments therefore would be an indication of a bottleneck in the nitrogen mineralisation process (Chapman *et al.*, 2006). There may also be a balance between immobilization of nitrogen by microbes and plant uptake, leading to competition between plants and microbes for different forms of nitrogen. The intensity of competition depends on the predominant nitrogen form, its availability and demand for it (Schimel and Bennett, 2004). As it is the depolymerisation of macromolecules that is thought to be the limiting factor in nitrogen mineralisation (Schimel and Bennett, 2004), understanding the dynamics of the breakdown of macromolecules may help explain nitrogen dynamics in this study.

There was also no significant differences found in the soil samples taken at the end of the experiment, with no significant difference in NH_4, NO_3 or TON between any of the planted treatments. By the end of the experiment the plants were exhibiting symptoms of nitrogen deficiency and remobilisation of nitrogen from the older leaves. This is supported by the significant decrease in total nitrogen in the planted soils compared to the bare soil controls. Therefore, all the planted treatments utilised the available nitrogen, with plants in isolation absorbing more nitrogen per unit biomass than those in competition until the nitrogen in the pots was depleted. The absence of promotion of an increase in RPE may have been due to the life stage of the plants. All the plants in this study had begun to produce flag leaves, the growth stage prior to grain production, a sign of a reduction in nitrogen uptake and an increase in nitrogen remobilisation. This may explain why nitrogen

deficiency did not lead to an increase in RPE. Soil solutions with weekly sampling are therefore unlikely to be an effective method to study nitrogen fluxes over a growing season, as fluxes occur over the timescale of hours and are likely to have been missed with weekly sampling.

4.4.2 - Effect on final soil nitrogen concentration

Planted soil samples at the end of the experiment had significantly lower concentrations of NO_3, TON and TN in planted treatments compared to the unplanted control. This demonstrates the depletion of soil nitrogen in the planted treatments as the plants grew. However, there was no difference between the planted treatments. This may be due to sampling at the end of the experiment when nutrients were likely to be severely limited. Therefore, to understand the temporal dynamics of soil nitrogen depletion, sampling throughout the experiment is likely to be required.

There was an opposing trend in final microbial biomass which was significantly higher in planted treatments compared to unplanted controls but not significantly different between planted treatments. This suggests a link between nitrogen depletion and microbial biomass but no significant effect of cultivar or planting density.

4.4.3 - Are soil processes temporally dynamic in response to plant-plant competition?

Most of the soil processes in this study did not show shifts in temporal dynamics in response to cultivar, intra- or inter-cultivar competition. In all treatments root respiration and microbial decomposition of SOM peaked between 21 and 29 days after planting. The lack of temporal dynamism in root respiration and priming do not support the previous study (Schofield *et al.*, 2019; Chapter 2), which found a reduction in biomass and nitrogen accumulation with competition and a delay in peak nitrogen uptake rate in Proctor intra-cultivar competition. The only temporally dynamic process found in this study was the soil solution concentration of TOC, indicating SOM breakdown by the soil microbial community occurred, primed by root activity.

4.5 - Conclusions

This study has found that the temporal dynamics of root derived respiration and RPE did not differ between the two cultivars and is not influenced by plant-plant competition. The evidence from this study suggests that this sampling method and frequency may not be able to detect changes in soil nitrogen temporal dynamics in response to plant-plant competition. However, total organic carbon in soil solutions was found to be a good indicator of the temporal dynamics of SOM breakdown in response to plant root activity. Although plant-plant competition did not affect the temporal dynamics of the soil processes studied here, soil processes are likely to have an important role in mediating the temporal dynamics of nutrient uptake.

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Chapter 5

Plant-plant competition influences temporal dynamism of soil microbial enzyme activity

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Contents

Abstract

Root-derived compounds can change rates of soil organic matter decomposition (rhizosphere priming effects) through microbial production of extracellular enzymes. Such soil priming can be affected by plant identity and soil nutrient status. However, the effect of plant-plant competition on the temporal dynamics of soil organic matter turnover processes is not well understood. This study used zymography to detect the spatial and temporal pattern of cellulase and leucine aminopeptidase activity, two enzyme classes involved in soil organic matter turnover. The effect of plant-plant competition on enzyme activity was examined using barley (*Hordeum vulgare*) plants grown in i) isolation, ii) intra- and iii) inter-cultivar competition. The enzyme activities of leucine aminopeptidase and cellulase were measured from portions of the root system at 18, 25 and 33 days after planting, both along the root axis and in the root associated area with detectable enzyme activity. The activities of cellulase and leucine aminopeptidase were both strongly associated with plant roots, and increased over time. An increase in the area of cellulase activity around roots was delayed when plants were in competition compared to in isolation. A similar response was found for leucine aminopeptidase activity, but only when in intra-cultivar competition, and not when in inter-cultivar competition. Therefore, plant-plant competition had a differential effect on enzyme classes, which was potentially mediated through root exudate composition. This study demonstrates the influence of plant-plant competition on soil microbial activity and provides a potential mechanism by which temporal dynamism in plant resource capture can be mediated.

5.1 - Introduction

One of the key processes governing plant nutrient acquisition is mineralisation of soil organic matter (SOM) mediated by microbial communities, a process that can be significantly influenced by plant roots (rhizosphere priming effects: Murphy *et al.*, 2017). Plant root exudates contain large quantities of labile carbon, and increase carbon availability to the soil microbial community (Kuzyakov *et al.*, 2000; Garcia-Pausas and Paterson, 2011). Addition of carbon causes an increase in the carbon to nitrogen to phosphorus ratio (C:N:P), leading to nutrient "mining" by the soil microbial community to restore the stoichiometry of these resources (Paterson, 2003), driven by extracellular enzyme production (Penton and Newman, 2007). These rhizosphere priming effects eventually lead to plant nutrient acquisition through turnover of the soil microbial community (Hodge *et al.*, 2000).

The breakdown of organic matter in the soil is driven by enzyme activity, the majority (90 - 95 %) of which is derived from the soil microbial community (Xu *et al.*, 2014), with some directly from plant roots (Spohn and Kuzyakov, 2013). Enzymatic activity is temporally dynamic, changing in response to the prevailing environmental conditions and associated plant community activity throughout the growing season (Bardgett *et al.*, 2005). The temporal dynamics of soil processes vary with abiotic conditions such as temperature (Steinweg *et al.* 2012) and nutrient availability (Mbuthia *et al.* 2015). Therefore, enzyme activity can be used as a measure of a range of soil microbial community activities and the influence of different factors on these processes, including plant-plant interactions, through time.

As a focus for assessing temporal dynamism in soil enzyme activity, and the impact on this of plant-plant interactions, this study chose two catabolic enzyme classes involved in SOM breakdown and nitrogen cycling, cellulase (EC number: 3.2.1.4) and leucine aminopeptidase (EC number 3.4.1.1). Both the spatial and temporal dynamics of catabolic enzymes, including cellulase and leucine aminopeptidase can be examined using zymography. This method uses fluorescently labelled substrates to measure extracellular enzyme activity in soil. The area and intensity of fluorescence can be calibrated and used for spatial quantification of enzyme activity (Spohn and Kuzyakov, 2014). As this method is non-destructive, it allows a range of enzymes to be studied spatially and temporally (Giles *et al.*, 2018), making it ideal to explore the impact of plant-plant competition on the temporal

dynamics of soil enzyme activity. Measuring enzyme activity is not a direct measure of nutrient cycling. An increase in enzyme activity could indicate an increase in turnover of SOM through mining by the soil microbial community or an increase in nutrient demand as the soil microbial community produce secrete more extracellular enzymes due to a lack of available nutrients.

The intensity of competition between plants for nutrients can vary spatiotemporally (Caffaro *et al.*, 2013); this can alter the temporal dynamics of nitrogen accumulation (Schofield *et al.*, 2019) when plants are in competition compared to isolation, with potential consequences for the temporal dynamics of soil microbial community enzyme activity. The temporal dynamics of nitrogen and biomass accumulation have been studied in barley (*Hordeum vulgare*) (Schofield *et al.*, 2019). A delay in peak nitrogen uptake was found when the Proctor cultivar was grown in intra-cultivar competition but not inter-cultivar competition. This response may be due to a change in the temporal dynamics of root associated soil enzyme activity influencing nutrient availability for plants. Therefore, to explore whether such changes in the timing of soil processes do occur, Proctor was chosen as the focal cultivar of this study.

As well as plant-plant competition, plants compete with microbes for resources (Schimel and Bennett, 2004), another factor that is likely to be temporally dynamic. There are periods of high competition between plants and microbes during periods of plant nitrogen uptake (Bardgett et al., 2003). This is likely to influence the temporal dynamics of extracellular enzyme production by the soil microbial community as microbes compete with plants for nitrogen but are also influenced by plant-plant competition. Another factor that influences exoenzyme activity is microbial biomass. An increase in exoenzyme production could be due to the existing microbes producing more enzymes or an increase in microbial biomass. In order to determine this, microbial biomass needs to be quantified alongside enzyme activity.

Two main approaches for analysing zymography images have emerged in the last decade. Spohn and Kuzyakov (2014) measured the root associated area of cellulase activity as a percentage of the total sampled area (root associated area) when assessing the activity of cellulases, chitinases and phosphatases in the presence of living and dead *Lupinus polyphyllus* roots. Alternatively, Giles *et al.* (2018) took a root-centric approach, measuring

phosphatase activity along *Hordeum vulgare* root axis (root axis). The Spohn and Kuzyakov (2014) method takes a subsection of the greyscale values, excluding the lightest and darkest pixels; in contrast Giles *et al.* (2018) used the total pixel range. The Spohn and Kuzyakov (2014) method excludes pixels that are extremely bright, which may skew the total dataset. However, by focussing on the extent of activity in terms of area instead of intensity of activity along the root axis, a relatively small proportion of the soil volume, subtle temporal dynamics of enzyme activity may be more easily detected.

This study aimed to determine the influence of plant-plant competition on the activity of the soil microbial community while keeping other environmental factors constant. Whilst this study has not measured the effect of plant-plant competition on plant-microbe competition directly, the former has been suggested to influence the latter (Hortal et al., 2017). This study took the opportunity to use both approaches for analysing zymography images. The aim was to determine the effect of plant-plant competition on the temporal activity dynamics of the two enzyme classes, outside of the zone of most intense competition. Plant root architecture can demonstrate a compensatory response to plant-plant competition (Caffaro *et al.*, 2013). It is expected that enzyme activity surrounding plant roots will show similar trends to root architecture, with increased enzyme activity surrounding roots outside the zone of most intense competition when the plants are in competition compared to isolation. As competition can be less intense between more closely related individual plants, due to changes in the temporal dynamics of resource capture, it is expected that interactions between more closely related individuals will promote less intense enzyme activity than inter-cultivar competition.

5.2 - Materials and methods

5.2.1 - Soil characterisation

Soil was collected from an agricultural field that had previously been cropped with spring barley (*Hordeum vulgare*) and had been subject to standard fertilisation conditions (500 kg of N ha^{-1} yr^{-1} in the ratio of N 22 : P 4 : K 14) (Balruddery Farm, Invergowrie, Scotland, 56.4837° N, 3.1314° W). The soil was then passed through a 3 mm sieve to homogenise the substrate and then stored at 4°C until planting. The soil had an organic matter content

(humus) of 6.2 % ± 0.3 % SEM (loss-on-ignition, n = 4) and a mean pH (in water) of 5.7 ± 0.02 SEM (n = 4), a total inorganic nitrogen concentration of 1.55 ± 0.46 mg g^{-1} (n = 4) and microbial C biomass (using a chloroform extraction) of 0.06 ± 0.002 SEM mg g^{-1} (n = 4). No fertilisation occurred during the experiment.

5.2.2 - Rhizobox preparation

Rhizoboxes (150 mm x 150 mm x 10 mm Perspex boxes with a removable side for access to roots) were packed to a bulk density of 1.26 g cm^{-3}, ensuring the soil was level with the edge of each box. Seeds of Proctor and Tammi barley (*Hordeum vulgare*) cultivars were pre-germinated on damp tissue paper in the dark at room temperature for two days before planting. Three replicates of each treatment: Proctor alone (P), Proctor in intra-cultivar competition (PP) and Proctor in inter-cultivar competition with Tammi (TP) were planted, as well as a bare soil control, giving 12 rhizoboxes in total. In the planted treatments, the germinated seeds were placed on the surface of the soil, ensuring contact between the emerging roots and soil surface, and then the side of the box was replaced and secured. In the planted treatments containing two plants, the germinated seeds were placed 2.5 cm apart to ensure no aboveground interaction between the two plants.

The rhizoboxes were wrapped in foil to exclude light from the roots and placed at a 45° angle to encourage root growth over the soil surface. The rhizoboxes were kept in a controlled environment cabinet (Jumo IMAGO 3000, Harlow, Essex, UK) at a constant 15°C, 65 % relative humidity and a 16/8 (day/night) (light intensity: 200 µmol m^{-2} s^{-1}) photoperiod for the duration of the experiment to mimic local springtime conditions. Each rhizobox was watered weekly with sufficient water to maintain soil moisture at field capacity and prevent root desiccation.

5.2.3 - Soil zymography

Enzyme activity was measured three times at weekly intervals between 18 and 39 days after planting. This is the period prior to and including peak barley nitrogen accumulation rate found in the previous study (Schofield *et al.* 2019; Chapter 2). Areas away from the

competition zone between the two plants were visually identified and labelled on the rhizobox rim to ensure measurements of soil enzyme activity occurred at a consistent location throughout the study. These were roots of the focal individual that consistently did not encounter roots of the other individual within the system. This setup was used to indicate whether a compensatory or systemic response to plant-plant competition could be detected in soil enzyme activity.

Two fluorescently labelled substrates were selected for this study; 4-methylumbellferyl ß-D-cellobioside, a substrate of cellulase which was imaged at 365 nm (excitation at 365 nm, emission at 455 nm) and L-leucine-7-amido-methylcoumarin hydrochloride, a substrate of leucine aminopeptidase that was imaged at 302 nm (excitation at 327 nm, emission at 349 nm) (Sigma-Aldrich, Reading, UK). Both substrates were diluted to a 6 mM concentration, the concentration used in previous studies using methylumbellferyl ß-D-cellobioside (Spohn and Kuzyakov, 2014) and the optimum concentration found during preliminary experiments (results not shown). A 47 mm diameter polyamide membrane (Whatman, GE Healthcare, Buckinghamshire, UK) was soaked in 300 µl of 6 mM of 4-methylumbellferyl ß-D-cellobioside or L-leucine-7-amido-methylcoumarin hydrochloride. On sampling days, the side of each rhizobox was removed and a 1 % agarose (Invitrogen, Carlsbad, CA, USA) gel of 1 mm thickness was placed on the soil surface to protect the membrane from soil particles which could adhere to it and disrupt the final image, whilst allowing the diffusion of extracellular enzymes (Spohn and Kuzyakov, 2014). The membrane was then placed on top of the gel and the foil was replaced over the top to exclude light and minimise moisture loss during enzyme assays.

Previous studies have incubated similar substrate soaked membranes for between 30 minutes and 3 hours (Spohn and Kuzyakov, 2014; Giles *et al.*, 2018). Therefore, a preliminary study was carried out which found that, for this system, an incubation of 1 hour gave a good level of resolution and UV intensity when viewed (results not shown). Following incubation (1 h), the membrane was placed onto a fresh 1 % agarose gel to minimise bubbling of the membrane during imaging. The membrane and gel were then placed in an UV imaging box (BioDoc-It^2 Imager, Analytik Jena, Upland, CA) and imaged at 365 nm (Spohn and Kuzyakov, 2014). This was repeated for L-leucine-7-amido-methylcoumarin hydrochloride, which was imaged at 302 nm (Ma *et al.*, 2018). This order of substrate

sampling was maintained throughout the experimental period (Spohn and Kuzyakov, 2014). The sampled area was marked on the rim of each rhizobox to ensure that the same area was sampled each time for both enzymes. After sampling, the rhizobox was watered and replaced in the controlled environment chamber.

5.2.4 - Calibration curves

Known dilutions of 4-methylumbelliferone (the fluorescent tag of 4-methylumbellferyl ß-D-cellobioside) and 7-amino-4-methylcoumarin (the fluorescent tag of L-leucine-7-amido-methylcoumarin hydrochloride) (1, 2, 4, 6 mM) were prepared and used to soak membranes, using the same procedure as the experiment (Giles *et al.*, 2018). The membranes were then imaged using the same method and settings as the samples. The images were used to calculate the substrate concentration per mm^2 and provide the calibration curve values from the sample images. This also informed the range of 8 bit greyscale values (the integer brightness value per pixel between 0 - 255) sampled in the percentage area analysis (Spohn and Kuzyakov, 2014).

5.2.5 - Root growth measurements

The roots of each rhizobox were photographed weekly from 4 - 39 days after planting using an iPhone 6 (8 - megapixel iSight camera with 1.5 µm pixels, Apple Inc). The root architecture photographs were then analysed using the SmartRoot plugin (Lobet *et al.*, 2011) of the ImageJ software (Schneider *et al.*, 2012). The roots of each plant were manually traced and labelled using the Trace tool. This was used to measure total root length over time. Dry root biomass was also recorded at the end of the experiment by drying roots at 100 ° C for 24 hours.

The effect of time and treatment on the measured root architecture parameters were assessed using a Generalized Least Squares model using the nlme package in R (R statistical software, R Core Team, 2016). Time and treatment were included as fixed factors as well as the interaction between treatment and time. A covariate of rhizobox number and treatment was included to account for autocorrelation caused by the repeated measures in

this study. This was followed by an ANOVA test (MASS package, R statistical software, R Core Team, 2016).

5.2.6 - Enzyme image analysis

The intensity and location of enzyme activity was analysed using two approaches: root axis activity (Giles *et al.*, 2018) and root associated area (Spohn and Kuzyakov, 2014). These two approaches differ in that the root axis activity records soil enzyme activity only along the root itself, whereas the root associated area measures soil enzyme activity in the surrounding rhizosphere as well. By comparing these two approaches the most appropriate image analysis method to study the temporal dynamics in root associated soil microbial activity can be determined. Root associated area was defined as the percentage of the total sampled area with greyscale values above a threshold defined by the calibration curves that indicated enzyme activity.

5.2.5.1 Root axis enzyme activity

For this approach, root axis image analysis technique developed by Giles *et al.* (2018) was used. Proctor roots contained within the sample area were tracked using the segmented line tool in the Fiji image analysis software (Schindelin *et al.*, 2012). The RProfile plugin developed by Giles *et al.* (2018) was then used to extract a profile of greyscale values along the sampled root. The nodes of the segmented line placed along the root were then centralised and placed evenly along the sampled root to refine the data using the Python script developed by Giles *et al.* (2018). The mean greyscale value was calculated for each root (subsequently referred to as 'root axis activity').

5.2.5.2 - Root associated area analysis

To measure the root associated area of enzyme activity, the approach developed by Spohn and Kuzyakov (2014) was used. Each image was first converted into an 8-bit greyscale image. The range of 80 - 170 grey values was extracted from each image (informed by the

calibration curves) then split into 10 grey value increments, and the area of each increment measured using Image J Software (Schneider *et al.*, 2012). This was then expressed as a percentage of the total membrane area (subsequently referred to root associated area). The percentage root associated area was then compared between treatments. The mean enzyme activity rate was the most common enzyme activity rate, i.e. the rate with the greatest percentage cover of the total sampled area.

5.2.7 - Statistical analysis

The effect of time and treatment on the root axis activity and root associated area were each assessed using a Generalised Least Squares model, accounting for repeated measures with an autocorrelation term, using the nlme package (Pinheiro *et al.*, 2016) in R (R Core Team, 2015). This was followed by an ANOVA test for significant differences using the MASS package (Venables and Ripley, 2002) in R (R Core Team, 2015). The interaction between treatment and time was included as a fixed factor, to detect differences between treatments in enzyme activity temporal dynamics, with an autocorrelation term for treatment and rhizobox number.

5.3 - Results

5.3.1 - Total root growth

Total root length increased over time for all treatments (Table 5.1). There was a significant effect of treatment ($F_{(2,52)} = 5.45$, $P = <0.01$) and time ($F_{(4,52)} = 45.04$, $P = <0.01$) on total root length but no significant interaction between treatment and time ($F_{(8,52)} = 1.27$, $P = 0.28$). There was no significant difference in total root biomass between the different treatments at 33 days ($F_{(2,10)} = 0.78$, $P = 0.48$).

Table 5.1 *– Mean total root length and biomass at 33 days after planting of Proctor barley plants in isolation (P), intra-cultivar competition (PP) and inter-cultivar competition (TP) (n = 3). Values in the brackets are the standard error of the mean (SEM).*

Treatment	Total root length (mm)	Root biomass (g)
P	158 (±23.2)	0.036 (±0.004)
PP	138 (±15.5)	0.191 (±0.004)
TP	153 (±42.4)	0.042 (±0.007)

5.3.2 - Root axis activity

Mean cellulase root axis activity at 33 days after planting ranged between 1.4 and 11.8 pmol mm^{-2} h^{-1} and leucine aminopeptidase between 4.5 and 6.3 pmol mm^{-2} h^{-1} (Figure 5.1). For cellulase activity there was a significant effect of treatment ($F_{(2,42)}$ = 5.03, P = 0.01) but no significant effect of time ($F_{(2,42)}$ = 0.51, P = 0.60) or interaction between treatment and time ($F_{(4,42)}$ = 0.94, P = 0.45). However, there was no significant effect of time ($F_{(2,63)}$ = 2.92, P = 0.06), treatment ($F_{(2,63)}$ = 2.74, P = 0.07) or the interaction between the two factors ($F_{(4,63)}$ = 1.02, P = 0.40) for leucine aminopeptidase activity.

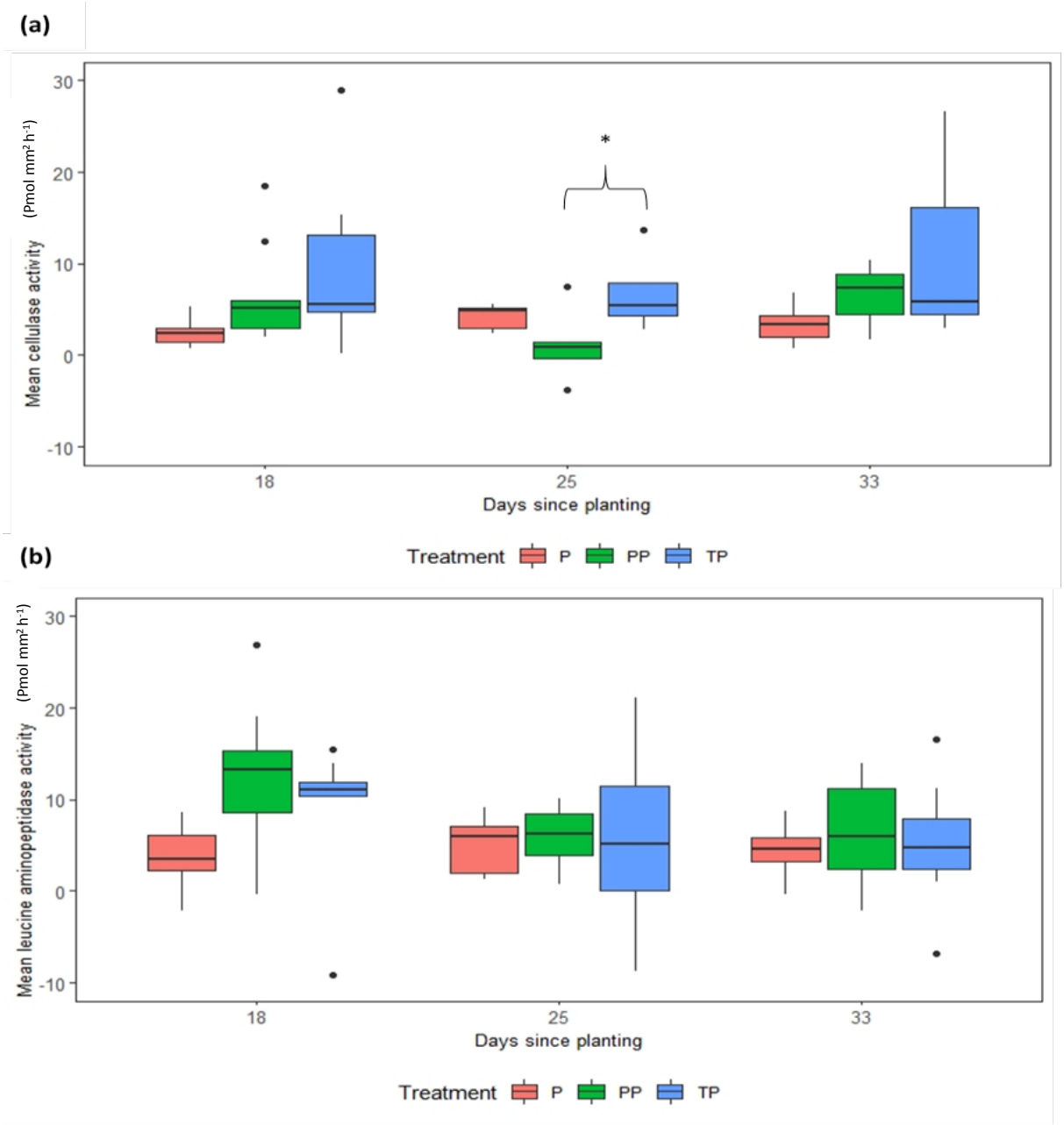

Figure 5.1 *– Mean cellulase and leucine aminopeptidase (pmol mm^{-2} h^{-1}) along the root axis of Proctor roots grown in isolation (P), intra- (PP) and inter- (TP) cultivar competition (n =12). A= Mean root axis cellulase activity, B = Mean root axis leucine aminopeptidase. Boxplots show the median, first and third quartiles and whiskers the maximum and minimum values. Significant differences (P = <0.05) denoted by asterisks.*

5.3.3 - Root associated area

The activity of both enzyme groups was highest nearest to the sampled roots, indicated by the brighter areas, and decreased with distance from them. The consistent sampling position is shown for each pot in Figure 5.2. Cellulase activity was not solely localised to the axis of sampled roots, and activity away from roots increased with time (Figure 5.3), with a mean root associated area activity of 0.57 -2.10 pmol mm^{-2} h^{-1} 33 days after planting. When Proctor was grown in isolation, the root associated area of cellulase activity was relatively constant (53 – 58 %) (Figure 5.5a). However, when Proctor was in inter- or intra- cultivar competition the initial percentage area was low (11 % in intra-cultivar competition and 13 % in inter-cultivar competition) but then rapidly increased to 25 days before stabilising at a similar percentage as Proctor in isolation (47 % in intra-cultivar competition and 58% in inter-cultivar competition) (Figure 5.5a). This shows a delay in the area of cellulase activity when Proctor was in competition compared to isolation. This is demonstrated in Figure 5.3, with darker images in the competition treatments at 18 days after planting compared to the isolation treatment. The root associated area in which cellulase activity occurred in the planted treatments showed a significant effect of treatment ($F_{(2,17)} = 4.72$, $P = 0.02$), time ($F_{(2,17)} = 44.98$, $P = <0.01$) and interaction between treatment and time ($F_{(2,17)} = 12.88$, $P = <0.01$).

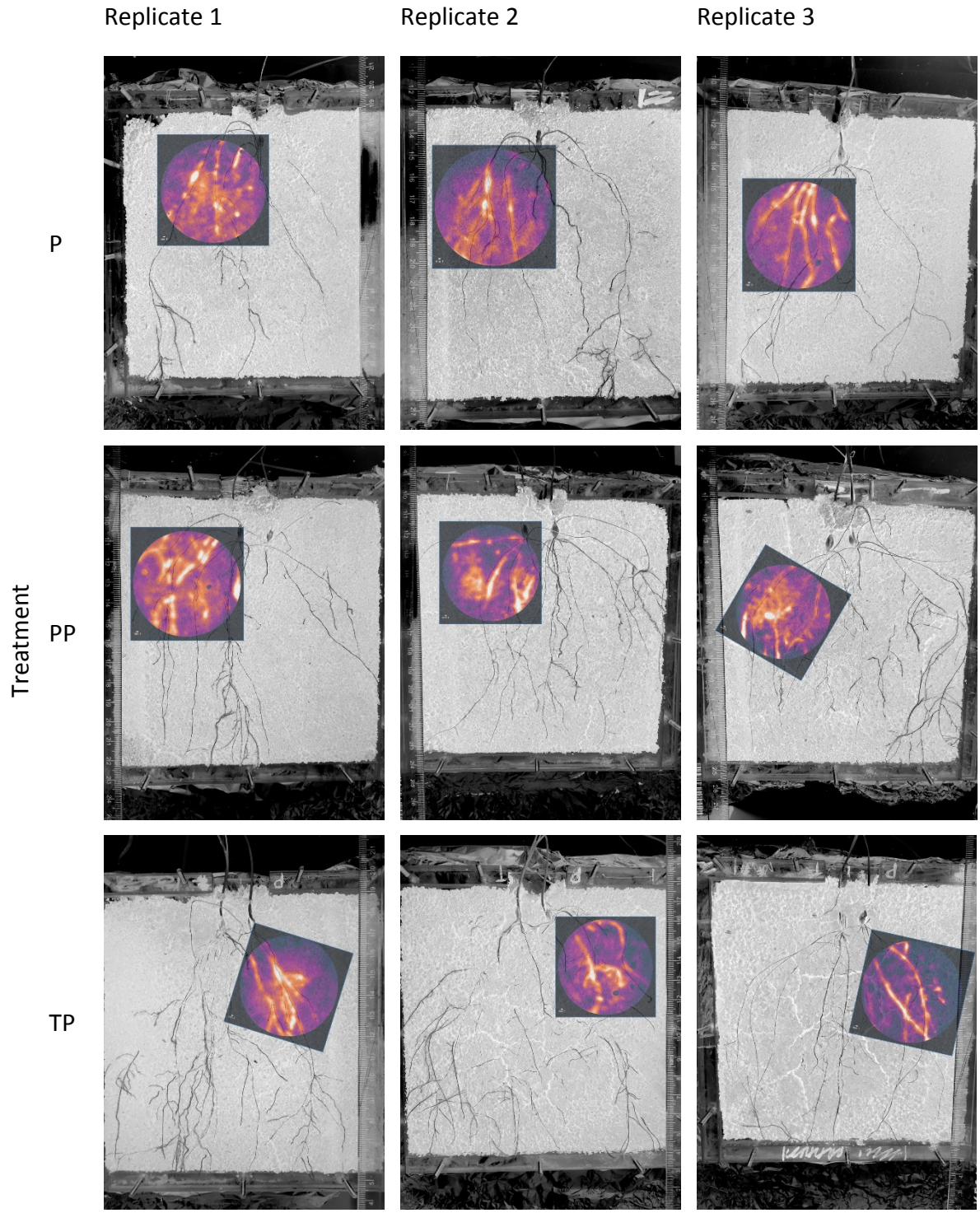

Figure 5.2 *– Images of the sampled rhizoboxes, showing the consistent sampling location used in this study and the relationship between root presence and soil enzyme activity.*

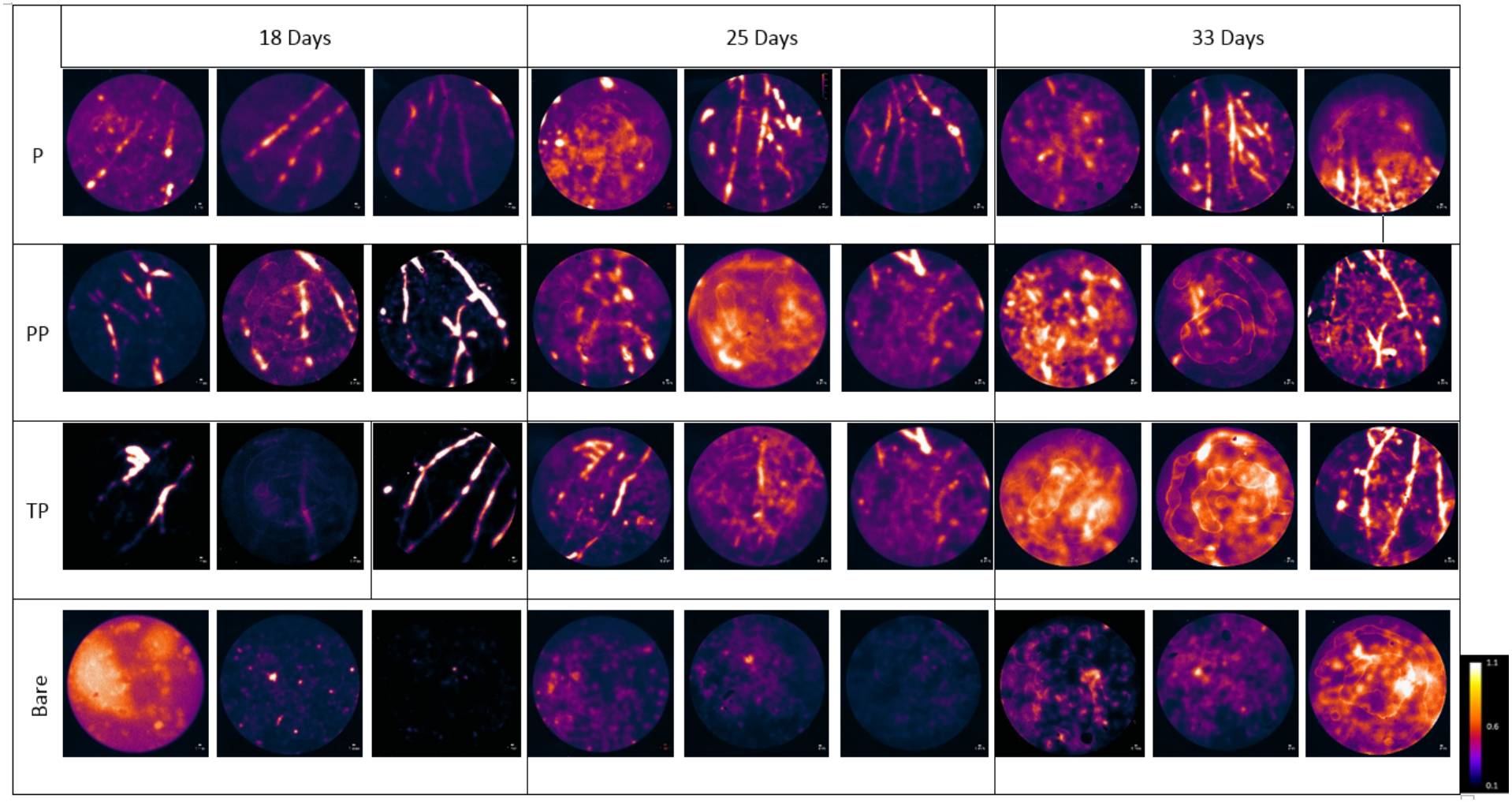

Figure 5.3 *- Soil zymography images showing (pmol mm^{-2} h^{-1}) cellulase activity around Proctor roots sampled from plants grown in isolation and competition as well as a bare soil control (n = 3). A. = Bare soil control, B. = Proctor, C. = Proctor and Proctor, D. = Proctor and Tammi*

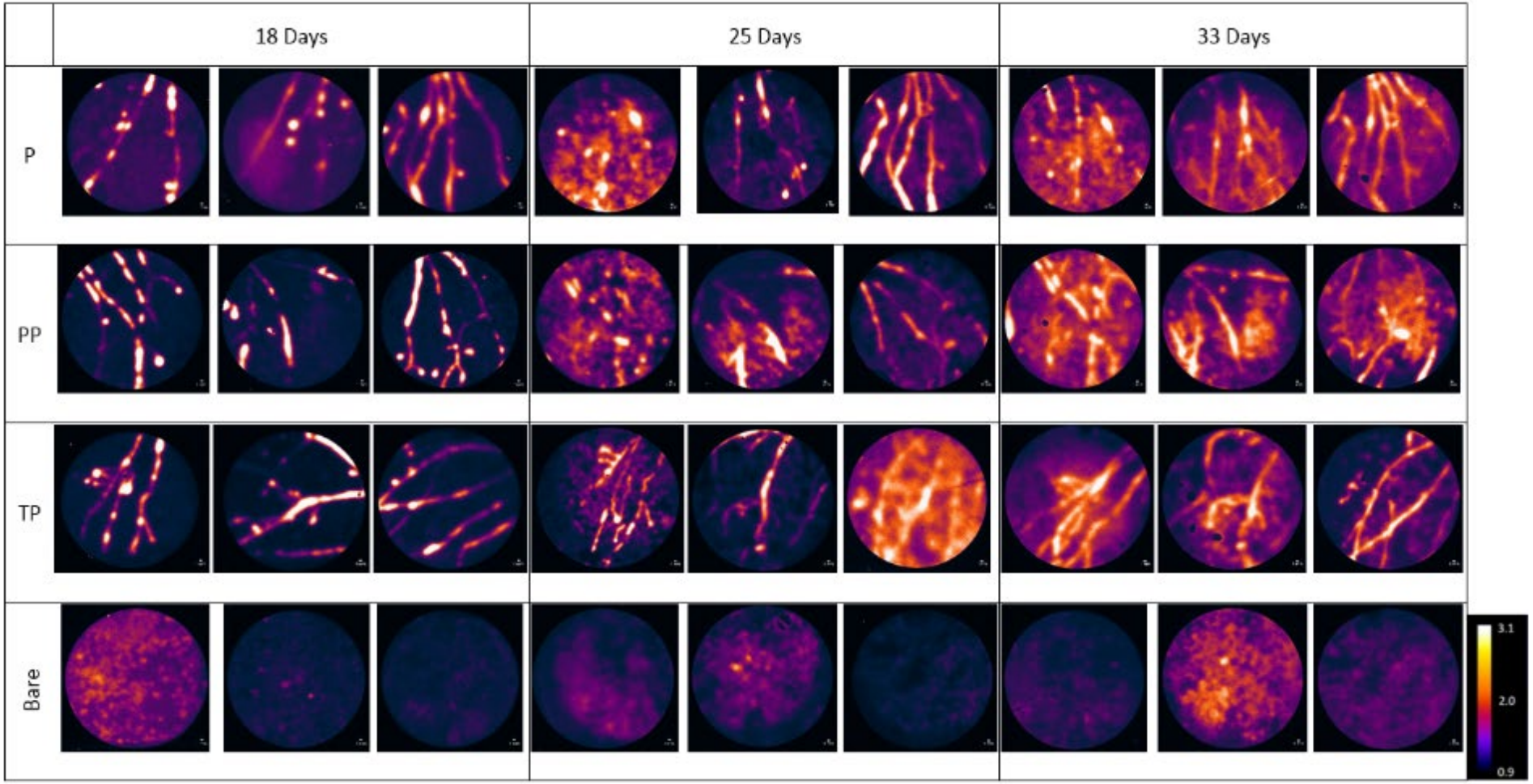

Figure 5.4 - *Soil zymography images showing (pmol mm^{-2} h^{-1}) leucine aminopeptidase activity around Proctor roots sampled from plants grown in isolation and competition as well as a bare soil control (n = 3). A. = Bare soil control, B. = Proctor, C. = Proctor and Proctor, D. = Proctor and Tammi*

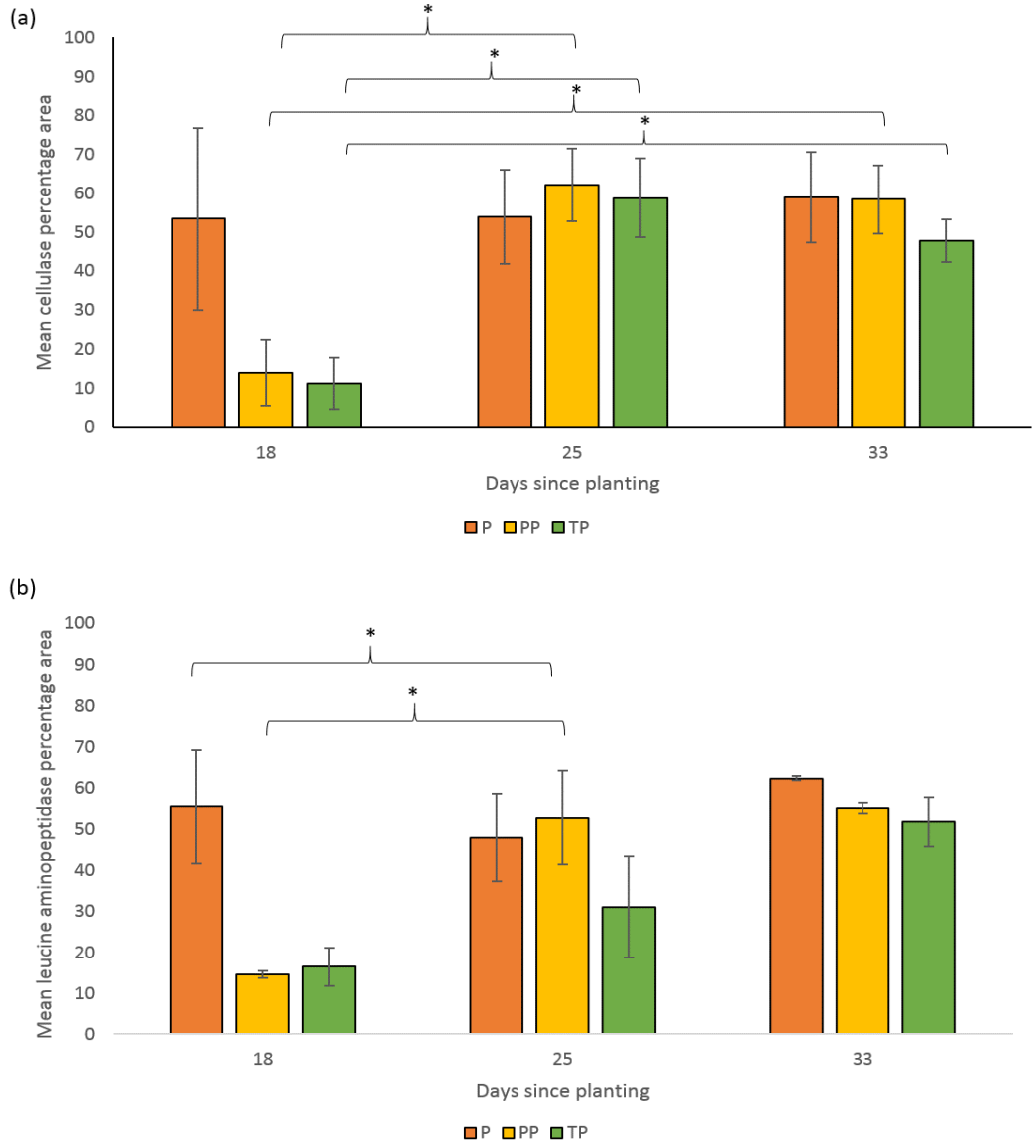

Figure 5.5 *– The mean percentage of sampled areas in which the activity of cellulase and leucine aminopeptidase were recorded (n = 12). Cellulase activity (a) and leucine aminopeptidase (b) activity were sampled surrounding Proctor roots outside the competition zone of plants grown in isolation, intra-cultivar competition and inter-cultivar competition. Significant differences (P = <0.05) denoted by asterisks.*

Leucine aminopeptidase activity occurred beyond the immediate rhizosphere (Figure 5.4). Mean root associated area activity at 33 days after planting ranged from 0.91 to 3.48 pmol mm^{-2} h^{-1}. When Proctor was grown in isolation and inter-cultivar competition, leucine

aminopeptidase root associated area steadily increased over time (Figure 5.5b). At 25 days, the intra-cultivar competition root associated area was lower (31 %) than in isolation (48 %) and inter-cultivar competition (52 %) (Figure 5.5b), indicating a delay in leucine aminopeptidase activity in intra-cultivar competition compared to isolation and inter-cultivar competition. This is demonstrated in Figure 5.4, with darker images in the intra-cultivar competition treatment at 18 days after planting compared to the isolation and inter-cultivar competition treatments. There was a significant effect of treatment ($F_{(2,17)}$ = 31.72, P = <0.01), time ($F_{(2,17)}$ = 30.36, P = <0.01) and a significant interaction between time and treatment on the root associated percentage area of leucine aminopeptidase activity ($F_{(2,17)}$ = 7.42, P = <0.01). Model details are in Appendix 2, Supplementary Figure A1.

5.4 - Discussion

This experiment aimed to determine the effect of plant-plant competition in barley on the temporal dynamics of nutrient cycling by measuring activity of cellulase and leucine aminopeptidase, two enzyme classes associated with nutrient turnover, specifically of carbon and nitrogen. Root axis activity for both enzyme classes was not significantly temporally dynamic (the interaction between time and treatment) when the focal plant (Proctor cultivar of barley) was in intra- and inter- cultivar competition compared to isolation. However, using the Spohn and Kuzyakov (2014) root associated area approach, cellulase activity was found to be delayed when in intra- and inter- cultivar competition compared to isolation (significant interaction between treatment and time). In contrast, leucine aminopeptidase root associated area was delayed when in intra-competition, but not inter-cultivar competition compared to isolation (significant interaction between treatment and time). This demonstrates that the temporal dynamics of soil enzyme activity were influenced by plant-plant competition independent of other environmental factors, that plant-plant competition did not have a uniform effect on different classes of soil enzymes, and that the observed effects are also dependent on the method of measurement.

5.4.1 - Root axis activity

Both cellulase and leucine aminopeptidase mean root axis activity was much higher than the whole sampled area, 3 - 4 times higher for leucine aminopeptidase and 4 - 6 times for cellulase. This is most likely due to the influence of plant root exudates, which provide a source of labile carbon, increase the rate of SOM mineralisation and, consequently, carbon and nitrogen cycling in the rhizosphere compared to bulk soil (Bengtson et al., 2012; C. J. Murphy et al., 2017). However, along root activity did not vary significantly over time for either enzyme class. The area of root system sampled was in the zone of maturation, a zone associated with a stable rate of nutrient uptake (Giles *et al.*, 2018). I hypothesised that plant-plant competition would have changed the temporal dynamics of root axis enzymatic activity, but it seems the inherent stability of this root zone was greater than the influence of plant-plant competition. Other root zones are associated with uptake of specific nutrients, for example the apical root zone is associated with iron absorption and the elongation zone with sulphur uptake (Travis S Walker et al., 2003). Therefore, depending on the root zone sampled and nutrient studied, there will likely be differing patterns of enzyme activity.

There is the potential for some enzyme activity to be produced by the plants themselves: up to 10 % (Xu *et al.*, 2014). Plant-derived leucine aminopeptidases genes have been detected in the plant genome, and found to have a role in protein turnover (Bartling and Weiler, 1992). Plants also have cellulases, but these are used for remodelling of cell walls and are not thought to be strong enough for large scale degradation of cellulose (Hayashi *et al.*, 2005). Therefore, due to their intra-cellular roles, it is unlikely that plant-derived enzymes contributed to the enzyme activity outside of the plant roots detected in this study.

5.4.2 - Root associated area

Cellulase and leucine aminopeptidase root associated area were not solely confined to the root axis, with increased activity across the sampled areas, including background soil activity. Cellulase root associated area was temporally dynamic, with a delay in peak enzyme activity (i.e. when the largest percentage area of membrane was recording either cellulase

or leucine aminopeptidase activity) when in competition compared to isolation. The zymography assay measured total cellulase activity of multiple microbial functional groups and did not differentiate between exo- and endo-glucanase activities. Exo-glucanases break glucose from the end of cellulase polymers, whilst endo-glucanases break bonds within the cellulose chains (Pappan *et al.*, 2011). There may have been differing dynamics if endo- and exo-glucanase activity were examined separately.

Leucine aminopeptidase root associated area also demonstrated a delay in activity but only when Proctor was in intra-cultivar competition. This delay in leucine aminopeptidase root associated area when in intra-cultivar competition echoes a similar trend to the delay of 14.5 days in Proctor peak above-ground nitrogen accumulation rate found in a previous study (Schofield *et al.*, 2019). The mechanism that links these two observations is not clear. Proctor plants may have delayed peak root exudate production when in intra-cultivar competition, influencing microbial activity to limit competition between the two plants. However, there may also be further mechanisms, for example involving plant-microbe signalling, already known to be important in recruitment of microbial symbionts and plant growth promoting rhizobacteria (Chagas *et al.*, 2018; Labuschagne *et al.*, 2018).

As the same area was sampled consistently over the experiment, the sampled area became increasingly far from the root tip, a known hotspot of soil microbial community enzyme activity. This may have influenced the activity of the two enzyme classes. Phosphatase activity has previously been found to vary with distance from the root tip (Giles *et al.* 2018), which may have influenced the results presented. However, there was no significant difference in root biomass or total root length between any of the treatments (Table 5.1), indicating that the relative sampling position remained consistent across treatments in this study. One benefit of sampling in the mature root zone is that it allows comparisons among treatments as the sampled areas were all a similar distance from the root tip at each time point. The zone of maturation is a region of the root with less exudation compared to the zone of elongation (Badri and Vivanco, 2009), but with root hairs that provide greater surface area for nutrient absorption (Gilroy and Jones, 2000). There may have also been an influence of root branching which occurred in some of the sampled areas due to plant foraging for nutrients (Forde, 2014). This hypothesis requires further

sampling of a greater proportion of the root system for a high resolution of spatiotemporal trends in microbial enzyme activity with root branching.

5.4.3 - What role could root exudates have in the temporal dynamics of enzyme activity?

The different patterns of soil enzyme activity associated with the three treatments may have been driven by differences in root exudation, with changes in root exudate composition then affecting microbial activity. Plants select for a specific microbial community through root exudates (Hu *et al.,* 2018; Shi *et al.,* 2011). Therefore, root exudates may do more than simply increase the rate of nitrogen mineralisation (Mergel *et al.,* 1998), and may also influence the timing of mineralisation by influencing soil microbial community composition.

Root exudation quality and quantity is known to change over time (van Dam and Bouwmeester, 2016) with root exudates increasing the carbon to nitrogen ratio in the rhizosphere, regulating mining of SOM by the soil microbial community (Chaparro *et al.*, 2012; Meier *et al.*, 2017). Exudates also act as a form of signalling between plants (van Dam and Bouwmeester, 2016), eliciting a change in root architecture (Caffaro *et al.*, 2013), branching (Forde, 2014) and biomass allocation (Schmid *et al.*, 2015). Therefore, the observed delay in soil enzyme activity could be regulated by temporally dynamic root exudation. Root branching would have also increased the total root area within the measurement areas, potentially increasing the total exudates available to the soil microbial community and promoting greater enzymatic activity. Consequently, the active control of root exudates instead of root biomass or surface area alone may be an important part of the mechanism behind the observed shifts in soil microbial community activity. Combining this research with measurements of microbial biomass in the rhizosphere soil would help determine if the increase in exudation is promoting an increase in exoenzyme production through priming of the soil microbial community (Dijkstra et al., 2013) or if increases in exoenzyme production are due to an increased microbial biomass. This is an avenue for future research.

5.4.4 - Temporal dynamics of enzyme activity in response to plant-plant competition

The soil enzyme classes in this study demonstrated different temporal patterns in activity in response to changes in plant-plant competition. Relative to the isolated-plant control, the temporal dynamics of cellulase root associated area were influenced by both intra- and inter-cultivar competition, whereas leucine aminopeptidase dynamics were only significantly influenced by intra-cultivar competition.

The influence of plant-plant competition on the temporal dynamics of root associated enzyme area occurred beyond the immediate zone surrounding the root. This contrasts with the results of Ma *et al.* (2018), who found a strong localisation of leucine aminopeptidase and cellulase activity close to plant roots across the whole root system. Furthermore, they found that the root associated area did not increase over time around lentil roots (*Lens culinaris*) and only began to increase around Lupin (*Lupinus albus*) roots eight weeks into the study (Ma *et al.* 2018). This is much later than the barley in this study, where sampling occurred in the first month of growth, the period prior to peak nitrogen accumulation rate in these barley cultivars (Schofield *et al.*, 2019). This is likely to be a period of soil microbial community priming to mine for nitrogen within soil organic matter and may account for the differences between Ma *et al.*'s and this study. In this study the extent of the rhizosphere and therefore activity of leucine aminopeptidase and cellulase may have increased over time, as labile carbon in root exudates diffused away from roots and the zone of nutrient depletion surrounding roots enlarged.

This study does however have its limitations. The rhizobox system is a very artificial setup with roots growing in a single plane, which would influence root growth and development. This does not account for the 3D nature of root growth and interactions with the soil particles and the soil microbial community. More complex interactions and temporally dynamic responses may be occurring in a 3D system through localised changes in the soil microbial community. Therefore, development of the zymography method in order to sample 3D root systems is a natural avenue for future research. There also need to be measures of nutrient concentration and microbial biomass as measurements of enzyme activity alone cannot be directly extrapolated as an indicator of nutrient cycling. Including these measures would determine if greater enzyme activity was due to an increase in microbial biomass or increased microbial demand for nutrients.

The temporal dynamics of enzyme activity are likely to be strongly influenced by environmental conditions including temperature (Steinweg *et al.* 2012), soil moisture (Barros *et al.* 1995) and soil nutrient concentration (Mbuthia *et al.* 2015). This study demonstrates that the temporal dynamics of the two groups of enzymes, both involved in nutrient turnover, were affected differently by plant-plant competition when grown in constant environmental conditions. This could be due to the composition of root exudates and concentration of secondary metabolites that selected for a soil microbial community with specific functions (Hu *et al.,* 2018; Shi *et al.,* 2016). Plants could have therefore regulated soil microbial community activity through the differing sensitivity of microbial taxa to root exudates (Shi *et al.,* 2011; Zhang *et al.,* 2017).

5.5 - Conclusions

Root axis activity of leucine aminopeptidase and cellulase was not temporally dynamic in response to plant-plant competition. Plant-plant competition influenced the root associated area of the two enzyme classes in this study differently. The extent of root associated cellulase area was delayed by inter- and intra-cultivar competition, whilst leucine aminopeptidase root associated area was only delayed by intra-cultivar competition. This may have been mediated through root exudates selecting for specific microbial functions. Therefore, conclusions concerning the temporal dynamics of nutrient cycling are likely to be dependent on the enzyme class being studied and method of image analysis used. Changes in these temporal dynamics may have been mediated through changes in the quantity and composition of root exudates by plants in competition, leading to a delay in peak soil enzyme activity. The extent of plant root influence was found to increase over time as exudates diffused away from roots, an important factor in studies of the soil microbial community activity. This study therefore demonstrates the close link between the temporal dynamics of plant and microbial resource capture and the influence each process has on the other.

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Chapter 6

Gene expression response to intra- and inter- cultivar competition and potential consequences for temporal dynamics of resource capture

Contents

Abstract

Belowground, plants are known to respond to the presence of a neighbouring individual through the modification of root architecture, changing patterns of root branching and the

temporal dynamics of nutrient uptake. However, to aid in elucidating the mechanisms behind a change in the temporal dynamics of nutrient uptake, the changes at a gene expression level require characterisation. This study aimed to characterise gene expression associated with intra-specific plant-plant competition during early plant growth. Barley (*Hordeum vulgare*, cv. Proctor) plants were grown in isolation, intra-cultivar and inter-cultivar competition for 19 days, then root material was harvested for gene expression analysis. A core set of genes were identified that were significantly differentially expressed in both competition treatments (17 total, 11 upregulated, 6 downregulated). Genes that were unique to each competition treatment were also identified. A greater number of genes were significantly differentially expressed in inter-cultivar competition (117 total, 58 upregulated, 59 downregulated) compared to intra-cultivar competition (41 total, 22 upregulated, 19 downregulated). The combination of up and down regulated genes in each competition treatment had a number of different identified functions. The majority of significantly differentially expressed genes were associated with plant growth and development, suggesting a growth pattern change in response to the presence of a competitor. This indicates a differential response at a gene expression level depending on the identity of a competing individual, and at a time which is likely to be prior to competition induced nutrient deficiency. Therefore, plants may be able to respond differently depending on how closely related they are to a competitor, to potentially favour those that are more closely related and compete more intensely with more distantly related individuals.

6.1 - Introduction

Plants respond to stress in a number of ways depending on the type of stress i.e. abiotic or biotic (Ramakrishna and Ravishankar, 2011; Schmid *et al.*, 2013) or the combination of stresses a plant is experiencing (Bowsher *et al.*, 2017). Reponses can include a change in shoot architecture due to shading stress (Cahill, 2003) or root architecture changes from nutrient deficiency stress as roots forage for nutrients (Caffaro *et al.*, 2013). Plants can also alter the partitioning of resources between roots and shoots in response to nutrient limitation and plant-plant competition (Berendse and Möller, 2009), as well as the timing of key processes, such as resource capture, to limit competition for common resources

(Schofield *et al.*, 2018). Changes in the temporal dynamics of resource capture have been found in response to plant-plant competition in annual (Schofield *et al.*, 2019) and perennial (Trinder *et al.*, 2012) species.

Mediation of plant responses to stress occurs at a molecular level through a series of plant growth regulators (Verma *et al.*, 2016). These cause changes in gene expression via transcription factors, which then mediates downstream responses to stress (Does *et al.*, 2013). The suite of genes up or down regulated are specific to the type of stress or combination of stresses being experienced by the plant (Suzuki *et al.*, 2014). Also, when there is a combination of stresses, for example heat and drought (which often co-occur), the profile of gene expression is not a combination of the two individual stresses, but a unique pattern of expression (Zandalinas *et al.*, 2018). These patterns may 'tailor' the response of a plant to specific environmental conditions, to minimise the negative impacts of stress.

A few studies have addressed the issue of gene expression patterns associated with plant-plant competition. These studies identified the accumulation of defensive secondary metabolites (Masclaux, Bruessow, Schweizer, Gouhier-Darimont, Keller, Reymond, et al., 2012) and pathogen related proteins in response to plant-plant competition (Schmid *et al.*, 2013). The role of pathogen related proteins in plant-plant competition is unclear, but the proteins have been associated with a biotic stress response (Schmid *et al.*, 2013). Also, genes associated with nutrient starvation, cold and salinity stress were upregulated in response to plant-plant competition (Schmid *et al.*, 2013). Bowsher *et al.* (2017), using *Trifolium* species grown in field soil, found a core set of genes associated with both biotic and abiotic stress expressed in response to plant-plant competition. In addition, differential gene expression was found in response to a heterospecific competitor compared to a conspecific competitor (Bowsher *et al.*, 2017). Conspecific competition has also been found to elicit a change in gene expression in a neighbouring individual (Subrahmaniam *et al.*, 2018). Intra-cultivar competition in barley has been found to elicit a temporally dynamic response in nitrogen accumulation rate (Schofield *et al.*, 2019; Chapter 2) but the impact of this at a molecular level has yet to be explored.

Barley (*Hordeum vulgare*) has been the subject of a concerted effort to sequence and annotate its genome, to identify genes with known functions (Schulte *et al.*, 2009). Using barley as a model plant provides information about plant-plant competition in an

ecologically (grasses) and economically (cereals) important group. In crop plants, competition between individuals is likely to influence yield. Therefore, in order to maintain or increase crop yields we need to better understand the mechanism behind interactions between neighbouring plants. The majority of gene expression studies have been carried out using the model plant *Arabidopsis thaliana* grown in lab conditions (Masclaux, Bruessow, Schweizer, Gouhier-Darimont, Keller, Reymond, et al., 2012; Schmid et al., 2013; Subrahmaniam et al., 2018). In contrast, the use of a crop species grown in soil, as in this study, provides information relevant to both agriculture and ecology.

Previous studies can help us pinpoint combinations of cultivars and key time points during intra-cultivar interactions which are likely to be of interest with respect to gene expression. Specifically, the previous study (Schofield *et al.*, 2019; Chapter 2) examined the temporal dynamics of peak nitrogen uptake rate in an early cultivar, Tammi, and late cultivar, Proctor, when the plants were in isolation, intra- and inter- cultivar competition. Peak nitrogen accumulation rate significantly shifted in both cultivars with intra-cultivar competition, but not inter-cultivar competition compared to isolation. At 19 days after planting, nitrogen accumulation rate peaked for Proctor plants in isolation and inter-cultivar competition but not intra-cultivar competition, which peaked at 33 days after planting (Schofield *et al.*, 2019; Chapter 2). Based on these data, I would expect differences in the pattern of gene expression at 19 days after planting between plants in intra-cultivar competition, compared to those in isolation or inter-cultivar competition, and the latter pair to be more similar to each other.

In this study, therefore, I examined the patterns of gene expression of Proctor plants grown in isolation, intra- and inter-cultivar competition at 19 days after planting. The aim of the study was to identify specific and common sets of differentially expressed genes when Proctor was grown under inter- and intra-cultivar competition conditions compared to when it was grown in isolation.

6.2 - Materials and methods

6.2.1 - Soil characterisation

Soil was collected from an agricultural field that had previously cropped spring barley (*Hordeum vulgare*), and had previously been cultivated using standard fertilisation practice (500 kg of 22N-4P-14K ha^{-1} yr^{-1}) (Sourced from Balruddery Farm, Invergowrie, Scotland, 56.4837° N, 3.1314° W). The soil was homogenised through a 6 mm sieve and thoroughly mixed, then stored at 4°C until planting. The soil organic content was 6.2 % ± 0.3 % SEM (loss-on-ignition, n = 4), with a mean pH of 5.7 ± 0.02 SEM (n = 4) in water.

6.2.2 - Experimental setup

Proctor plants were grown in isolation (P), intra-cultivar competition (PP) and inter-cultivar competition with Tammi (TP), with four replicates of each of the three treatments, giving 12 pots in total. Pots (diameter = 102 mm, depth = 135 mm) were filled to a bulk density of 1 g cm^{-3}. Proctor and Tammi plants were pre-germinated on damp tissue paper at room temperature in the dark for three days prior to planting. Germinated seeds were planted 25 mm deep and approximately 50 mm apart within the pot. Pots were grown in a greenhouse (18 °C with supplementary lighting) for 19 days with weekly watering to 60 % water holding capacity to limit competition for water. Mesh screens (45 x 16 cm, mesh size 0.08 mm (Harrod Horticulture, Lowestoft, UK)) were inserted into pots to ensure competition only occurred underground.

Harvesting was carried out after 19 days in replicate blocks. This was during the period of peak nitrogen accumulation rate, and prior to peak biomass accumulation rate and grain filling (Schofield *et al.*, 2019; Chapter 2). The plants were removed from pots, separated and roots rinsed. The washed roots of each individual were placed into vials, sealed and placed into liquid nitrogen within three minutes of harvesting. The vials were then stored at -80 °C until RNA was extracted.

6.2.3 - RNA microarray analysis

This method uses short pieces of DNA, complementary to known genes (identified through genome sequencing) that are individually printed on a glass slide known as a microarray. The microarray slide in this study had 61,000 complementary sequences printed on it. RNA was extracted from the samples, cleaned and converted into cDNA. The cDNA was then labelled using a fluorescent dye and added to the slide. The slides were incubated overnight and then scanned for fluorescence at each printed gene. The level of fluorescence was then used to determine whether the expression of the gene increased or decreased (Kaliyappan *et al.*, 2012) in each of the competition treatments compared to plants in isolation.

RNA was extracted using a Qiagen RNeasy Mini Kit (Qiagen, Manchester, UK), as recommended by the manufacturer with an additional clean-up step consisting of a phenol-chloroform extraction (Toni *et al.*, 2018). RNA was quantified using a NanoDrop™ 2000/2000c (Thermo Fisher Scientific, UK) and quality measured using a Bioanalyser (Agilent Technologies UK, Edinburgh UK).

Microarray processing was performed using a custom-designed barley Agilent microarray (A-MEXP-2357; www.ebi.ac.uk/arrayexpress). One microarray was used for each of the twelve replicates. The microarray contains c. 61,000 60-mer probes derived from predicted barley transcripts and full-length cDNAs (IBGSC, 2012). Samples were labelled using an Agilent One Colour Low Input Quick Amp Labelling Kit (Agilent, Santa Clara, CA, USA) and hybridised to microarrays as recommended according to the 'One-Color Microarray-Based Gene Expression Analysis' protocol (v 6.5, Agilent Technologies). Scanning was performed using an Agilent G2565CA Microarray Scanner (Agilent, Santa Clara, CA, USA) at 3 µm resolution.

6.2.4 - Data analysis

Data from the scanned microarray images were extracted using Feature Extraction Software (v. 10.7.3.1; Aglient, Santa Clara, CA, USA). Following visual quality control, data from each microarray was imported into GeneSpring software (v. 7.3; Aglient, Santa Clara, CA, USA) for data analysis. Data were normalised using default Agilent FE one-colour settings in

GeneSpring. In addition, data were filtered to remove inconsistent probe data flagged as absent in more than one replicate per sample. Following filtering using feature flags to assess outliers in the data, reliable data from 26,006 probes were taken forward for statistical analysis. Putative differentially expressed genes (≥ two-fold change) were identified through pairwise analysis (Student's t-test) of Proctor plants in isolation (P) with intra-specific competition (PP: 41 genes) or inter-specific competition (TP: 117 genes). Gene lists were compared to identify those which are common or specific to each competition type. Corrections for multiple testing did not allow any genes though the filtering process, due to the subtle nature of the expected changes in gene expression.

Differentially expressed genes were categorised by function using the rice annotation description in the UniProt database (www.uniprot.org). Based on the rice described function, the genes were placed into five categories: growth and development; plant stress; genome rearrangement; gene expression control; and those of unknown function.

6.2.5 - Validation of microarrays using qRT-PCR

A technical validation was carried out to validate the trends in gene expression observed in the microarrays using quantitative reverse transcription-PCR (qRT-PCR). For this, five stress response genes that were significantly differentially expressed in the microarrays were selected (MLOC_74116.1 – *Chalcone synthase*, MLOC_25773.1 – *Jasmonate induced*, MLOC_23705.2 – *Jacalin lectin like protein*, MLOC_81765.1 – *WIP wounding protein*, MLOC_44884.1 – *Zinc finger protein*, plus *protein phosphatase 2* (PDF2), a commonly used housekeeping gene (Warzybok and Migocka, 2013) as an internal control. As stress genes were predicted to be upregulated in response to plant-plant competition, these were chosen for the validation to confirm the observed microarray gene expression patterns. Primer pairs were designed for each of the five genes (Table 6.1) with an internal probe using the Roche Universal ProbeLibrary (Roche, Basel, Switzerland). RNA from each treatment was pooled, treated with a DNase (DNase I kit, Thermo-Fisher Scientific, Manchester, United Kingdom) and converted to cDNA (TaqMan® cDNA kit, Thermo-Fisher Scientific, Manchester, United Kingdom).

Primer pair efficiencies were initially tested using serial dilutions which were then analysed using StepOne thermocycler (ThermoFisher Scientific (Applied Biosystems), Waltham, MA, USA). Only three primer pairs were efficient enough to be carried forward to the validation: MLOC_23705.2 – *Jacalin lectin like protein*, MLOC_25773.1 – *Jasmonate induced* and MLOC_74116.1 – *Chalcone synthase*. These were taken forward to the qRT-PCR assay and analysed using a StepOne thermocycler (ThermoFisher Scientific (Applied Biosystems), Waltham, MA, USA) (15 min 95°C, followed by 40 cycles of 10 seconds 95°C and 60 seconds at 60°C) with three technical replicates for each treatment. The results were then normalised to the isolation (P) treatment. These results were compared to the gene expression patterns from the microarray to confirm the magnitude and direction (up or down regulation) of gene expression.

Table 6.1 *- qRT-PCR primers used in this study.*

Gene name	Forward primer	Reverse primer	Reference gene
Chalcone synthase	cagaagacgaggtgggtgat	gcagaaggccatcaagga	MLOC_74116.1
Jasmonate induced	ttgttaaaggcgagcttgagt	acaagacgtcccgtatggag	MLOC_25773.1
Jacalin lectin like protein	ggaaatggaggggtgataa	cgagccactgctaactgtgat	MLOC_23705.2
WIP wounding protein	atgcatgggaaatcagtggt	attgatttcggttgcgtttt	MLOC_81765.1
Zinc finger protein	cctacagagcatgcatagttgc	aggaaaaaggattttccgatg	MLOC_44884.1

6.3 - Results

6.3.1 - Common competition genes

A core set of genes was identified by comparing the list of genes significantly differentially expressed in each competition treatment. This identified 17 genes common to both competition treatments (Figure 6.1). Six of these genes were downregulated and 11

upregulated (Table 6.2). Of these, four upregulated genes were associated with plant stress response, including genes induced by jasmonate production, abiotic stress, reactive oxygen species production and genes involved in flavonoid production (Table 6.2). The majority of the significantly differentially expressed genes identified that were common to both competition treatments had functions linked with plant metabolism and growth, with one gene identified as being involved in the control of gene expression. The remaining genes were of unknown function. Two genes were found to be differentially downregulated in intra-cultivar competition and upregulated in inter-cultivar competition. One was associated with abiotic stress tolerance, whereas the other had a role in general plant metabolism.

6.3.2 - Intra-cultivar competition genes

A total of 41 genes (listed in Appendix 3) were significantly differentially expressed ($P \leq 0.05$ with ≥ 2 fold change in expression) only when the plants were in intra-cultivar competition compared to plants grown in isolation. The identified genes consisted of those associated with plant growth and development (34 %), stress (24 %), gene expression regulation (9 %) and genome rearrangement (7 %) (Figure 6.1). The majority of the stress associated genes were downregulated, with only three genes upregulated (Figure 6.1). Genes associated with biotic and abiotic stress were downregulated, as well as genes associated with fungal pathogen response. Plant growth and development associated genes were both up and down regulated, with similar mixed patterns for gene expression and genome rearrangement. Of the ten genes with unknown function, seven were upregulated and three downregulated (Figure 6.1).

6.3.3 - Inter-cultivar competition genes

A total of 117 genes (listed in Appendix 3, Table A2) were significantly ($P \leq 0.05$ with ≥ 2 fold change in expression) differentially expressed only in the inter-cultivar competition compared to isolation, 76 more than Proctor in intra-cultivar competition. The identified genes significantly differentially expressed were from a range of functional groups, with the majority (53 %) associated with plant growth and development. A further 15 % were

associated with control of plant gene expression, 6 % with genome rearrangement and 9 % with plant stress (Figure 6.1). Those involved in gene expression control were predominantly transcription factors or DNA binding proteins. The differentially expressed genes associated with plant stress response were predominantly associated with a general stress response, instead of a response to a specific stressor. Half of the genes associated with plant stress were upregulated and half downregulated. This was the same pattern in genes associated with growth and development and gene expression control, with similar proportions of both up and down regulated genes (Figure 6.1). There were 27 genes identified of unknown function, of which 14 were downregulated and 13 upregulated.

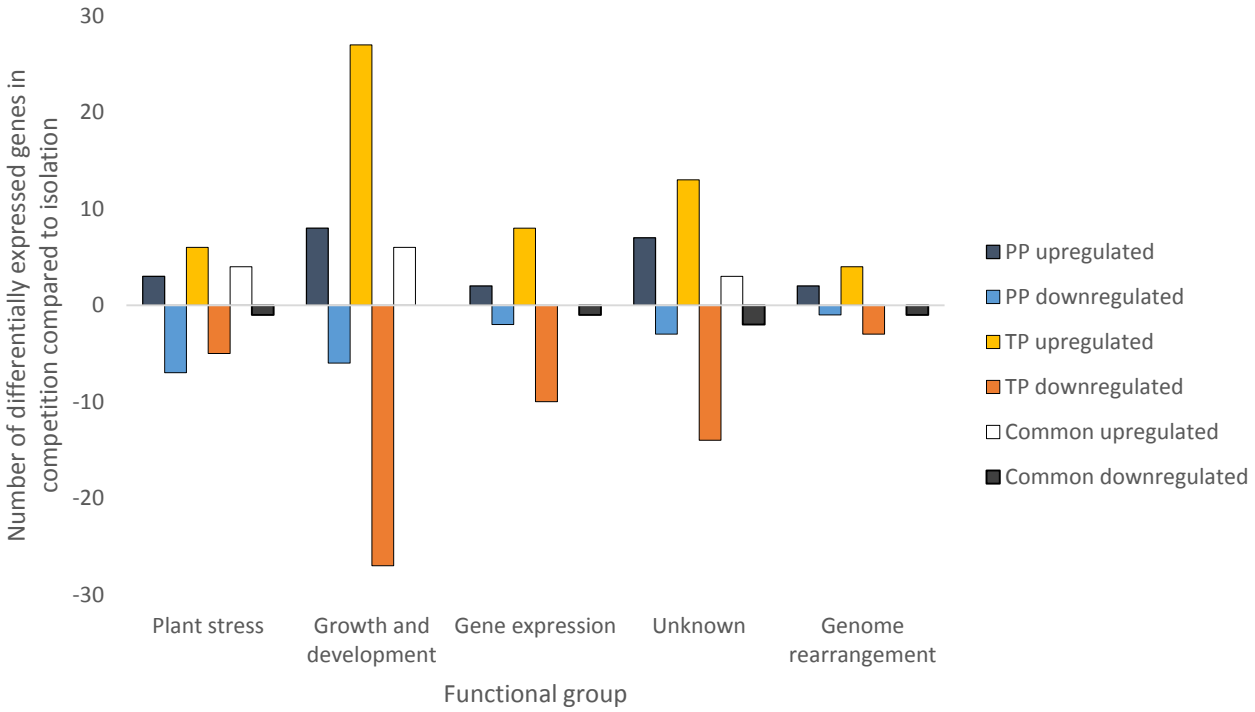

Figure 6.1*- Functional groups of genes significantly (P ≤ 0.05, with a ≥ 2 fold change in expression) differentially expressed in competition treatments compared to Proctor plants in isolation. Plants were grown in inter-cultivar competition (TP) and intra-cultivar competition (PP), and genes that were common to both competition treatments were also identified (common) compared to plants in isolation.*

Table 6.2 *- List of significantly (P ≤ 0.05 with ≥ 2 fold change in expression) differentially expressed genes common to both competition treatments, with annotated functions from the UniProt database.*

Primary Accession	Rice description	Function	Up (↑)/down (↓) regulated
Plant stress			
MLOC_74116.1	Chalcone synthase, putative, expressed	Initial step of flavonoid production pathway - plant secondary metabolite production	↑
MLOC_25773.1	Jasmonate-induced protein, putative	Ribosomal inactivating protein thought to be part of plant defence	↓
MLOC_17545.2	Laccase precursor protein, putative, expressed	Abiotic stress tolerance including drought and salinity	↓(intra-cultivar competition) ↑(inter-cultivar competition)
AK373696	Leucoanthocyanidin reductase, putative, expressed	Enzyme involved in flavenoid production	↑
MLOC_64053.1	Metal cation transporter, putative, expressed	Involved in zinc and iron uptake. Can also transport cadmium, cobalt, zinc and to a lesser extent nickel and copper. Also involved in response to ROS	↑
Metabolism, growth and development			
MLOC_53163.1	Profilin domain containing protein, expressed	Involved in cell development, cytokinesis, membrane trafficking, and cell motility	↑

MLOC_42095.1	No apical meristem protein, putative, expressed	Plant development protein	↑
MLOC_44922.1	RING-H2 finger protein, putative, expressed	Protein modification role	↑
MLOC_6963.5	Plant PDR ABC transporter associated domain containing protein, expressed	ATP production	↑
AK364469	Hydrolase, alpha/beta fold family domain containing protein, expressed	General role in metabolism	↓ (intra-cultivar competition) ↑ (inter-cultivar competition)
MLOC_54094.1	Hexokinase, putative, expressed	Involved in glucose metabolism	↑
Gene expression control			
MLOC_9821.2	SWIB/MDM2 domain containing protein, expressed	Chromatin modification to control transcription	↓
Unknown function			
MLOC_52935.1	DUF567 domain containing protein, putative, expressed	Unknown function	↓
MLOC_41636.1	Expressed protein	Unknown function	↓
MLOC_64800.1	Expressed protein	Unknown function	↑
AK367837	Expressed protein	Unknown function	↑
MLOC_41796.1	Hypothetical protein	Unknown function	↑

6.3.4 - Validation of expression patterns using qRT-PCR

The expression of the chosen validation genes followed the same patterns in both the microarray and qRT-PCR analyses (Table 6.3). This validates the pattern of expression found in the microarray analysis. The results are normalised to the isolation treatment which is represented as 1.00 in Table 6.3.

Table 6.3 *– Comparison of the expression patterns of three genes selected for validation measured by microarray and qRT-PCR in the three treatments. Expression patterns were normalised to plants in isolation (P) and gene expression in this category is therefore represented as 1.00. Plants were grown in intra-cultivar competition (PP) and inter-cultivar competition. Values under 1.00 indicate down-regulation and above 1.00 up-regulation. Similar values between microarray and qRT-PCR indicate a similar magnitude of gene expression change.*

Primary Accession	Rice description	P		PP		TP	
		Micro-array	qRT-PCR	Micro-array	qRT-PCR	Micro-array	qRT-PCR
MLOC_23705	Jacalin-like lectin domain containing protein, putative, expressed	1.00	1.00	0.09	0.13	0.29	0.45
MLOC_25773	jasmonate-induced protein, putative	1.00	1.00	0.41	0.50	0.41	0.50
MLOC_74116	chalcone synthase, putative, expressed	1.00	1.00	2.44	2.40	2.84	2.65

6.4 - Discussion

This exploratory study aimed to investigate potential markers for inter- and intra- cultivar competition and patterns in gene expression. Barley cv. Proctor plants were grown in isolation, inter- and intra-cultivar competition for 19 days in agricultural soil. A core set of 17

genes were significantly differentially expressed in both competition treatments, but there were also genes that were uniquely expressed in each of the competition treatments. A total of 117 (58 upregulated, 59 downregulated) genes were differentially expressed in inter-cultivar competition, compared to 41 (22 upregulated, 19 downregulated) in intra-cultivar competition. The majority of genes were associated with growth and development but there were others associated with plant stress and the control of gene expression.

6.4.1 - Genes of unknown function

A total of 42 differentially expressed genes were of unknown function, with a mixture of up and down regulation. The current barley whole genome sequence was completed in 2012 and is yet to be fully annotated (Mayer et al., 2012a). Consequently the rice genome was used for many of the functional annotations in this study: rice was the first complete crop genome to be published in 2006 and is well annotated (Jackson, 2016). The genes in this study that are of unknown function have either not been annotated in rice, are not similar enough to the rice homologues, or are unique to barley. Future barley annotation projects may allow the function of these genes to be identified. This includes transcriptome sequencing to validate gene annotation, using closely related species as a reference genome (Mayer et al., 2012a).

6.4.2 - Patterns of gene expression

Gene expression differed between the two competition treatments in terms of the total number of genes significantly differentially expressed, the pattern of up and down regulation and the function of the genes. The observation of a core set of 'competition genes' differentially expressed in both competition treatments, as well as uniquely expressed genes in each competition treatment, has been found previously by Bowsher *et al.* (2017) in *Trifolium fucatum,* and Schmid *et al.* (2013) *in Arabidopsis thaliana*. This included genes associated with disease resistance (Bowsher *et al.*, 2017), gene expression and transcription factors (Schmid *et al.*, 2013). In these earlier studies, core genes represented multiple functions, the same trend as found in this study. It is thought that

these are involved in the recognition of a neighbouring individual regardless of its identity (Bowsher *et al.*, 2017), something again supported by this study.

In this study, the upregulated core 'competition genes' included those involved in abiotic stress response, indicating that simply categorising genes as involved in plant stress may be too simplistic, as they are part of a larger signalling pathway which can also be involved in responses to plant-plant interactions. There was also an unexpected lack of nutrient deficiency associated genes expressed in the competition treatments. This suggests that the gene-level response to plant-plant competition is not the same as nutrient deficiency. Notably the sampling point for this study may have been before nutrient deficiency responses occurred. At 19 days after planting, there were minimal physical root-root interactions and peak nitrogen accumulation rate occurred (Schofield *et al.*, 2019; Chapter 2). Further experiments with sampling throughout the growth period could be used to characterise dynamics of competition interactions as nutrient deficiency becomes increasingly apparent.

The plant stress genes identified in this study (Appendix 3, Tables 1A and 2A) have been associated with both biotic and abiotic stress responses. Stress responses linked to pathogen defence have been found in *Arabidopsis thaliana* plants grown in both inter- and intra- specific competition (Masclaux, Bruessow, Schweizer, Gouhier-Darimont, Keller, Reymond, et al., 2012; Schmid et al., 2013). Defence responses, in particular increased jasmonate associated gene expression, have been found to lead to the upregulation of nutrient deficiency response genes (Schmid *et al.*, 2013). However, this study found a mixture of up and down regulation of plant stress associated genes. In addition, there were differing patterns of gene expression in terms of gene identity, and up or down regulation between the inter- and intra-cultivar competition treatments. The combination of up and down regulation of a number of genes may mediate the intensity of a plant response to a neighbouring plant, depending on the identity of a neighbour. The combination of gene function and level of expression may consequently 'tailor' the response to a neighbouring plant, as discussed below.

The majority of significantly expressed genes were categorised as part of a growth and development response to competition. Many of these genes have only generalised identified functions or are involved in a large range of cellular processes (The UniProt

Consortium, 2018). Therefore, characterising the growth response in each competition treatment is difficult. Further studies with more frequent sampling over the growth period would provide more evidence of a link between the observed gene expression patterns and growth responses such as biomass and nutrient accumulation.

A number of the identified genes were associated with the control of gene expression through histone modifications and transcription factors (The UniProt Consortium, 2018). The expression pattern of these genes mimicked the pattern seen in the growth and development genes and may therefore be part of the same growth processes. Changes in the control of gene expression could indicate changes in the physiology and morphology of Proctor roots in competition (Malamy, 2005).

6.4.3 - Multiple testing and validation

The expression of genes in this study were subtle and did not pass through a multiple testing correction filter. This suggests that at this stage of growth there are only subtle changes in gene expression. However, the direction and magnitude of expression in the microarrays were confirmed in the qRT-PCR validation. This demonstrates that the gene expression patterns were unlikely to be an artefact of the microarray data analysis. The magnitude of these gene expression responses to plant-plant competition may increase over time or with greater environmental stress, allowing genes to pass through the filter. The inclusion of multiple time points in future studies would allow this hypothesis to be tested.

6.4.4 - What does this mean in terms of plant-plant competition?

Competition between plants has traditionally been characterised as a scramble for limited available resources, with plant responses occurring due to resource depletion (Schenk, 2006). At the early stage of plant growth investigated in this study, during the period of peak nitrogen accumulation and prior to peak biomass accumulation rate and grain production, there was a lack of genes differentially expressed that were associated with nutrient stress or foraging. However, there were changes in the expression of genes

associated with plant growth and other stress roles. These changes in gene expression patterns potentially indicate the detection of a neighbouring individual occurred before nutrients became limiting, suggesting a form of neighbour recognition. Furthermore, fewer gene expression changes were found in intra-cultivar competition compared to inter-cultivar competition, indicating that not only the presence but also the identity of a neighbour can be detected by the plants. Such an effect has previously been found in *Trifolium* (Bowsher *et al.*, 2017) in response to a congeneric individual compared to a heterospecific individual. These studies included competition for light which likely to have led to different gene expression patterns to those in this chapter, where shading was limited using mesh screens. If this study was repeated with competition aboveground also allowed, the pattern of differentially expressed genes would likely be different due to the additional effect of shading.

Such responses may indicate a form of kin recognition. Kin recognition is the modification of plant behaviour depending on the identity of a neighbouring plant (Dudley *et al.*, 2013). Underlying mechanisms include root exudates (Semchenko *et al.*, 2014), volatile compounds (Delory *et al.*, 2016) or via the soil microbial community (Hortal et al., 2017a) prior to physical root contact. The unique chemical fingerprint of an individual can be recognised by a neighbouring plant, which can then respond depending on neighbour identity (Karban *et al.*, 2013; Depuydt, 2014). The differences in gene expression patterns of plants in inter- and intra- cultivar competition in this study provide evidence for differing responses depending on neighbour identity. Differences in gene expression between plants in isolation and intra-cultivar competition also suggest that the mechanism of recognition of a closely related individual (self/non-self-recognition) is different to recognition of its own roots (self-recognition) (Biedrzycki *et al.*, 2010; Depuydt, 2014).

Kin recognition is thought to lead to reduced strength of competition between closely related individuals (Dudley *et al.*, 2013), although there is no direct evidence for this in this study. The identity of the genes differentially expressed in intra-cultivar competition is likely to form part of the response to a closely related neighbouring individual, and gene identity may be of greater importance than simply the number of differentially expressed genes. Future research, using more quantitative methods such as RNA sequencing (Pounds,

2006) and into the function of genes identified in this study may elucidate the mechanism behind the observed differences, and confirm the patterns observed here.

6.5 - Conclusions

This study demonstrates plant recognition of the identity of a neighbour at a molecular level before it is reflected through changes in nitrogen and biomass accumulation dynamics. This is characterised by a change in expression of genes predominantly associated with growth and development, plant stress and gene expression control. A core set of genes was identified associated with both inter- and intra- cultivar competition. The gene expression patterns in this study indicate differences in responses depending on the identity of a neighbouring individual. This may be more dependent on the function of the genes than simply the number of differentially expressed genes, but the power of detection is weak. These findings suggest differential root growth responses depending on the identity of a neighbouring plant, providing further evidence for kin recognition in barley. No nutrient stress associated genes were found to be differentially expressed in this study, but the differential expression of growth and development genes may occur prior to nutrient stress. Therefore, it is likely that detection of a neighbouring competitor occurs prior to nutrient deficiency.

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Chapter 7

The temporal dynamics of salicylic acid and jasmonic acid production in response to early stage plant-plant competition

Contents

Abstract

Plant-plant competition, both inter- and intra- specific, has been found to change the temporal dynamics of resource capture. This may be a missing factor in explaining plant coexistence in complex plant communities. The mechanisms behind a temporally dynamic response to a neighbouring plant are unclear but is likely to be mediated via plant growth

regulators. To address this, an experiment was conducted to examine how plant growth regulators, common indicators of plant stress, vary with plant-plant competition over time. Two target plant stress hormones were selected, salicylic acid and jasmonic acid, which moderate the response of plants to both biotic and abiotic stress. Barley was chosen as a model plant and was grown in isolation, and in intra- and inter- cultivar competition. Plants were harvested at intervals between 15 – 27 days after planting, covering the period up to and including plant peak nitrogen accumulation rate. Jasmonic and salicylic acid were extracted from roots and analysed using a High Performance Liquid Chromatography High-Resolution Quadrupole Time-of-Flight Mass Spectrometer. Whilst jasmonic acid was not detected in the samples, the concentration of salicylic acid in plants in isolation was higher than basal levels measured in previous studies, suggesting that all the plants in this study were under some level of stress. The concentration of salicylic acid varied over time but there were no statistically significant effects of the treatments. At 21 days after planting, there was a trend towards a greater concentration of salicylic acid in the competition treatments and a greater concentration in intra-cultivar competition compared to inter-cultivar competition. This supports the theory that salicylic acid potentially has a role in the acclimation of plants to competition stress.

7.1 - Introduction

Plants respond to competition from neighbouring plants in a number of ways including changes in physiology (Trinder *et al.*, 2013), biochemistry (Laliberté, 2016) and the temporal dynamics of resource capture (Trinder *et al.*, 2012). The timing and rate of nutrient capture have been shown to change with intra- (Schofield *et al.*, 2019) and inter- specific plant-plant competition (Trinder *et al.*, 2012). A change in the temporal dynamics of nutrient uptake is thought to reduce direct competition and promote coexistence between plants (Schofield *et al.*, 2018). However, the mechanism that regulates changes in the temporal dynamics of nutrient capture in response to plant-plant competition is unclear.

As plant roots grow they release a range of compounds including volatile organic compounds (VOCs) (Ninkovic *et al.*, 2016) and water soluble compounds in root exudates (Yang *et al.*, 2013). These chemical signatures are often unique at the species and genotype

level, allowing inter- and intra- specific recognition of neighbouring individuals (Chaparro *et al.*, 2012). The presence of a competing individual or root exudates of a competitor have been found to induce a change in plant root architecture (Caffaro *et al.*, 2013). At a molecular level, a neighbouring plant can cause upregulation of stress associated plant growth regulators (PGRs), including salicylic acid (SA) and jasmonic acid (JA) (van Dam and Bouwmeester, 2016). However, the temporal relationships between SA and JA production and nutrient capture when plants are in competition have yet to be explored.

The presence of a neighbouring plant belowground is initially detected by receptors embedded in the root epidermis (Trewavas, 2002). The signal is transduced via the production of reactive oxygen species (ROS) and calcium ions within cells (Tuteja and Mahajan, 2011). Under abiotic stress and some cases of biotic stress this has been found to cause an increase in abscisic acid concentration (Rejeb *et al.*, 2014). This in turn induces the production of SA and JA (Verma *et al.*, 2016). These PGRs are involved in the mediation of responses to biotic stress such as pathogen and pest attack (An and Mou, 2011) and abiotic stresses including drought and salinity (Ahmad *et al.*, 2016; Zhu, 2016). Salicylic acid production has been found to be associated with a response to drought (Khan *et al.*, 2015) and biotrophic pests (Glazebrook, 2005), whereas jasmonic acid production has been more frequently associated with necrotrophic pest response (L. Zhang et al., 2017). The relative proportion of these two PGRs moderate a specific response to a stressor through crosstalk, each mediating the expression of the other PGR to produce either an antagonistic or synergistic response (Does *et al.*, 2013). The balance between JA and SA is specific to each stressor or combination of stresses being experienced (Zandalinas *et al.*, 2018). This may include the stress of a neighbouring individual competing for a limited pool of resources, for example soil nitrogen, a hypothesis which has yet to be tested.

Due to its importance as a crop plant, barley (*Hordeum vulgare*) has been the focus of previous research, including characterisation of the timing and rates of nitrogen and biomass accumulation (Schofield *et al.*, 2019; Chapter 2). Proctor was chosen as the focal cultivar for this study as in a previous study (Schofield *et al.*, 2019; Chapter 2) it demonstrated large temporal shifts in nitrogen accumulation in response to a neighbour. Specifically, it shifted peak nitrogen accumulation 14.5 days later when in intra-cultivar competition compared to plants in isolation and inter-cultivar competition, from 19.5 to 33

days after planting (Schofield *et al.*, 2019; Chapter 2). At a molecular level, plant-plant competition in Proctor has also been examined at a single time point (Chapter 6) using microarrays to characterise gene expression in Proctor roots of plants in inter- and intra-cultivar competition. At 19 days after planting there were more genes differentially expressed under inter-cultivar competition compared to intra-cultivar competition. The genes that were up- and down-regulated had a range of roles in defence, growth and development, and the control of gene expression. These processes are often regulated by plant hormones and the crosstalk between them (Masclaux, Bruessow, Schweizer, Gouhier-Darimont, Keller, Reymond, et al., 2012; Verma et al., 2016). Therefore, measuring the concentration of plant growth regulators could be used to investigate the timing and magnitude of plant-plant competition at a molecular level.

This study focussed on the period surrounding peak nitrogen accumulation rate, when competition is expected to be the most intense, and tested how two plant stress indicators (JA and SA) varied with plant-plant competition treatments and over time in barley (*Hordeum vulgare* cv. Proctor) roots. Concentrations of JA and SA were measured in Proctor roots grown in intra- and inter- cultivar competition compared to plants grown in isolation. Samples were analysed between 15 to 27 days of growth, which covers the period surrounding peak nitrogen accumulation rate (19 days) in this cultivar. It is expected that: 1) there will be higher concentrations of JA and SA in the competition treatments compared to plants in isolation, indicating that these plants are experiencing elevated levels of stress; 2) the concentration of SA and JA will be temporally dynamic and will increase over time as nutrients become depleted, and that the relative concentration of SA compared to JA will also increase over time as nutrients become depleted, mimicking the pattern of SA and JA seen in nutrient deficiency stress (Khan *et al.*, 2015); 3) inter-cultivar competition with Tammi will produce the greatest of the two stress responses (i.e. the highest concentration of SA and JA) in Proctor plants, as in a previous study (Chapter 2) the individuals of this cultivar did not alter the temporal dynamics of nitrogen accumulation to potentially reduce the stress of plant-plant competition (Schofield *et al.*, 2019).

7.2 - Materials and methods

7.2.1 - Soil characterisation

Soil was used from an agricultural field that had contained spring barley (*Hordeum vulgare*) and had been subjected to standard fertilisation conditions previously (500 kg of 22N-4P-14K ha^{-1} yr^{-1}) (Sourced from Balruddery Farm, Invergowrie, Scotland, 56.4837° N, 3.1314° W). Upon collection, the soil was homogenised and passed through a 6 mm sieve, then stored at 4°C prior to planting. It had an organic matter content of 6.2 % ± 0.3 % SEM (loss-on-ignition, n = 4) and a mean pH (in water) of 5.7 ± 0.02 SEM (n = 4).

7.2.2 - Experimental setup

For this study I used the same cultivars of barley (*Hordeum vulgare*) as were used by Schofield *et al.* (2019) (Chapter 2). Proctor plants were grown in isolation (P), intra- (PP) or inter- cultivar competition with Tammi (TP), with three replicates of the three treatments for each of the five harvests (45 pots total). Cylindrical 2 litre pots (diameter 152 mm, height 135 mm) were filled with field soil. Seeds of both cultivars were pre-germinated on damp tissue paper for two days before planting. In pots containing two plants, seeds were planted approximately 5 cm apart and an aboveground mesh screen placed between the two individuals (45 x 16 cm, mesh size 0.08 mm (Harrod Horticulture, Lowestoft, UK)) to ensure competitive interactions only occurred belowground. The presence of a screen was unlikely to have led to differences in shoot development as the foliage was upright; therefore screens were only inserted into pots with two plants.

To account for potential positional effects, the pots were randomised and then grown in a controlled environment room (Conviron, Isleham, UK). The rooms were kept at 15°C constantly with an 8/16 (day/night) hour photoperiod and 65 % relative humidity, to mimic local spring conditions.

7.2.3 - Harvesting

Five harvests were carried out between 15 and 27 days after planting. This covered the majority of the nitrogen uptake period (17 – 33 days after planting; Schofield *et al.*, 2019). At each harvest all plants were harvested within 2 hours of each other. The plants were removed from pots, the roots washed, then shoot and root material separated. The roots were then flash frozen in liquid nitrogen within 3 minutes of harvest to halt metabolic activity. Material was stored at -80°C prior to freeze drying, and then stored at room temperature until extraction of JA and SA.

7.2.4 - Extraction of jasmonic acid and salicylic acid

The extraction and analysis method developed by Forcat *et al.* (2008) was used in this study. Sampled root material was ground and extracted using a 10% methanol, 1% acetic acid (Sigma-Aldrich, Poole, United Kingdom) extraction solution. Internal deuterated standards of 5000 ng ml^{-1} salicylic acid-D6 and jasmonic-D5 acid (Sigma-Aldrich, Poole, United Kingdom) were added to measure percentage recovery during analysis whilst differentiating from native sources of JA and SA in the samples. The extracts were filtered through a 0.2 µm filter (Fisher Scientific, Loughborough, United Kingdom) to remove any remaining particulates. A solvent exchange was then carried out based on initial trial data (not presented) to improve SA and JA peak shape. The 10 % methanol extraction solution was replaced with 95 % distilled water 5 % acetonitrile (Sigma-Aldrich, Poole, United Kingdom) to match the mobile phase of the solvent. The extracts were then stored in 50 µl of this solvent in glass vials at 4°C prior to analysis.

The analytical instrument in this study differed from that used by Forcat *et al.* (2008). An Accucore 3 µm C18 100 mm x 2.0 mm column (Thermo Scientific, Waltham, Massachusetts, USA) was used at 35°C. Samples (50 µl) were analysed using an Agilent 1260 series Agilent 6540 UHD Accurate-Mass High Performance Liquid Chromatography High-Resolution Quadrupole Time-of-Flight Mass Spectrometer (HPLC-HRqTOFMS) (Aglient, Santa Clara, California, USA). The solvent gradient was 5% A (95% H_2O: 5% CH_3CN: 0.1% CHOOH), 95% B (95% CH_3CN: 5% H_2O: 0.1% CHOOH) to 95% A, 5% B over 15 min. To avoid contamination in the instrument, the first 2 min of the run was directed to waste. A needle

wash and blank was run after every sample to avoid contamination between samples. The solvent flow rate was 0.2 ml min^{-1}. The HPLC-HRqTOFMS ion source was Dual Agilent Jet Stream Electrospray Ionization (AJS ESI) with a negative ion polarity. The method was optimised to the following conditions: gas temperature 325°C, gas flow 5 l min^{-1}, Gas Sheath Flow 10 (arbitrary units), fragmentor voltage 80V.

7.2.5 - Data quantification and analysis

Undeuterated standard SA and JA (Sigma-Aldrich, Poole, United Kingdom) were run at concentrations of 0.05 - 99 pg μl^{-1} with internal deuterated standard at 50 pg μl^{-1} to determine the detection limit of the instrument. This found that the range of 0.2 – 20 pg μl^{-1} of the undeuterated standard could be reliably quantified by the instrument. The inclusion of internal deuterated standards at a known concentration allowed the quantification of SA and JA whilst differentiating from the SA and JA present in the sample. Deuteration, the addition of deuterium, a heavy form of hydrogen, gave the standards a slightly different mass compared to the compounds being analysed, allowing them to be differentiated within the samples. This allowed the areas of the peaks to be compared and the concentration of the undeuterated SA and JA determined. Standard concentrations of undeuterated and deuterated SA and JA were run at the start and end of the instrument run and blanks run between each sample to monitor instrument performance over the course of running the samples.

The effects of time, treatment and interactions between these two factors were examined with a Generalised Least Squares model using the nlme package (Pinheiro *et al.*, 2016) in R (R Core Team, 2015). Repeated measures were accounted for using an autocorrelation term. This was followed by an ANOVA test for significant differences using the MASS package (Venables and Ripley, 2002) in R (R Core Team, 2015).

7.3 - Results

7.3.1 - Salicylic acid

The concentration of salicylic acid in the roots varied between 5 and 12 ng g^{-1}. At 15 days after planting, the concentration of SA was similar between the treatments at around 10 ng g^{-1} (Figure 7.1). At 21 days after planting, when compared to plants in isolation (6 ng g^{-1}), SA was higher in Proctor plants in competition (9 ng g^{-1} in inter-cultivar competition), particularly in intra-cultivar competition (13 ng g^{-1}). By 27 days after planting the concentration of salicylic acid had fallen in all the treatments to around 6 ng g^{-1}. Despite these trends there was no statistically significant effect of time ($F_{(4,30)}$ = 0.68, P = 0.51), treatment ($F_{(2,30)}$ = 1.95, P = 0.12) or the interaction of these two factors ($F_{(8,30)}$ = 1.05, P = 0.42).

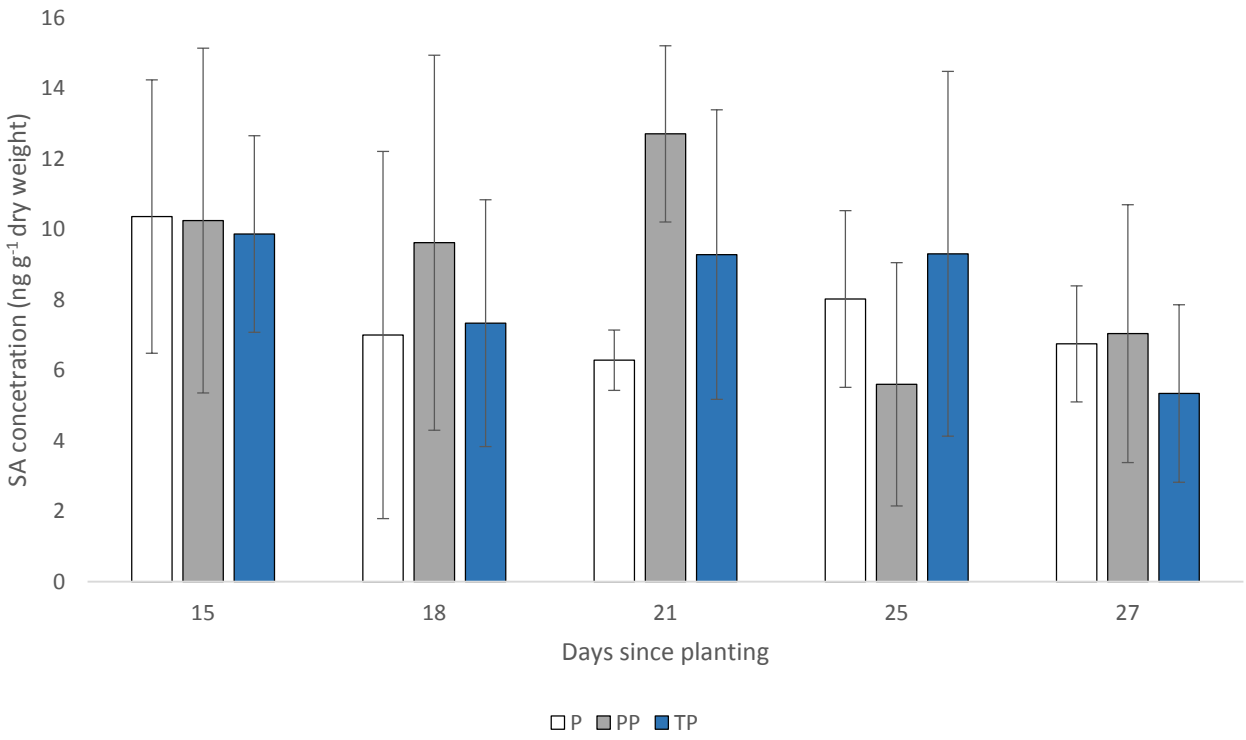

***Figure 7.1** – Concentration of salicylic acid extracted from roots of Proctor sampled over the first month of growth. Proctor plants were grown in isolation (P), intra-cultivar competition (PP) or inter-cultivar competition (TP). Error bars are twice the standard error of the mean.*

7.3.2 - Jasmonic acid

Jasmonic acid was not detected in any sample at a concentration above the instrument detection limit of 0.2 ng g^{-1}, possibly due to the presence of isobaric compounds, which are those with a similar mass to JA that may have increased the background signal and obscured any JA peaks.

7.4 - Discussion

This study aimed to test how plant stress hormone (SA and JA) concentrations varied with plant-plant competition over time. Jasmonic acid was not detected in root samples but salicylic acid was detected in all of the samples. Salicylic acid demonstrated some temporally dynamic trends in concentration, although these trends were non-significant, with increased concentration at 21 days after planting and then declining, making this a potentially interesting time point for more detailed future studies.

7.4.1 - Salicylic acid

The concentration of salicylic acid (SA) in this study ranged from 5 to 12 ng g^{-1}. This suggests that plants in both competition and isolation experienced some level of stress during the study. However, direct comparisons between plant-plant competition stress and other forms of stress are limited due to the lack of data on endogenous SA production in barley, in particular barley roots. The majority of SA studies in the last five years which use barley have focussed on the exogenous application of SA to improve stress tolerance (Khan *et al.*, 2015; Mutlu *et al.*, 2016; Kim *et al.*, 2017; Guo *et al.*, 2019), with only a few studies addressing endogenous SA concentration in response to stress (Chaman *et al.*, 2003; Rivas-San Vicente and Plasencia, 2011). This is therefore an important area for future work to determine the role of SA in moderating different forms and intensities of plant stress. This would allow plant stresses to be better characterised, and the development of plant breeding and management to limit stress in crops, potentially improving productivity.

7.4.2 - Jasmonic acid

The concentration of jasmonic acid was below the detection limit of the instrument in this study. This may be due to interference from isobaric compounds, which had the same mass as JA present in the sample. There are thousands of metabolites in a plant at any one time (Wang *et al.*, 2019), which may have interfered with the detection of JA in barley. Studies of jasmonic acid should include the development of a clean-up step prior to the solvent exchange step to remove potential isobaric compounds.

7.4.3 - Potential link with temporal dynamism in nutrient uptake

This study indicated that SA concentration in barley may demonstrate temporally dynamic trends during the first month of growth. Although the results were not statistically significant, a trend towards a higher SA concentration in intra-cultivar competition compared to inter-cultivar competition at 21 days after planting indicates that this may be a potentially crucial time for spring barley plant-plant interactions. In order to study this further, more power is required for statistical analysis. Increasing both the sampling frequency to daily sampling, and the number of replicates sampled around this time point, would provide greater temporal resolution.

A previous study (Schofield *et al.*, 2019; Chapter 2) found that peak nitrogen accumulation rate occurred for Proctor plants grown in isolation and inter-cultivar competition at 19 to 22 days after planting; for plants grown in intra-cultivar competition this occurred at 33 days. In this study, the higher concentration of SA in the intra-cultivar competition treatment compared to the other treatments suggests a differential response to competition mediated at a molecular level.

An observed increase in SA concentration is often considered to be a response to oxidative stress (Verma *et al.*, 2016). However, SA has been found to have roles in plant processes beyond plant defence, including germination (Rajjou *et al.*, 2006), response to cadmium toxicity (Krantev *et al.*, 2008), and photosynthesis regulation (Rivas-San Vicente and Plasencia, 2011). It has been found to also have a role in plant growth and programmed cell death (Rivas-San Vicente and Plasencia, 2011), development, and plant-microbe

interactions (Herrera Paredes *et al.*, 2016; Chagas *et al.*, 2018). Another potentially important role of SA is in acclimatisation to stress i.e. improving plant tolerance to stress by limiting damage. The accumulation of SA has been found to acclimatise plants to drought through the induction of defensive and antioxidant compound production to limit reactive oxidative stress damage (Sharma *et al.*, 2017). Salicylic acid also promoted the accumulation of unsaturated fatty acids and antioxidant production to protect against cold induced cellular damage (Pál *et al.*, 2013). Salinity stress SA mediated responses include a reduction in photosynthetic pigments and an accumulation of carotenoids and sucrose to protect against oxidative stress (Szepesi *et al.*, 2009). There may therefore be a role of SA in acclimatising plants to the stress of plant-plant competition for nutrients.

7.4.4 - Understanding the mechanism of plant-plant competition at a molecular level

The experimental approach in this study aimed to measure plant hormone concentrations to understand plant stress responses to plant-plant competition. However, as no clear patterns were detected, the system may be more complex and changes more subtle than expected. It may also be the case that signalling compounds other than SA and JA are involved in responses to plant-plant competition. Salicylic acid interacts with auxin during vegetative growth, and with JA, abscisic acid, gibberellins and ethylene during growth and development (Rivas-San Vicente and Plasencia, 2011). Genes that were upregulated at 19 days after planting included those involved in flavonoid production (Chapter 6). Therefore, flavonoids may be another potential indicator of plant-plant competition. Flavonoids have a range of roles in plants including root-rhizosphere communication, in particular root nodulation (Liu and Murray, 2016) as well as defence against pathogens and environmental stress (Treutter, 2005). The role of flavonoids in plant stress is of particular relevance when studying plant-plant competition as it likely contains a stress response component.

Measuring the temporal dynamics of multiple PGRs and other metabolites simultaneously using a metabolic screen may provide a better indication of the type of response at a molecular level and the downstream consequences that lead to the temporally dynamic change in nutrient uptake rate. At 21 days after planting, there is an indication of differences between the treatments, and a metabolomic screen using either

mass spectrometry or NMR methods, as detailed by Balmer *et al.*, (2013), would provide a more comprehensive view of the response to a competitor at a molecular level. The metabolic profiles of barley under biotic stress, specifically pest and pathogen attack, have been characterised using HPLC-DAD (high performance liquid chromatography with diode-array detection) (Balmer *et al.*, 2013) and could be used to draw comparisons between different forms of plant stress, identifying commonalities and differences.

A series of linked studies would be required to examine the effect of plant-plant competition on gene expression, secondary metabolite production, and to then link this to physiological changes. Experimental approaches including studies of gene expression using microarrays or transcriptome sequencing (Liu *et al.*, 2007) can then be combined with proteomic and metabolomic studies using mass spectrometry (Griffiths and Wang, 2009). This would provide an idea of the cascade of processes from detection of a neighbouring plant to a physiological or growth response. The integration of such datasets would involve functional analysis and topological network analysis, as well as multivariate and regression approaches (Bartel *et al.*, 2013; Haider and Pal, 2013; Wang *et al.*, 2016)

There is also a need to test multiple plant tissues to identify tissue specific responses to plant-plant competition. This study focussed on root tissue as screens in the study allowed only interactions between roots. However, a systemic response to plant-plant competition may involve different responses in multiple tissues which vary during growth. Sampling multiple tissues over time would provide information about spatial and temporal variation in plant-plant competition responses within individual plants. Such studies could answer key questions about the role of plant hormones in plant-plant interactions.

7.5 - Conclusions

In this study I found temporally dynamic trends in the concentration of salicylic acid in response to growth stage, particularly when in intra-specific competition, however these trends were not statistically significant. At 21 days after planting – when SA concentrations were higher in intra-cultivar competition compared to inter-cultivar competition – is a potentially important time point that may be crucial for determining plant-plant competition in spring barley, warranting future investigation. Jasmonic acid was not

detected above the instrument detection limit, potentially due to the presence of isobaric compounds. This suggests that the extraction method may require a further clean-up step to improve the detectability of JA. However, to understand the plant stress response to plant-plant competition, measuring multiple metabolites simultaneously is a logical next step. This would allow the detection of subtle stress signalling in response to temporally dynamic plant-plant competition

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Chapter 8

Has temporal dynamism in resource capture been lost in modern barley cultivars?

Contents

Abstract

The timing and rate of resource capture is a potentially important factor in coexistence within plant communities and may therefore also be an important factor in the design of crop mixtures. The temporal dynamism of nitrogen accumulation has been found to be affected by plant-plant competition in the Proctor cultivar of barley (*Hordeum vulgare* cv. Proctor), with a delay of 14.5 days in peak nitrogen accumulation rate when in intra-cultivar competition. There is potential to use this ability to shift the timing of peak nitrogen accumulation in crop mixtures to improve complementarity and resource use efficiency. However, it is not known if temporal dynamism in nitrogen and biomass accumulation have been conserved in the modern cultivar descendants of Proctor. Three cultivars that are descendants with increasing genetic distance from Proctor - Krona, Annabell and Chanson - were selected and grown in isolation, inter- and intra- cultivar competition. Sampling occurred every five days between 20 and 55 days after planting, and plant shoot biomass, nitrogen concentration and C:N were measured at each time point. The detected temporally dynamic trends in nitrogen accumulation of Proctor differed from the trends in a previous study by up to 16.5 days. Proctor, Krona, Annabell and Chanson all demonstrated a 2 – 3 day earlier peak in nitrogen accumulation rate timing when in competition compared to isolation. All cultivars apart from Annabell also had lower total accumulated nitrogen when in competition compared to isolation. These results demonstrate that temporal dynamism is conserved in modern cultivars of barley, and indicates the potential to utilise the temporal dynamics of resource capture in the development of temporally complementary crop mixtures.

8.1 - Introduction

Intercropping and crop mixtures have been used for millennia to improve crop yield stability and reduce inputs of fertiliser, herbicide and pesticides (Brooker *et al*., 2015). Complementarity between intercrops is often based on root and shoot architecture (Postma and Lynch, 2012; Zhu *et al.*, 2016) and the ability of one crop to fix nitrogen (Bedoussac *et al.*, 2015). However, the temporal dynamics of resource capture are likely to be another factor in intercrop complementarity. By occupying different temporal niches, plants can

reduce direct competition for resources whilst occupying the same spatial niche, promoting coexistence between individuals (Schofield *et al.*, 2018). In an agricultural system, complementarity in the temporal dynamics of resource capture is likely to lead to increased resource use efficiency, potentially increasing yield whilst reducing input of fertilisers (Ghaley *et al.*, 2005). The temporal dynamics of resource capture have been explored in intercrops, including complementary canopy growth in relay intercropping of wheat (*Triticum aestivum*) and cotton (*Gossypium hirsutum* L) (Zhang *et al.*, 2008), as well as complementary temporal dynamics of nutrient uptake in wheat (*Triticum aestivum* L.) and faba bean (*Vicia faba* L.) (Li *et al.*, 2014).

Complementarity has also been explored to some extent at an intra-specific level. A recent meta-analysis found that intraspecific crop mixtures on average increase crop yield amount and stability, whilst reducing the negative impact of pests and diseases (Reiss and Drinkwater, 2018). However, plasticity in temporal dynamics of resource capture in response to plant-plant competition of spring barley mixtures has yet to be explored.

Plasticity in the temporal dynamics of resource capture rate has not been actively selected for during breeding of spring malting barley in the last 100 years, where the focus has been on maximising yield, pest and disease resistance and malting quality (Friedt *et al.*, 2011). However, crop domestication has been found to increase the competitiveness of individuals and reduce complementarity in mixtures (Milla *et al.*, 2017). This is due to mostly inadvertent (apart from selection for weed suppression, e.g. Benaragama *et al.*, (2014)) selection for competitive traits in crops and against complementary behaviour (Milla *et al.*, 2014). The ability to shift the temporal dynamics of resource capture might therefore have been lost as part of this selection process. This may have occurred through the accumulation of random mutations in the protein coding regions of these genes, which were not under selection pressure to be functionally maintained in the genome (Lahti *et al.*, 2009).

The temporal dynamics of nitrogen and biomass accumulation have previously been studied in barley (*Hordeum vulgare*). Schofield *et al.* (2019) (Chapter 2) used the Tammi and Proctor cultivars to investigate temporal dynamism of nitrogen and biomass accumulation rate with plant-plant competition. A shift in peak nitrogen accumulation rate timing was found in both cultivars: when in intra-cultivar competition, but not in inter-cultivar

competition, Tammi advanced peak nitrogen accumulation rate by 0.5 days and Proctor delayed it by 14.5 days. As Proctor showed the greatest shift in peak nitrogen accumulation rate timing with intra-cultivar competition, it was chosen to further investigate the potential heredity of resource capture temporal dynamics.

Proctor is a spring malting barley cultivar developed in the 1940s and first introduced commercially in the UK in 1955 (Hayward, 1958). This was prior to the widespread use of recurrent selection, introduced to increase the speed of new cultivar production in barley by using a small genetic base (McProud, 1979). Proctor was favoured due to its high yield and malt quality (Gothard *et al.*, 1978) and is the ancestor of many spring malting barley cultivars (Friedt *et al.*, 2011). This raises the question of whether the observed change in temporal dynamics of peak nitrogen accumulation rate of Proctor in intra-cultivar competition has been maintained in its descendants, including modern barley cultivars.

The three spring barley cultivars selected have increasing genetic distance from Proctor as detailed in Figure 8.1. Krona is the result of a complex cross including Proctor and at least four other cultivars (Hatz *et al.*, 2002; von Bongsong, 2014). It is a malting cultivar first introduced in 1989 and was popular in Germany for brewing for the period of roughly twenty years from its introduction (Oliver, 2014). Annabell is a cross of Krona and another cultivar, ST 900 14DH (Vratislav Psota *et al.*, 2009), and therefore a second generation descendent of Proctor. It was first introduced in 1999 (Xu *et al*., 2018), and was popular due to the high quantity of malt produced (Friedt *et al.*, 2011). Chanson is a seventh generation descendent of Proctor and is also the result of a complex cross. It is a modern malting cultivar that has been on the AHDB Recommended list since 2017 (Stein and Muehlbauer, 2018).

This study investigated if shifts in the temporal dynamics of nitrogen and biomass accumulation rate in response to plant-plant competition have been maintained or lost in Krona, Annabell and Chanson, three spring barley descendants of Proctor. It is expected that as this trait has not been actively selected for, temporal shifts will be less apparent in Krona, Annabell and Chanson compared to Proctor. Barley breeding for monocultures may have selected against collaborative behaviours (Milla *et al.*, 2014) such as temporally dynamic shifts in nutrient uptake rate to promote plant community coexistence. Therefore, the genes that control temporally dynamic shifts might have been lost during the breeding of

modern barley cultivars. This may have led to a reduction in the ability of modern cultivars to shift resource capture dynamics in response to competition pressure.

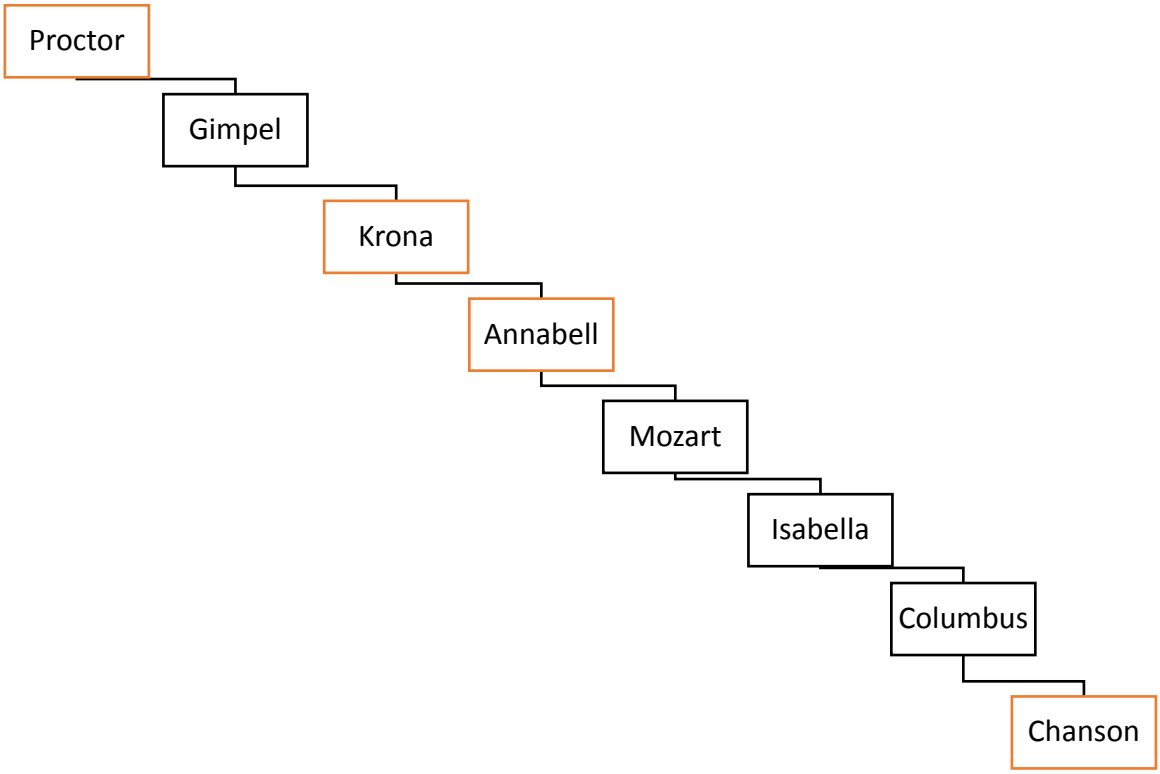

Figure 8.1 *– The pedigree of the four cultivars used in this study highlighted in orange and generations between them (Dr Bill Thomas, personal communication).*

8.2 - Materials and Methods

8.2.1 - Soil characterisation

Soil was sourced from an agricultural field in July 2019 (Balruddery Farm, Invergowrie, Scotland, 56.4837° N, 3.1314° W) that had previously contained spring barley (*Hordeum vulgare*) and had been subject to standard management for barley production (including fertiliser addition at a rate of 500 kg of 22N-4P-14K ha^{-1} yr^{-1}). The soil had an organic matter content (humus) of 6.2% ± 0.3% SEM (loss-on-ignition, n = 4) and a mean pH (in water) of 5.7 ±0.02 SEM (n = 4), a total inorganic nitrogen concentration of 1.55 ± 0.46 mg g^{-1} (n = 4) and microbial C biomass (using a chloroform extraction) of 0.06 ± 0.002 SEM mg g^{-1} (n = 4)

(analysed by Konelab Aqua 20 Discrete Analyser (Thermo Scientific, Waltham, MA USA)). Before use, the soil was passed through a 6 mm sieve, then stored at 4°C until planting occurred. No fertilization of the soil occurred during the experiment.

8.2.2 - Experimental setup

Pots (diameter 152 mm, height 135 mm) were planted with one of the four focal cultivars (Proctor (P), Krona (K), Annabell (A) or Chanson (C)) in one of three treatments: isolation, inter-cultivar competition or intra-cultivar competition. Inter-cultivar treatments were grown in competition with Tammi (T), an early spring barley cultivar with no known shared heritage with any of the focal cultivars, to provide a baseline competitive response to a neighbouring plant that was not a descendent of Proctor (P, PP, TP, K, KK, TK, A, AA, TA, C, CC, TC). Three replicates for each treatment for each of the eight planned harvests were planted, giving a total of 288 pots. This experimental design was influenced by the statistical analysis in Chapter 3 whilst accounting for practical constraints of space and cost. Barley seeds were germinated in the dark on damp paper at room temperature for three days prior to planting. Germinated seeds were planted at a depth of 2 cm and those in the competition treatments were planted 6 cm apart. Pots were arranged randomly to avoid potential positional effects. Mesh screens (45 x 16 cm, mesh size 0.08 mm (Harrod Horticulture, Lowestoft, UK) were inserted between competing plants, to ensure competition only occurred belowground. The foliage was relatively upright and would have been unlikely to be affected by a screen, therefore screens were only inserted in competition treatments.

8.2.3 - Harvesting and sample processing

Three pots of each treatment were selected randomly at each successive harvest, every 5 days from 20 to 55 days after planting. Shoot material was cut at soil level and dried at 100°C to a constant mass. Shoots of the focal cultivars were milled and analysed for carbon and nitrogen concentration (Flash EA 1112 Series, Thermo Scientific, Bremen, Germany).

8.2.4 - Statistical analysis

8.2.4.1 - Carbon and nitrogen temporal dynamics

The non-linear least squares with bootstrapping approach developed in Chapter 2 was used to analyse the data (Schofield *et al.*, 2019; Chapter 2). Logistic growth curves were modelled using non-linear least squares (nls) models (R Core Team, 2015). This allowed the estimation of peak accumulation rate timing and absolute maximum accumulated biomass and nitrogen. Significant differences in peak rate timing and maximum accumulation were determined from the difference in bootstrapped 95 % confidence intervals of the model outputs.

8.2.4.2 - Carbon to nitrogen ratio

At the final harvest (55 days after planting) differences between the four cultivars and between the competition treatments were analysed using an ANOVA test from the MASS package (Venables and Ripley, 2002) in R (R Statistical Software, R Core Team, 2015). The fixed factor in this analysis was treatment or cultivar, with C:N as the response variable. A Tukey post-hoc test was carried out to compare the treatment groups.

8.3 - Results

8.3.1 -Temporal dynamics of biomass accumulation

Biomass accumulated steadily over the growing period in all the cultivars in this study (Figure 8.2). There was a lag period until 35 days after planting, then biomass accumulation rate increased rapidly until the end of the experiment. The biomass accumulation rate derived from the non-linear least squares model peaked at between 50 – 65 days after planting for all the cultivars grown in isolation. Details of the confidence interval differences between the treatments and cultivars are detailed in Tables A1 and A2 of Appendix 4. Plant-plant competition led to a significant shift in the timing of peak biomass accumulation rate in Proctor. Proctor also demonstrated a significant decrease in absolute maximum biomass accumulation in both inter- and intra- cultivar competition compared to isolation,

accompanied by a significantly earlier peak biomass accumulation rate timing (Figure 8.3a). When Proctor was in inter-cultivar competition, peak biomass accumulation rate timing was 11.5 days earlier than plants in isolation or intra-cultivar competition. Krona (Figure 8.3b) did not demonstrate significant shifts in peak biomass accumulation rate timing or absolute maximum accumulated biomass in inter- and intra- cultivar competition. For Annabell, there were no significant shifts in peak biomass accumulation rate timing in inter- or intra- cultivar competition, but absolute maximum accumulated biomass was significantly lower when the plants were in inter-cultivar competition compared to plants in isolation (Figure 8.3c). Chanson (Figure 8.3d) also did not demonstrate significant shifts in peak biomass accumulation rate timing or absolute maximum accumulated biomass when in either inter- or intra- cultivar competition.

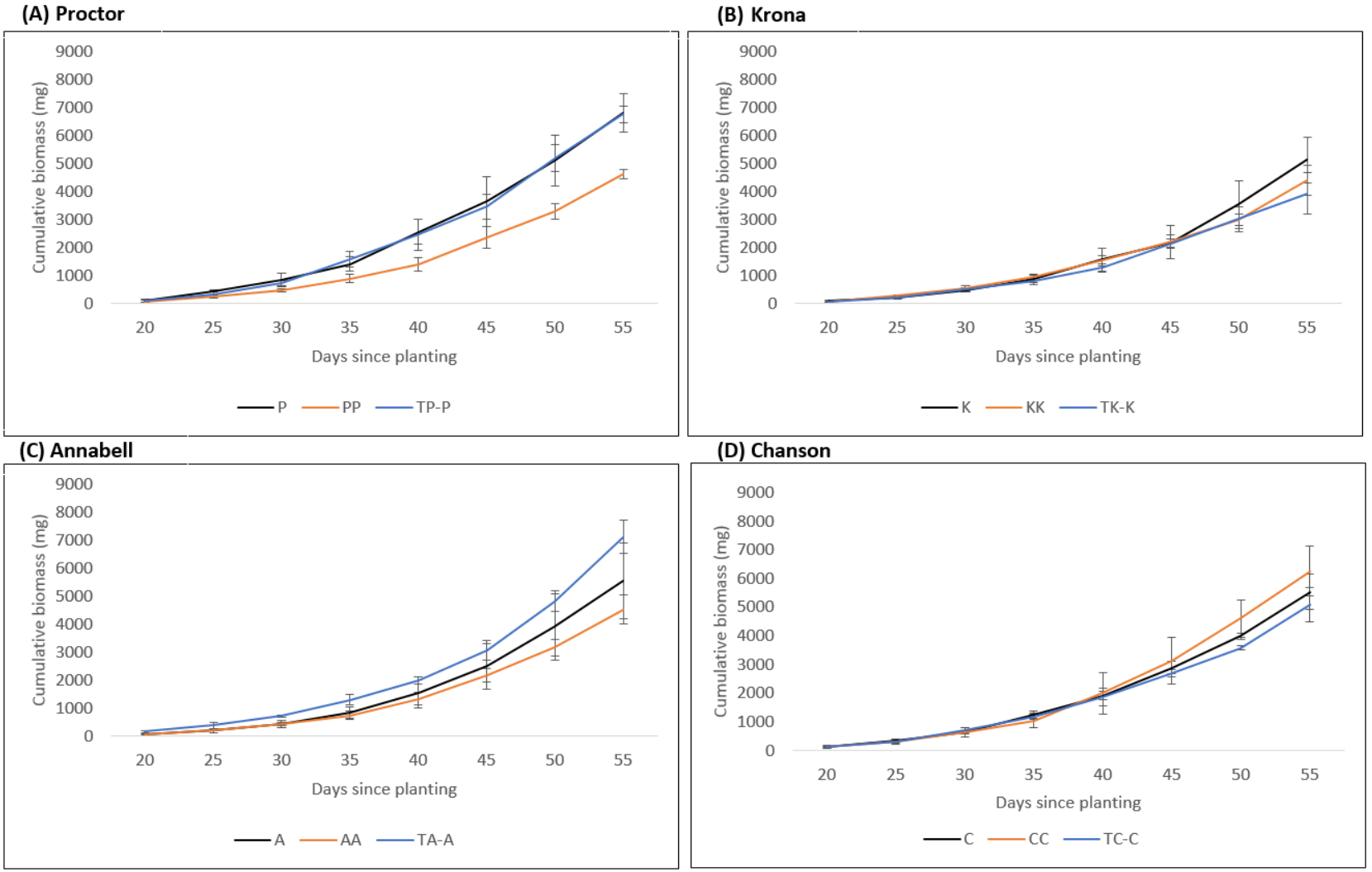

Figure 8.2 *– Cumulative shoot biomass accumulation of the four barley cultivars in this study; Proctor (a), Krona (b), Annabell (c) and Chanson (d). Plants were grown in isolation (Proctor = P, Krona = K, Annabell = A, Chanson = C), intra-cultivar competition (Proctor = PP, Krona = KK, Annabell = AA, Chanson = CC) and inter-cultivar competition (Proctor = TP-P, Krona = TK-K, Annabell = TA-A, Chanson = TC-C). Error bars are two times the standard error of the mean (SEM).*

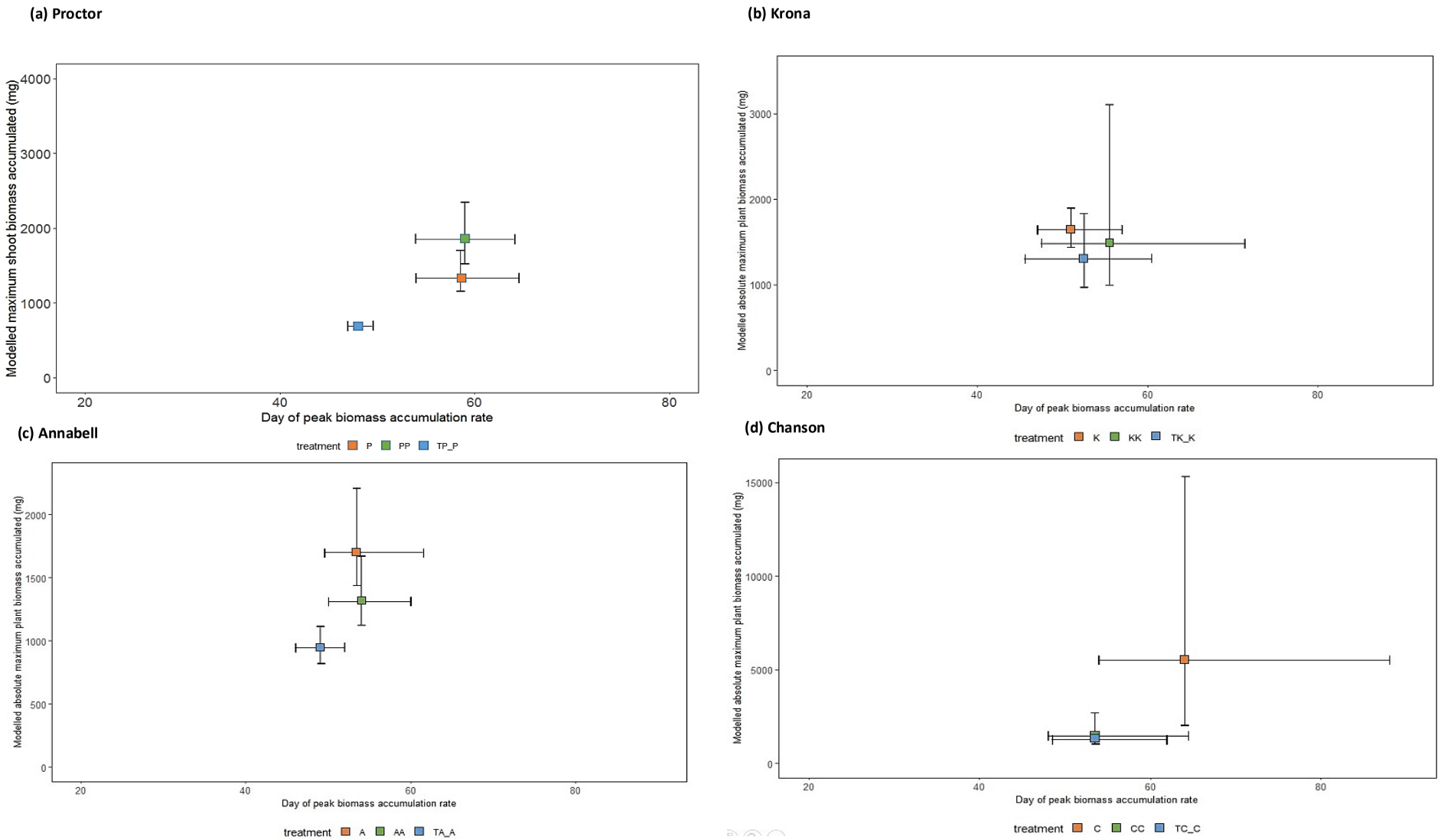

Figure 8.3 *– The absolute maximum biomass accumulation and timing of peak biomass accumulation rate of Proctor (a), Krona (b), Annabell (c) and Chanson (d). Plants were grown in isolation (Proctor = P, Krona = K, Annabell = A and Chanson = C), inter-cultivar competition (Proctor = PP, Krona = KK, Annabell = AA and Chanson = CC) and intra-cultivar competition (Proctor = TP_P, Krona = TK_K, Annabell = TA_A and Chanson = TC_C). Error bars represent the 95% confidence intervals derived from the non-linear least squares model.*

8.3.2 - Temporal dynamics of nitrogen accumulation

Nitrogen accumulation increased rapidly until 35 days when it then began to plateau, which then continued for the remainder of the experiment (Figure 8.4). Details of the confidence interval differences between the treatments and cultivars can be found in Tables A1 and A2 of Appendix 4. Nitrogen accumulation rate peaked at 24.5 - 28 days after planting for all cultivars and treatments. The absolute maximum accumulated nitrogen and the timing of peak accumulation rate were both shifted by plant-plant competition, with differences among the cultivars. The timing of peak nitrogen accumulation rate in Proctor was significantly earlier in both inter- and intra- cultivar competition compared to Proctor in isolation. When in intra-cultivar competition the timing of peak accumulation rate was earlier by 2.5 days and 3.5 days earlier in inter-cultivar competition. There was also a significantly lower absolute maximum accumulated nitrogen concentration in both competition treatments compared to plants in isolation (Figure 8.5a). This trend was very similar to Krona, which significantly shifted peak nitrogen accumulation rate timing earlier by 2 days in response to both inter- and intra-cultivar competition. There was also a significantly lower level of absolute maximum accumulated nitrogen when the plants were in competition compared to isolation (Figure 8.5b).

Annabell demonstrated similar changes in nitrogen temporal dynamics compared to the other cultivars when in competition. When in inter-cultivar competition, peak nitrogen accumulation rate timing shifted, with a significant advancement of 3 days compared to plants in isolation and a significantly lower absolute maximum accumulated nitrogen. However, when in intra-cultivar competition maximum nitrogen accumulation was significantly higher than Annabell in isolation and peak nitrogen accumulation rate timing was 2 days earlier than plants in isolation (Figure 8.5c).

The temporal dynamics of nitrogen accumulation in Chanson, the most modern of the cultivars, were similar to the other cultivars. When in intra-cultivar competition peak nitrogen accumulation rate timing was significantly earlier by 2.5 days than Chanson in isolation and 3 days earlier when in inter-cultivar competition (Figure 8.5d). The biomass and nitrogen responses to intra- and inter- cultivar competition for each cultivar are detailed in Table 8.1.

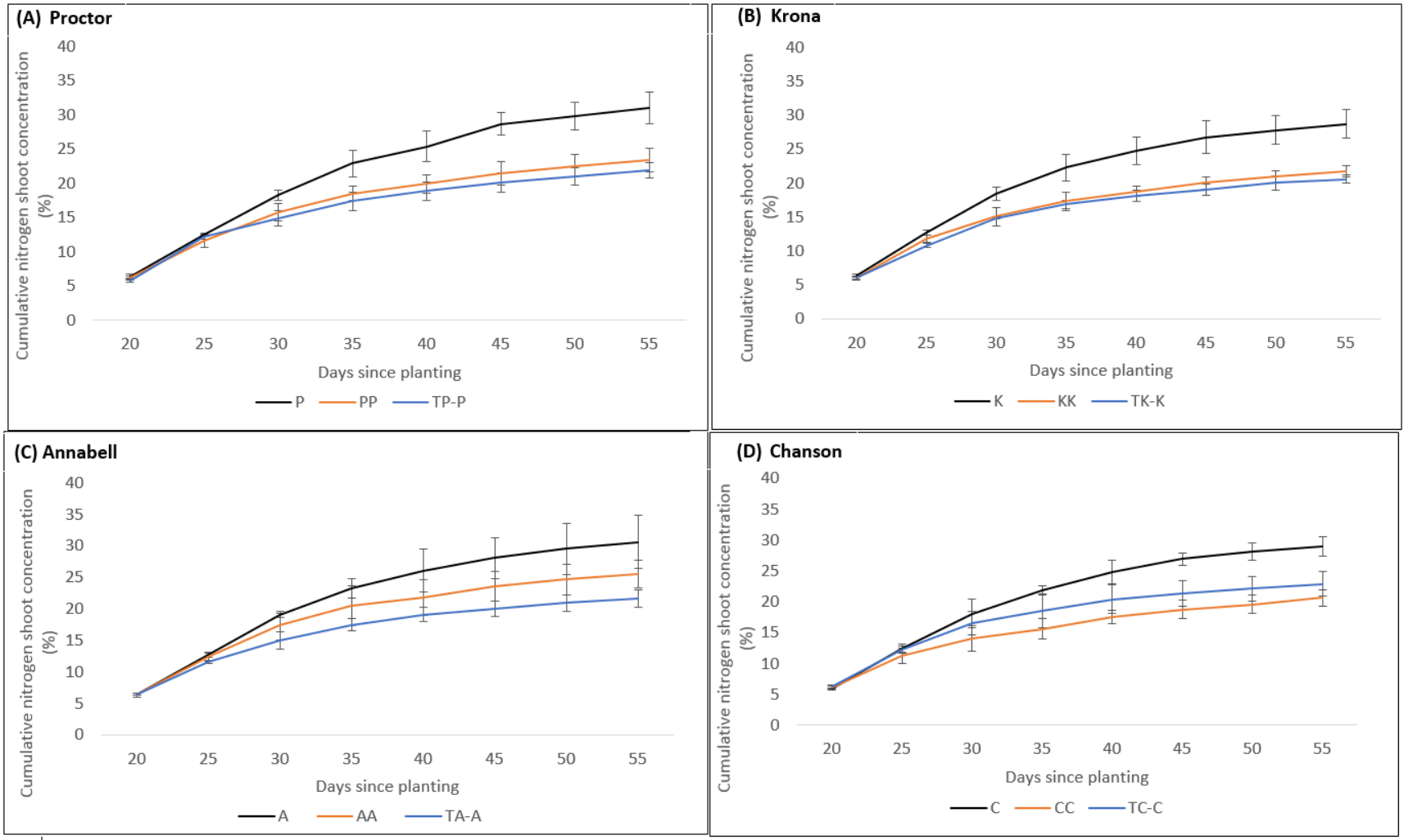

Figure 8.4 *– Cumulative shoot nitrogen concentration of Proctor (a), Krona (b), Annabell (c) and Chanson (d) barley cultivars used in this study. Plants were grown in isolation (Proctor = P, Krona = K, Annabell = A, Chanson = C), intra-cultivar competition (Proctor = PP, Krona = KK, Annabell = AA, Chanson = CC) and inter-cultivar competition (Proctor = TP, Krona = TK, Annabell = TA, Chanson = TC). Error bars are twice the standard error of the mean (SEM).*

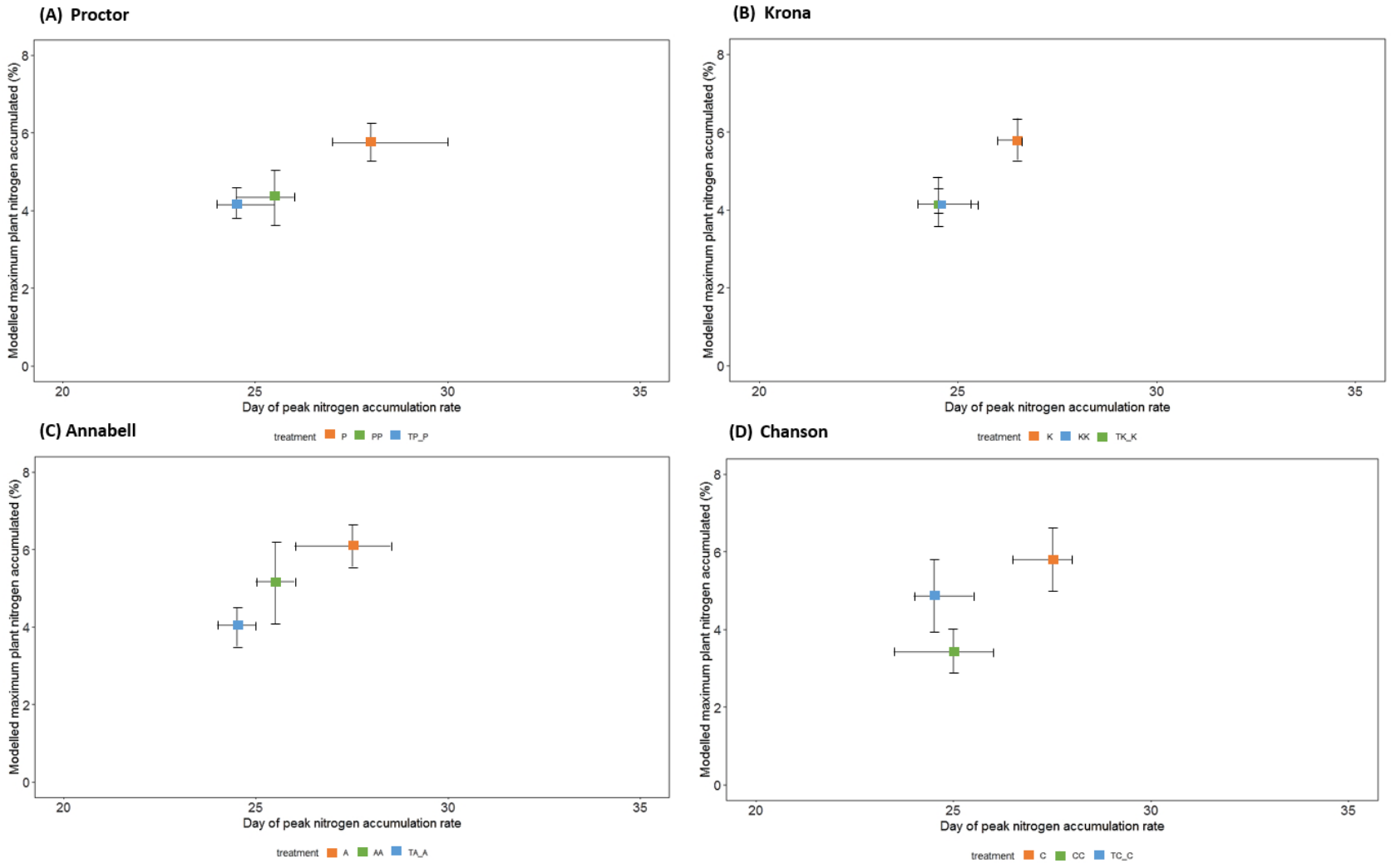

Figure 8.5 *- The absolute maximum nitrogen accumulation and timing of peak nitrogen accumulation rate of Proctor (a), Krona (b), Annabell (c) and Chanson (d). Plants were grown in isolation (Proctor = P, Krona = K, Annabell = A and Chanson = C), inter-cultivar competition (Proctor = PP, Krona = KK, Annabell = AA and Chanson = CC) and intra-cultivar competition (Proctor = TP_P, Krona = TK_K, Annabell = TA_A and Chanson = TC_C). Error bars represent the 95% confidence intervals derived from the non-linear least squares model.*

8.3.3 - C:N ratio

The carbon to nitrogen ratio increased over time as the plants accumulated relatively less nitrogen than carbon. However, there were no significant differences between the four cultivars grown in isolation at the end of the experiment ($F_{(3,8)} = 1.28$, $P = 0.35$). There was also no significant difference between the competition treatments at the end of the experiment ($F_{(11,23)} = 2.03$, $P = 0.07$). Details of the Tukey tests are detailed in Appendix 4, Table A3.

Table 8.1 *– Summary of the biomass and nitrogen responses to intra- and inter- cultivar competition.*

Cultivar	**Biomass timing shift in competition**	**Biomass maximum accumulation change with competition**	**Nitrogen timing shift in competition**	**Nitrogen maximum accumulation change with competition**
Proctor	Earlier peak in inter-cultivar competition only	Lower in both competition treatments	Earlier peak in both competition treatments	Lower in both competition treatments
Krona	Earlier peak in both competition treatments	No significant differences	Earlier peak in both competition treatments	Lower in both competition treatments
Annabell	No significant shifts	Lower in inter-cultivar competition only	Earlier peak in both competition treatments	Higher when in inter-cultivar competition only
Chanson	No significant shifts	No significant differences	Earlier peak in both competition treatments	Lower in both competition treatments

8.4 - Discussion

This study aimed to determine if the ability to shift the temporal dynamics of biomass and nitrogen in barley in response to plant-plant competition had been maintained or lost from the descendants of Proctor as a result of selective breeding. The absolute maximum accumulated biomass was significantly lower in Proctor in both inter- and intra- cultivar competition and Annabell when in inter-cultivar competition. The timing of peak biomass accumulation rate shifted significantly earlier only in Proctor when in inter-cultivar competition. Peak nitrogen accumulation rate timing was significantly earlier in both intra-

and inter- cultivar competition in Proctor, Krona, Annabell and Chanson. Proctor, Krona and Chanson also had significantly lower absolute maximum accumulated nitrogen in competition treatments compared to plants in isolation. However, Annabell had a significant increase in absolute maximum accumulated nitrogen when in intra-cultivar competition. This demonstrates that the ability to shift the temporal dynamics of nitrogen accumulation rate has not been lost during the breeding of modern barley cultivars, and the potential remains for developing temporally complementary crop mixtures using modern cultivars.

8.4.1 - General patterns of temporal dynamics of nitrogen and biomass accumulation

Nitrogen accumulation rate peaked at 23 - 28 days after planting, 27 – 32 days before peak biomass accumulation rate. However, the temporal dynamics of biomass and nitrogen accumulation demonstrated different shifts in response to inter- and intra- cultivar competition. This follows the trend seen in the previous temporal dynamism study, which also found a lack of consistent responses to plant-plant competition between biomass and nitrogen accumulation dynamics (Schofield *et al.*, 2019; Chapter 2).

Several plant processes have been found to be closely linked during growth including: light interception and carbon assimilation (Van Heerden *et al.*, 2010), as well as nutrient uptake and carbon assimilation (Lamaze *et al.*, 2003). Although these processes are linked most of the time, under certain conditions these processes can uncouple. For example, cumulative intercepted solar radiation and biomass accumulation in sugar cane (*Saccharum officinarum*) increase linearly but uncouple at high levels of cumulative solar irradiation interception, when biomass accumulation is limited by a high sugar concentration and cooler temperatures (Van Heerden *et al.*, 2010). Nitrogen and biomass accumulation rate have also been found to uncouple during spring growth of evergreen shrubs to temporarily alleviate competition stress (Lamaze *et al.*, 2003). This is one potential explanation for the differing shifts in biomass and nitrogen accumulation dynamics in this experiment. Plant recognition processes or competition stress early in the growth cycle of barley may lead to a similar response, as plants temporarily uncouple these two processes to reduce competition for resources.

Despite differing shifts in peak biomass and nitrogen accumulation rate timing among the cultivars, there were no significant differences in shoot C:N at the end of the harvesting period. Grain C:N is used as an indicator of malting quality, with a low nitrogen content desired for malting (Janković *et al.*, 2011). Grain nitrogen concentration has been strongly selected for in the development of malting barley cultivars (Munoz-Amatriain *et al.*, 2010). The requirement for a grain low in nitrogen may constrain shoot C:N just prior to grain production and nitrogen remobilisation, to ensure the grain has the desired C:N. At the end of this study the barley plants had produced a flag leaf, the stage prior to grain filling that occurs towards the end of the nitrogen uptake period (Spink *et al.*, 2015). Shoot nitrogen in barley peaks at the grain filling stage, whilst carbon accumulation continues over the whole growing season (Haugen-Kozyra *et al.*, 1993). Therefore, barley may be able to uncouple nitrogen and biomass accumulation rate during the early stages of growth but the difference in carbon and nitrogen accumulation dynamics ensure that shoot C:N is maintained towards the end of vegetative growth.

8.4.2 - *Biomass temporal dynamism cultivar differences*

Proctor was the only cultivar that demonstrated a significant shift in the timing of peak biomass accumulation rate when in inter-cultivar competition. This suggests that there is no linear relationship between temporal dynamics of biomass accumulation and relatedness to Proctor. However, genetic relationships among cultivars are unlikely to be linear, with complex crosses involved in the lineages of the cultivars in this study (Kim, 2014).

Krona did not demonstrate significant shifts in the temporal dynamics of biomass accumulation. Although this may suggest that the temporally dynamic changes in biomass accumulation have not been under selection during modern breeding, biomass has previously been found to be a poor indicator of the temporal dynamics of resource capture (Schofield *et al.*, 2019; Chapter 2). Therefore, this may not answer the question of whether shifts in temporal dynamism in nitrogen accumulation has been bred out of modern barley cultivars. Also, many of the estimates derived from the model have peak biomass accumulation rate timing estimates that peak after the end of the experiment. Therefore,

extending the length of the study may allow more accurate estimates of peak biomass accumulation rate timing.

8.4.3 - Nitrogen temporal dynamism cultivar differences

Peak nitrogen accumulation timing was similar among the cultivars in this study, and occurred between 24.5 and 28 days after planting. All of these cultivars have been bred for modern agriculture in a northern European climate (Friedt *et al.*, 2011), with the same growing season length and growing conditions. Therefore, there are unlikely to be substantial differences in the nutrient uptake dynamics between the cultivars in this study. However, significant shifts in peak nitrogen accumulation rate timing with plant-plant competition were observed in this study. Proctor, Krona, Annabell and Chanson demonstrated a significantly earlier shift in the timing of peak accumulation rate when in competition compared to isolation by 2 – 3 days. This was accompanied by a decrease in absolute nitrogen accumulated. However, Annabell demonstrated the opposite trend, with an increase in the percentage nitrogen in the plant shoots when the plants were in inter-cultivar competition. This suggests that either the plants in inter-cultivar competition accumulated more nitrogen than Proctor plants in other treatments, or accumulated less biomass when in competition with Tammi than the other treatments. There was a significant decrease in the maximum accumulated biomass in this study when the plants were in competition, suggesting the latter explanation. The mechanism of this is unclear but supports the idea that biomass and nitrogen accumulation dynamics were uncoupled in this experiment.

The differing nitrogen uptake response to plant-plant competition may have been due to the influence of the ST 900 14DH cultivar, crossed with Krona in the breeding of Annabell (Vratislav Psota et al., 2009). ST 900 14DH may have differing patterns of biomass and nitrogen accumulation in response to plant-plant competition compared to Krona. The cascade of gene expression involved in nitrogen uptake and associated processes including root growth and biomass accumulation have been characterised using gene regulatory network analysis (Varala *et al.*, 2018; Knoch *et al.*, 2020). The nitrogen and biomass accumulation patterns may have a genetic component, which can be inherited in

subsequent generations. The combination of ST 900 14DH and Krona may have led to the mixing of these genes, and consequently a change in nitrogen and biomass accumulation in response to plant-plant competition in Annabell compared to Krona. Therefore, in order to test this hypothesis, the patterns of nitrogen and biomass accumulation with plant-plant competition in ST 900 14DH need to be measured.

8.4.4 - Why might this study differ from the previous study?

The temporal dynamics of biomass and nitrogen accumulation of Proctor in this study are not the same as the trends seen in Schofield *et al.* (2019) (Chapter 2). In the study described in Chapter 2 there was no significant effect of competition on the temporal dynamics of biomass accumulation and a delay of 14.5 days in peak nitrogen accumulation rate timing when in intra-cultivar competition. However, in this study the shifts in peak nitrogen accumulation rate timing were different, with a shift of 2.5 – 3.5 days earlier when in intra- and inter-cultivar competition compared to plants in isolation. These differences in temporal dynamism estimates may have been due to the different length and replicate number of the two experiments. The study in Chapter 2 was 15 – 60 days sampling length with five replicates, whereas this study was 20 – 55 days in length with three replicates. This may have affected the model estimates of peak biomass and nitrogen accumulation rate timing, an issue explored in Chapter 3. This chapter found that the sampling frequency and replicate number both have a significant effect on estimates of peak biomass accumulation rate timing.

The difference between 3 and 5 replicates under idealised conditions was 2 days in peak biomass accumulation rate timing (Chapter 3). Therefore, the differences in the two experimental designs are likely to have contributed to the observed differences in temporal dynamism shifts between the studies. However, this alone is insufficient to explain the differences. This experiment was carried out two years after the one in Chapter 2. Therefore, other genotype by environment factors such as seed age and soil variation may also be important. Further investigation into the mechanism of shifts in the temporal dynamics of nitrogen accumulation may provide other factors that affected the timing of peak nitrogen accumulation rate timing.

8.4.5 - Have shifts in temporal dynamism of nitrogen accumulation been lost in modern cultivars?

The fact that the four cultivars in this study have a similar pattern of shifted peak nitrogen accumulation rate timing suggests that temporal dynamism in nitrogen accumulation rate has not been accidently lost from the gene pool when these cultivars were developed. Historically the focus of barley breeding has been on grain quantity and quality (Bringhurst, 2015). This includes grain cell wall composition modifications to improve malt quality (Bamforth and Kanauchi, 2001), as well as maximising sugar and alcohol extraction from grain (Jamar *et al.*, 2011). As the temporal dynamics of nitrogen and biomass accumulation have not been under such selection pressure in the past, it provides an untapped potential to improve crop resource use efficiency by altering the timing of key processes.

The complete cascade of gene expression that contribute to shifts in the temporal dynamics of nitrogen accumulation in response to plant-plant competition has yet to be identified. A temporally dynamic shift is likely to include several components within nutrient uptake, plant growth, plant growth regulator production and stress responses, based on the gene expression data presented in Chapter 6. This is likely to involve a range of genes, similar to the quantitative trait loci trait of fermentability (Thomas, 2003; Rostoks *et al.*, 2006; Bringhurst, 2015). Studies that use microarrays and qRT-PCR such as that described in Chapter 6 can be used to identify candidate genes involved in the temporal dynamics of resource capture (Masclaux, Bruessow, Schweizer, Gouhier-Darimont, Keller, Reymond, et al., 2012; Janská et al., 2013). These can then inform breeding of temporally dynamic crops or temporally complementary crop mixtures.

The shifts in peak nitrogen accumulation rate timing were statistically significant but were small. In barley 2 – 3 days is only 3.3 – 5 % of the total lifecycle of the plant, a relatively small shift in terms of the total 60 day period of nutrient uptake of spring barley (Spink *et al.*, 2015). These are statistically significant shifts but may not represent highly biologically significant temporal shifts. This study was carried out under ideal growing conditions, and greater shifts in nitrogen accumulation dynamics may occur under less favourable conditions to limit potential stress damage experienced by the plant. Nelissen *et al.*, (2019) compared studies in growth chambers to those in field conditions and found that the baseline expression of some stress tolerance genes in the field were higher than stressed

plants grown in growth chamber conditions. Differences in environmental conditions in the field can significantly impact the outcome of studies with the same setup (Nelissen *et al.*, 2019). Therefore, future work using plants grown in stress conditions may find that greater plant stress induces greater temporal shifts in resource capture dynamics.

8.4.6 - Does this have consequences for future breeding and crop mixtures?

The magnitude of temporal shifts in nitrogen accumulation rate in this study are small but demonstrate the presence of temporally dynamic shifts in response to plant-plant competition in modern barley cultivars. Growing barley under stress conditions may lead to greater shifts in resource accumulation dynamics with plant-plant competition. This is important with the predicted increase in weather variability over the next 50 years (Mahmood *et al.*, 2019) and an increasing need for crops resilient to climate change (Newton *et al.*, 2011). Crops that are resilient under climatic variability are likely to produce yield stability across a number of variable years (Powell *et al.*, 2012). The ability to shift the timing of key processes may therefore be an adaptive advantage in an uncertain climate.

Increasing resilience in barley using temporal dynamism of resource capture could occur in two ways. One method involves breeding new barley cultivars that show high levels of temporal plasticity in resource capture. Repeated crossing of elite barley lines is the conventional method of barley breeding (Munoz-Amatriain *et al.*, 2010). This can be guided by gene expression and mapping studies to locate candidate genes in marker assisted breeding (Fang *et al.*, 2019). However, this relies on the identification and mapping of key genes to be crossed (Ren *et al.*, 2016). Many of the candidate genes identified in Chapter 6 had generalised functions and may have a range of functions beyond the temporal dynamics of nitrogen accumulation. This may have consequences for breeding as there may be unintended impacts on other stages of growth.

The other method involves the utilisation of crop mixtures. Simultaneously growing multiple cultivars or species in an area has been found to improve crop yield and yield stability between years (Brooker *et al.*, 2015). Barley cultivar mixtures have been found to improve complementarity, stress tolerance and resource use efficiency (Creissen *et al.*, 2016). Also, mixtures that have evolved together show greater facilitation and reduced

competition compared to monocultures (Schöb *et al.*, 2018). However, the traits that contribute to this have yet to be identified. One of these may be the ability to shift the temporal dynamics of resource capture in response to competition. Yield stability could be further improved by using barley cultivars with complementary patterns of resource capture temporal dynamics. There has been much focus on enhancing complementarity using legume intercropping to improve nitrogen and phosphorous use efficiency (Duchene *et al.*, 2017), but limited focus on temporal complementarity. This is a potential area to improve crop mixture resource use efficiency, yield and yield stability.

8.5 - Conclusions

Temporally dynamic shifts in peak nitrogen accumulation rate were found to be conserved in all the descendants of Proctor in this study. Krona, Annabell and Chanson had a similar response to inter- and intra- cultivar competition as Proctor. As temporal dynamism of nitrogen accumulation rate has been conserved in Chanson, a modern barley cultivar, it demonstrates the potential for breeding barley cultivars that have highly plastic temporal dynamics of resource capture or cultivar mixtures that are temporally complementary. The temporal shifts in peak nitrogen accumulation rate were small, most likely due to being grown under ideal, low stress conditions. Increased plant stress may lead to greater shifts in peak nitrogen accumulation rate timing. Future studies using more realistic field conditions would explore the potential for temporally complementary crop mixtures.

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General conclusions

The temporal dynamism of key processes is a potentially important missing factor in our understanding of coexistence in plant communities. Currently, coexistence cannot be explained in complex, species-rich ecosystems through processes such as niche differentiation, as many plants seemingly occupy the same niches. The inclusion of temporal dynamism allows plants to occupy the same spatial niche but different temporal niches, promoting coexistence. One important temporally dynamic process, and the focus of this thesis, is the temporal dynamics of nutrient uptake, specifically nitrogen. This work demonstrates that the use of novel techniques, and refinement of previously utilised approaches, can be used to study temporal dynamism of plant nitrogen uptake. The main finding of this thesis is that the timing and rate of nutrient uptake is affected by intra-specific competition between neighbouring plants and, critically, that this response depends on the identity of the competitor.

The ability to shift the temporal dynamics of key processes such as nutrient uptake is intrinsically linked to the concept of niche differentiation as shifting the timing of peak accumulation of nutrients would lead to a species or genotype occupying a different temporal niche. Therefore, temporal dynamism and temporal niche plasticity could be seen as the same concept, as a temporal niche is the timing of an activity or behaviour (Terradas *et al.,* 2009). However, temporal dynamism also accounts for rate (in this case the rate of nutrient uptake) as well as timing (Schofield *et al.,* 2019). Therefore, temporal dynamism can be seen as part of the concept of niche differentiation that accounts for both the timing and rate of a process.

The studies presented in this thesis used barley (*Hordeum vulgare*) as a model plant, and, as a result, the outcomes of my work have implications for both fundamental ecological research and sustainable agriculture. Barley is an ideal model plant as its genetics (Mayer et al., 2012b), morphology (Spink et al., 2015), physiology (Adem et al., 2014) and growth dynamics (Neumann et al., 2017) have been well studied in an agricultural setting. In addition, there has been an increasing cross-over between ecological and agricultural studies (Brooker et al., 2015), with ecological principles informing sustainable agricultural

practices and vice versa. Consequently, this type of research on barley sits at a key point of interface between fundamental ecological and applied agricultural research.

This body of work aimed to identify temporally dynamic shifts in the nitrogen and biomass accumulation rate of plants, and the activity of the associated soil microbial community in response to plant-plant competition. The results of these studies are summarised in Figure 9.1. Nitrogen accumulation was found to be temporally dynamic in response to intra-specific competition (Schofield *et al.*, 2019; Chapter 2). Peak nitrogen accumulation rate shifted in barley when in intra-cultivar but not inter-cultivar competition. However, the logistic model design and software program used for analysis can affect the estimate of peak accumulation rate (Chapter 3). These findings are applicable to temporal dynamism studies in which the data can be fitted with a logistic curve, including the biomass and nutrient accumulation of plants with deterministic growth and grain filling in cereal crops.

There were no significant timing shifts in soil processes associated with plant-plant competition when measured at the pot level (Chapter 4). However, at a smaller spatial scale the temporal dynamics of soil enzyme activity were affected by plant-plant competition. Peak cellulase area activity was delayed by plant-plant competition, whereas leucine aminopeptidase activity was delayed only in intra-specific competition (Chapter 5). Therefore, plant-plant competition differentially affected the activity of soil enzymes with different roles and at different spatial scales, most likely due to differing scales and methodologies of measurement.

Going into detail with plant belowground processes, plant root gene expression was affected by plant-plant competition at an early growth stage, and prior to obvious signs of nutrient stress, suggesting plant competitive interactions begin early in plant growth and development (Chapter 6). A core set of genes expressed in both inter- and intra- cultivar competition indicates that plant-plant competition caused a general response in a neighbouring plant. However, a set of genes unique to each competition treatment indicates that the response to plant-plant competition also has a neighbour-dependant component. A greater number of genes were differentially expressed in barley plants when in inter-cultivar competition compared to intra-cultivar competition. This supports the idea of a differential response depending on the identity of a competing individual, most likely

mediated via plant-plant communication such as root exudates, volatile organic compounds or via the soil microbial community. The mechanisms behind this process are an area for future research including root exudate and volatile organic compound sampling, as well as more detailed gene expression studies at multiple time points.

The link between gene expression and stress hormone production was less clear (Chapter 7). Salicylic acid concentration was highly variable and thus no statistically significant trends in the temporal dynamics were seen. A greater number of replicates and more sampling time points may yield a clearer pattern of stress hormone concentration changes over time. Twenty-one days after planting is a potentially interesting time point, when the concentration of salicylic acid was higher in inter-cultivar competition than in intra-cultivar competition (Chapter 7). Further studies focussing on the period around 21 days after planting with additional markers of competition, such as more metabolites, potentially the flavonoids and signalling compounds identified in the microarray analysis (Chapter 6), would provide a clearer picture of the molecular level responses to plant-plant competition.

Against expectations, the temporal dynamics of nitrogen accumulation rate was conserved in the modern barley cultivars; Krona, Annabell and Chanson (Chapter 8). All of the modern cultivars showed temporally dynamic shifts in nitrogen accumulation rate in response to plant-plant competition. Krona, Annabell and Chanson demonstrated the same trends as Proctor, shifting earlier in response to inter- and intra- cultivar competition. This suggests that the ability to shift peak nitrogen accumulation rate in response to plant-plant competition may be a heritable trait. Further studies to map the genes involved in this response could support this theory. The trends in nitrogen accumulation dynamics differed

from those in Chapter 2 by up to 16.5 days. These differences are potentially due to unmeasured factors, such as seed age and soil factors and this merits further investigation.

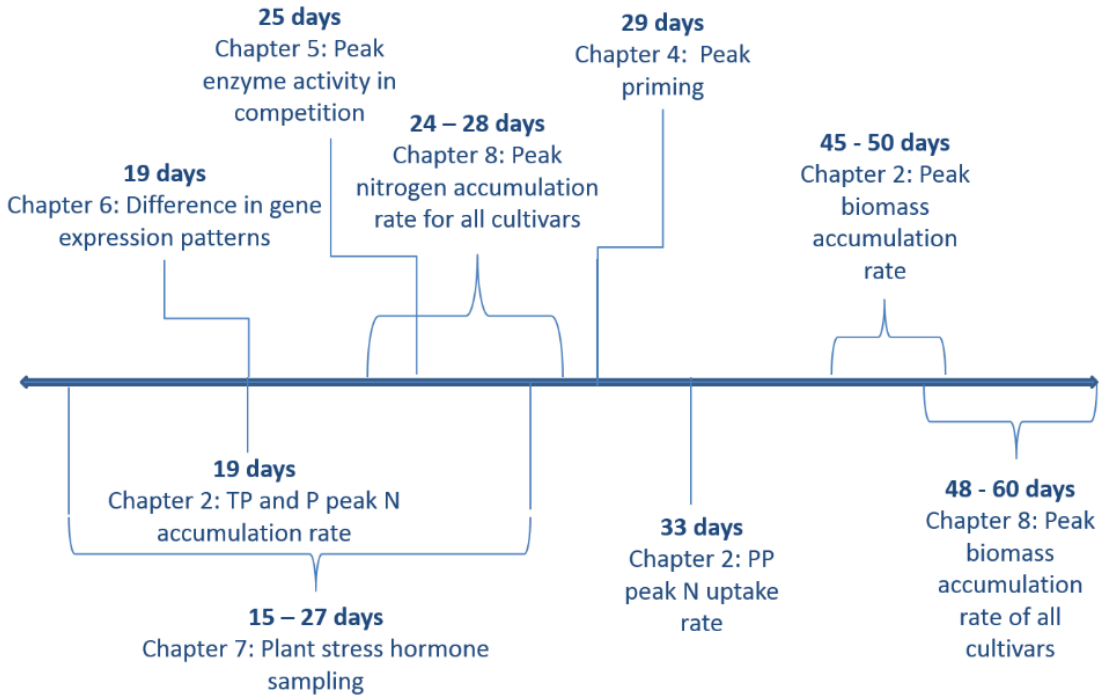

Figure 9.1 *– A summary of the core studies carried out in this thesis, detailing the timing of each study and how they relate to each other within the first 60 days of barley growth.*

Tentative links can be made between the studies in this thesis, displayed in Figure 9.2. It is likely that the initial step of a temporally dynamic response is the perception of a neighbour through water soluble exudates and VOCs (Semchenko et al., 2014). This leads to a change in stress hormone (Chapter 7) production and gene expression changes (Chapter 6). There may then be a change in the quality or quantity of root exudation to prime the soil community to mine for nutrients (Mwafulirwa et al., 2016). This induces changes in the temporal dynamics of the activity of the soil microbial community as seen in Chapter 5. The plant would detect changes in nutrient availability, leading to further changes in gene expression. Ultimately the physiological response of a shift in the timing of peak nitrogen accumulation rate results from these molecular level changes (Chapter 2). There are also likely to be a number of feedback processes that moderate the process over time (Figure 9.2). Therefore, some of the studies in this thesis can be linked but there are still missing pieces of the puzzle that are avenues for future research.

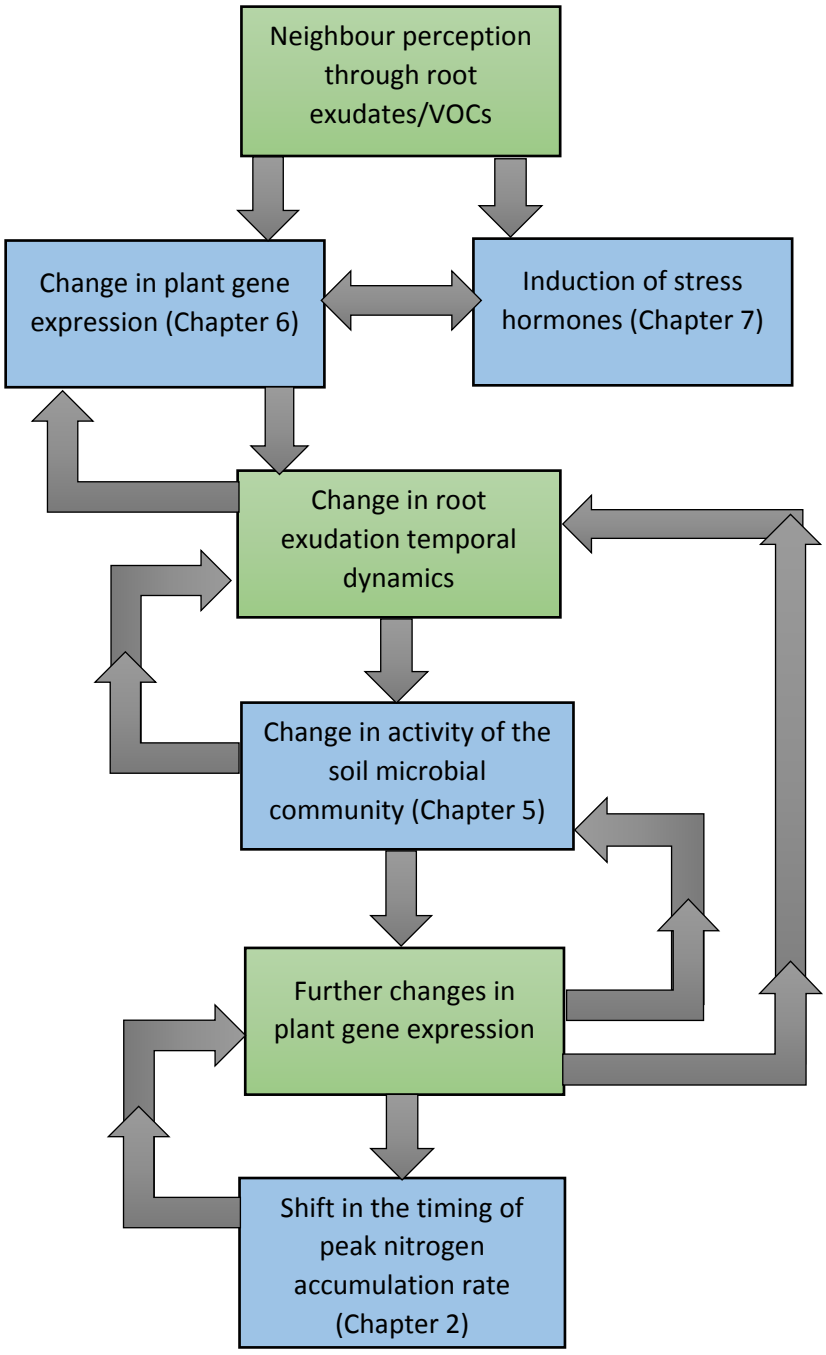

Figure 9.2 *– Links between the studies in this thesis based on experimental evidence and existing literature. Blue boxes indicate work carried out in this thesis, green boxes indicate potential links based on existing knowledge of plant competitive processes. Grey arrows show the expected order of these processes and the feedback loops between them.*

Future work

Using barley as a model plant creates two future avenues of research: the development of both sustainable agriculture and our understanding of plant community coexistence.

There has been much focus on the role of plant mixtures in sustainable agriculture. Mixtures have been found to improve crop yield, quality and maintain overall yield between years i.e. improve yield stability (Brooker et al., 2015). Sustainable agriculture has previously explored spatial complementarity between intercrops (Postma et al., 2014; Zhu et al., 2016) and the inclusion of legumes in crop mixtures (Ghaley et al., 2005; Bedoussac and Justes, 2010), identifying these as important for sustainable production. The data in this thesis demonstrates that the temporal dynamics of plant nutrient uptake is another mechanism with potential for exploitation in sustainable agriculture. By understanding the effect of plant-plant competition on the temporal dynamics of nutrient uptake, temporally complementary crop mixtures can be developed. Although this thesis has focussed on intra-specific plant interactions, the same principles can be applied to inter-specific crop mixtures. For example, the successive harvesting experimental setup used in this thesis can be applied to a range of agricultural and ecological studies using different species and time periods. The experimental design can also be adapted to specific circumstances using the sampling frequency and replicate number framework developed in Chapter 3. However, if these studies are to be carried out on a large scale, this form of nitrogen analysis is destructive, requiring large scale studies with multiple samples, increasing experimental costs. Therefore, the development of a non-destructive proxy measure of plant nitrogen content such as leaf spectrometry using a chlorophyll meter would make these studies more feasible, as well as being useful for field-based plant nitrogen measurements.

Many of the studies in this thesis could be extended in order to further understand the link between the plant and soil components of the temporal dynamics of resource capture. This forms a vital missing factor in plant community coexistence theory. The microarrays used in the gene expression study of Chapter 6 only captured one time point. Future work to expand on this would include sampling gene expression at multiple time points during the growth cycle. This would indicate how gene expression in response to plant-plant interactions changes over time. It may be found that over time expression of genes associated with nutrient deficiency increases, as has been found in *Arabidopsis*

thaliana (Masclaux, Bruessow, Schweizer, Gouhier-Darimont, Keller, Reymond, et al., 2012). The characterisation of differences in gene expression may provide information about the mechanism of temporally dynamic shifts in resource accumulation. This could also generate target genes for future marker assisted breeding programs. Another important factor to consider is if the pattern of gene expression changes with different cultivars of barley. This may be detected at a gene expression level by characterising the expression patterns associated with differing nitrogen accumulation dynamics.

Root exudation quality and quantity is known to vary depending on location within the root zone, with greater exudation near the root tip and in the zone of elongation (Travis S. Walker et al., 2003). This is impacted by, and impacts on, the activity of the soil microbial community (Canarini et al., 2019). The zymography sampling in Chapter 5 only sampled at one root zone location, i.e. in the zone of maturation. The temporal dynamics of soil enzyme activity associated with plant roots in the zone of elongation or at the root tip may differ from the zone of maturation. Therefore, sampling the activity of multiple enzyme classes across the root zones would provide a view of both the temporal and spatial dynamics of soil enzyme activity, allowing further understanding the fundamental link between the soil and plant dynamics in plant community coexistence.

The focus on salicylic acid and jasmonic acid in Chapter 7 could be expanded to include a full metabolomic screen using a method such as mass spectrometry. Twenty one days after planting has been identified as a potentially interesting time point for further studies. By investigating other potentially important compounds, molecular indicators of plant-plant competition could be identified and used to track plant competition stress over time. Combined with the gene expression data from a time series of microarrays this can be used to characterise plant-plant competition responses over time, linking the observed physiological and molecular level responses.

The work in Chapter 8 illustrates the conservation of a temporally dynamic nitrogen accumulation rate in response to plant-plant competition in the descendants of Proctor. The next step in future work would be to examine the temporal dynamics of resource capture under field and stress conditions to determine the potential benefit of temporally dynamics shifts in these circumstances. This can then be used to develop crop mixtures with

temporally complementary resource capture dynamics, which can then be tested under field conditions.

Initial steps

The previous section details a large number of potential future avenues for research. The work in this thesis has demonstrated methods to detect temporally dynamic shifts in processes associated with nutrient uptake in response to plant-plant competition. It also addresses the interactions between the plant and soil processes involved in temporally dynamic shifts of key processes. However, in order to add temporal dynamism as a factor in models of coexistence in complex plant communities and crop mixtures, I suggest focusing on understanding the mechanisms of temporally dynamic responses to plant-plant competition. This would initially involve expanding the gene expression study to include multiple time points, covering the barley lifecycle. The gene expression data would provide information about how plant responses to competition for nutrients vary over time. This type of study would need to be combined with a metabolome study to begin linking gene expression to physiological and biochemical responses to plant-plant competition. These studies would form the basis for a mechanistic approach to understanding plant-plant interactions at a molecular level and link it to the physiological changes detailed in this thesis.

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Appendix 1

Supporting R Code 1

```
n.col <- ncol(Tammi)
n.row <- nrow(Tammi)-1 # line one is time data so subtract

Tammi.time <- as.numeric(Tammi[1,]) # extract the times
Tammi.time <- rep(Tammi.time,each=n.row)
Tammi1 <- ts(Tammi[-1,]) #needs to be a time series to bootstrap correctly

#resampling bootstrap
TammiBoot <- list() #creates list to put values in
for(i in 1:1000){
  TammiBoot[[i]] <- Tammi1 # copy the original data to a list entry for TammiBoot (so we ge
t the right size object)
  for(j in 1:n.col){
    # replace each column of TammiTemp with a resample of the n.row data points at that t
ime
    TammiBoot[[i]][,j] <- sample(Tammi1[,j], size=n.row, replace=TRUE)
  }
}

TammiBoot

#make data cumulative
TammiBootCumul <- list()
TammiBootforAnalysis <- list()
for(i in 1:1000){
  TammiBootCumul[[i]] <- (t(apply(TammiBoot[[i]],1,cumsum)))
  TammiBootforAnalysis[[i]] <- c(TammiBootCumul[[i]]) # convert to vector form for nls
}

n.models <- 1000
```

```
# run the nls on the bootstrap resamples
Tammi.a <- array(NA,dim=c(n.models,4))
Tammi.b <- array(NA,dim=c(n.models,4))
Tammi.c <- array(NA,dim=c(n.models,4))
TammiBootModels <- list()
for(i in 1:n.models){
   Tammi.temp <- TammiBootforAnalysis[[i]]
   TammiBootModels[[i]] <- nls(Tammi.temp ~ SSlogis(Tammi.time, a, b, c)) # need to be abl
e to loop this for every line of the matrix
   Tammi.a[i,] <- summary(TammiBootModels[[i]])$coef["a",]
   Tammi.b[i,] <- summary(TammiBootModels[[i]])$coef["b",]
   Tammi.c[i,] <- summary(TammiBootModels[[i]])$coef["c",]
}
colnames(Tammi.a) <- colnames(summary(TammiBootModels[[1]])$coef)
colnames(Tammi.b) <- colnames(summary(TammiBootModels[[1]])$coef)
colnames(Tammi.c) <- colnames(summary(TammiBootModels[[1]])$coef)

# Maximum points
# Mean peak time from the bootstrap (x-axis)
mean(Tammi.b[,"Estimate"])
# CI for peak time from the bootstrap (x-axis)
quantile(Tammi.b[,"Estimate"],probs=c(0.025,0.975)) # 95%
# Calculate vector of bootstrapped peak heights of rate per day (y-axis)
Tammi.peaks <- Tammi.a[,"Estimate"]/(4*Tammi.c[,"Estimate"])
# Mean peak rate from the bootstrap (y-axis)
mean(Tammi.peaks)
# CI for peak rate from the bootstrap (y-axis)
quantile(Tammi.peaks,probs=c(0.025,0.975)) # 95%

#significant differences in timing
Tammi.peaks.T <- Tammi.b
```

```
Tammi.peaks.TT <- Tammi.b
Tammi.peaks.TP <-Tammi.b
Proctor.peaks.P <- Tammi.b
Proctor.peaks.TP <- Tammi.b
Proctor.peaks.PP <- Tammi.b

Tammi.peaks.T.minus.Tammi.peaks.TP <- Tammi.peaks.T[sample(1000)] - Tammi.peaks.TP[sample(1000)]
quantile(Tammi.peaks.T.minus.Tammi.peaks.TP,probs=c(0.025,0.975))

Tammi.peaks.T.minus.Tammi.peaks.TT <- Tammi.peaks.T[sample(1000)] - Tammi.peaks.TT[sample(1000)]
quantile(Tammi.peaks.T.minus.Tammi.peaks.TT,probs=c(0.025,0.975))

Proctor.peaks.P.minus.Proctor.peaks.PP <- Proctor.peaks.P[sample(1000)] - Proctor.peaks.PP[sample(1000)]
quantile(Proctor.peaks.P.minus.Proctor.peaks.PP,probs=c(0.025,0.975))

Proctor.peaks.P.minus.Proctor.peaks.TP <- Proctor.peaks.P[sample(1000)]- Proctor.peaks.TP[sample(1000)]
quantile(Proctor.peaks.P.minus.Proctor.peaks.TP,prob=c(0.025,0.975))

#Testing for significant accumulation differences in bootstrapped samples
Tammi.acc.T <- Tammi.peaks
Tammi.acc.TT <- Tammi.peaks
Tammi.acc.TP <- Tammi.peaks
Proctor.acc.P <- Tammi.peaks
Proctor.acc.PP <- Tammi.peaks
Proctor.acc.TP <- Tammi.peaks

Tammi.acc.T.minus.Tammi.acc.TT <- Tammi.acc.T[sample(1000)] - Tammi.acc.TT[sample(1000)]
```

```
quantile(Tammi.acc.T.minus.Tammi.acc.TT,prob=c(0.025,0.975))

Tammi.acc.T.minus.Tammi.acc.TP <- Tammi.acc.T[sample(1000)] - Tammi.acc.TP[sample(10
00)]
quantile(Tammi.acc.T.minus.Tammi.acc.TP,prob=c(0.025,0.975))

Proctor.acc.P.minus.Proctor.acc.PP <- Proctor.acc.P[sample(1000)] - Proctor.acc.PP[sample(
1000)]
quantile(Proctor.acc.P.minus.Proctor.acc.PP,prob=c(0.025,0.975))

Proctor.acc.P.minus.Proctor.acc.TP <- Proctor.acc.P[sample(1000)] - Proctor.acc.TP[sample(
1000)]
quantile(Proctor.acc.P.minus.Proctor.acc.TP,prob=c(0.025,0.975))
```

Supporting R Code 2

```
CN_65days$treatment <- (CN_65days$treatment)
CN_65days$ID <- (CN_65days$ID)

Res <- aov(CN ~ treatment, data = CN_65days)
fit <- aov(Res)
TukeyHSD(fit)
```

***Table A1** – Model parameters of the logistic growth curve fitting using a nls model of biomas s and nitrogen accumulation of Proctor and Tammi barley varieties grown in isolation, intra- and inter- cultivar competition. 95% confidence intervals are shown in brackets.*

Treatment	Peak timing (days since planting)	Absolute maximum (mg)
<u>Biomass</u>		
T	48.0 (44.5 - 51.5)	1527.3 (1342.5 - 1707.2)
TT	47.0 (45.0 - 49.0)	1069.5 (986.6 - 1149.3)
TP-T	47.0 (44.5 - 50.5)	1221.8 (1068.8 - 1368.6)
P	51.5 (49.5 - 54.5)	1125.1 (1042.6 - 1207.0)

PP	48.5 (46.0 - 52.5)	705.0 (630.7 - 785.1)
TP-P	47.0 (42.0 - 54.0)	530.9 (530.9 - 687.4)
Nitrogen		
T	19.0 (18.5 - 20.0)	210.0 (190.0 - 220.0)
TT	17.5 (17.0 - 18.0)	160.0 (150.0 - 180.0)
TP-T	18.5 (17.5 20.0)	160.0 (150.0 - 170.0)
P	19.5 (18.5 - 20.5)	210.0 (190.0 - 230.0)
PP	35.0 (33.5 - 36.0)	120.0 (100.0 - 140.0)
TP-P	20.5 (19.5 - 21.0)	170.0 (150.0 - 190.0)

Table A2 - *Bootstrapped confidence interval differences of timing of peak biomass and nitrogen accumulation of Proctor (P) and Tammi (T) barley plants grown in isolation (T, P), inter-cultivar competition (TT, PP) and inter-cultivar competition (Tammi: TP-T, Proctor: TP-P). Asterisks indicate significant differences.*

Treatment	CI differences in timing of peak accumulation rate
Biomass	
T vs. TT	-9.0, 11.0
T vs. TP-T	-10.0, 11.0
P vs. PP-	-9.0, 16.5
P vs. TP-P	-8.0, 13.0
Nitrogen	
T vs. TT	11.5, 14.0*
T vs. TP-T	-12.0, 13.0
P vs. PP	-13.0, 12.5
P vs. TP-P	-33.0, -29.5*

Table A3 - *Bootstrapped confidence interval differences of absolute maximum biomass and shoot nitrogen accumulation of Proctor (P) and Tammi (T) barley plants grown in isolation (T, P), inter-cultivar competition (TT, PP) and inter-cultivar competition (Tammi: TP-T, Proctor: TP-P). Asterisks indicate significant differences.*

Treatment	CI differences in maximum accumulation
Biomass	
T vs. TT	268.10, 653.69*
T vs. TP-T	89.81, 547.62*
P vs. PP	312.00, 523.62*

P vs. TP-P	441.20, 728.07*
TP-P vs. PP	-8.82, 302.41
TP-T vs. TT	-332.64, 19.17
Nitrogen	
T vs. TT	0.03, 0.07*
T vs. TP-T	0.03, 0.07*
P vs. PP	0.06, 0.11*
P vs. TP-P	0.01, 0.06*
TP-P vs. PP	1.98, 2.02*
TP-T vs. TT	-0.02, 0.02

Table A4 *– Model parameters of the ANOVA analysis carried out on shoot C:N of Proctor and Tammi barley varieties grown in isolation, intra- and inter- cultivar competition.*

Proctor					
	Degrees of Freedom	Sum of squares	Mean of squares	F value	P value
Treatment	2	203.3	101.64	1.44	0.26
Residuals	17	1196.7	70.39		
Tammi					
Treatment	2	2915	1457.6	2.74	0.09
Residuals	17	9053	532.5		

Appendix 2

Figure A1 *– Details of the Generalised Least Squares model used to analyse root associated area and root axis activity. * denotes significant results.*

	Time	Treatment	Time*Treatment
Cellulase root associated area	$F_{(2,17)}$ = 44.98, P = <0.0001*	$F_{(2,17)}$ = 4.71, P = <0.0001*	$F_{(2,17)}$ = 12.88, P = 0.0001*
Leucine aminopeptidase root associated area	$F_{(2,17)}$ = 30.36, P = <0.0001*	$F_{(2,17)}$ = 31.72, P = <0.0001*	$F_{(2,17)}$ = 7.42, P = 0.0012*
Cellulase root axis activity	$F_{(72,63)}$ = 0.51, P = 0.60	$F_{(72,63)}$ = 5.03, P = 0.01 *	$F_{(72,63)}$ = 0.94, P = 0.45
Leucine aminopeptidase root axis activity	$F_{(72,63)}$ = 2.74, P = 0.07	$F_{(72,63)}$ = 2.92, P = 0.06	$F_{(72,63)}$ = 1.02, P = 0.40

Appendix 3

Table A1 *- List of significantly (P ≤ 0.05 with ≥ 2 fold change in expression) differentially expressed genes expressed in intra-cultivar competition, with annotated functions from the UniProt database.*

Primary Accession	Rice description	Function	Up/down regulated
Plant defence			
MLOC_23705.2	Jacalin-like lectin domain containing protein, putative, expressed	Biotic and abiotic stress response, specifically fungal resistance	↓
MLOC_47908.1	Jasmonate-induced protein, putative, expressed	Induced by jasmonate production	↓
AK359282	Jacalin-like lectin domain containing protein, putative, expressed	Biotic and abiotic stress response, specifically fungal resistance	↓
MLOC_74229.1	Ribosome inactivating protein, expressed	Common plant defence protein thought to defend against viral and fungal attack	↓
MLOC_33768.7	Stress responsive A/B Barrel domain containing protein, expressed	Thought to be involved in plant stress response including salt stress	↑
MLOC_29656.1	HEV3 - Hevein family protein precursor, expressed	General stress response - drought, salt, fungus, herbivore, virus and systematic acquired resistance	↓
MLOC_53527.1	HEV3 - Hevein family protein precursor, expressed	General stress response - drought, salt, fungus, herbivore, virus and systematic acquired resistance	↓
MLOC_22174.2	Laccase precursor protein, putative	Abiotic stress tolerance including drought and salinity	↑

AK359587	1-aminocyclopropane-1-carboxylate oxidase protein, putative, expressed	Enzyme involved in ethylene biosynthesis	↓
MLOC_15369.1	Verticillium wilt disease resistance protein, putative, expressed	Putative verticillium wilt disease resistance protein	↑
Metabolism, growth and development			
MLOC_56921.1	Cytochrome P450, putative, expressed	Role in general metabolism	↓
MLOC_58866.1	Pyridoxal-dependent decarboxylase protein, putative, expressed	Active form of vitamin B6, involved as a co-enzyme in many metabolic reactions including amino acid biosynthesis	↓
AK249901.1	BBTI4 - Bowman-Birk type bran trypsin inhibitor precursor, expressed	Serine-type endopeptidase inhibitor activity - inhibits activity of endopeptidases	↑
MLOC_20612.1	Transferase family protein, putative, expressed	Production of glucose polymers	↑
AK363287	Serine esterase, putative, expressed	Hydrolysis of polypeptides	↓
MLOC_74633.2	Citrate transporter, putative, expressed	Mitrochondrial transporter protein	↑
MLOC_62337.1	Helix-loop-helix DNA-binding domain containing protein	Transcription factor	↓
MLOC_13480.1	Glycerophosphoryl diester phosphodiesterase family protein, putative, expressed	Lipid metabolism	↓
AK368375	OsFBX64 - F-box domain containing protein, expressed	Protein interactions, cell cycle, protein ubiquitination	↑
MLOC_23023.1	Membrane-associated 30 kDa protein, chloroplast precursor, putative, expressed	Chloroplast membrane protein	↑
AK369652	Ribulose bisphosphate carboxylase small chain, chloroplast precursor, putative, expressed	Protein in chloroplast stroma part of the Calvin cycle	↑
MLOC_47977.1	Preprotein translocase subunit secY, putative, expressed	Protein transmembrane transporter and signal transduction	↑

MLOC_32850.1	T-complex protein, putative, expressed	Protein folding and ATP binding	↑
MLOC_9983.1	Integral membrane protein DUF6 containing protein, expressed	Potential membrane proteins with some signalling potential from animal and microbial orthologs	↑
MLOC_12671.1	Peptidyl-prolyl cis-trans isomerase, FKBP-type, putative, expressed	Family of molecular chaperones that regulate cellular processes	↑
Gene expression control			
MLOC_2169.1	la domain containing protein, putative, expressed	Histone modification and chromatin remodelling	↑
MLOC_75618.1	DEAD-box ATP-dependent RNA helicase 7, putative, expressed	RNA, helicase and ATP binding protein	↑
MLOC_34063.1	la domain containing protein, putative, expressed	Histone modification and chromatin remodelling	↓
Genome rearrangement			
MLOC_46646.1	Retrotransposon protein, putative, unclassified	Genome rearrangement	↑
MLOC_59110.1	Transposon protein, putative, unclassified, expressed	Genome rearrangement	↓
MLOC_32827.1	Retrotransposon protein, putative, unclassified	Genome rearrangement	↑
Unknown function			
MLOC_63825.1	No hits found	Unknown function	↓
MLOC_61312.1	Conserved hypothetical protein	Unknown function	↑
MLOC_8387.1	Hypothetical protein	Unknown function	↑
MLOC_26566.1	Uncharacterized 50.6 kDa protein in the 5region of gyrA and gyrB, putative, expressed	Unknown function	↑
MLOC_36029.1	Expressed protein	Unknown function	↓
MLOC_18226.2	Conserved hypothetical protein	Unknown function	↑
MLOC_75527.1	Expressed protein	Unknown function	↑
MLOC_34210.1	No hits found	Unknown function	↑
MLOC_60892.1	No hits found	Unknown function	↓
MLOC_15135.2	Expressed protein	Unknown function	↑

Figure A2 *- List of significantly (P ≤ 0.05 with ≥ 2 fold change in expression) differentially expressed genes expressed in inter-cultivar competition, with annotated functions from the UniProt database.*

Primary Accession	Rice description	Function	Up/down regulated
Plant defence			
MLOC_5633.1	Respiratory burst oxidase, putative, expressed	Production of ROS in response to plant pathogen attack	↓
MLOC_24632.1	DUF567 domain containing protein, putative, expressed	Plant defence against pathogens	↓
MLOC_55663.1	Peroxidase precursor, putative, expressed	Stress response to environmental stresses such as wounding, pathogen attack and oxidative stress. These functions might be dependent on each isozyme/isoform in each plant tissue	↓
AK252327.1	GDSL-like lipase/acylhydrolase, putative, expressed	Tissue dependent, stress response in roots	↓
MLOC_54129.1	Peroxidase precursor, putative, expressed	General stress response	↓
AK354203	14-3-3 protein, putative, expressed	Regulation of pathogen defense-related proteins and modulate signal transduction	↓
MLOC_76289.1	Protein kinase PKN/PRK1, effector, putative, expressed	Potential role in plant defence in rice, plasma membrane protein	↑
MLOC_26919.1	Cupin domain containing protein, expressed	Role in plant development and defence	↑
MLOC_70601.1	Heat shock protein, putative	Heat shock response	↑
MLOC_59149.1	Stress responsive protein, putative, expressed	General stress response	↑
MLOC_67097.1	HVA22, putative, expressed	ABA or stress-inducible gene expression, for	↑

		dehydration protection	
MLOC_81765.1	WIP1 - Wound-induced protein precursor	Endopeptidase inhibitor involved in herbivore defence	↑
Metabolism, growth and development			
MLOC_65390.1	Pectinesterase, putative, expressed	Plant cell wall modification and subsequent breakdown	↓
MLOC_9250.2	Cysteine synthase, putative, expressed	Cysteine production	↓
MLOC_1081.1	Mitochondrial chaperone BCS1, putative, expressed	Part of mitochondrial respiratory chain	↓
MLOC_8151.2	CBS domain-containing protein, putative, expressed	Transmembrane protein or DNA binding protein	↑
AK362212	CSLC1 - cellulose synthase-like family C, expressed	Cellulose synthesis	↓
MLOC_21213.1	Ergosterol biosynthetic protein 28, putative, expressed	Protein binding	↓
MLOC_12426.1	Gibberellin receptor GID1L2, putative, expressed	Gibberellin receptor involved in the regulation of plant development and growth	↓
MLOC_54679.5	Alpha/beta hydrolase fold, putative, expressed	Plant cuticle production process	↓
MLOC_44884.1	CW-type Zinc Finger, putative, expressed	Bind DNA, RNA, protein and/or lipid substrates	↓
AK370749	Receptor-like protein kinase precursor, putative, expressed	Precursor to RLKs - involved in hormonal response pathways, cell differentiation, plant growth and development, self-incompatibility, and symbiont and pathogen recognition.	↓
AK371913	Phosphoesterase family protein, putative, expressed	Phosphate breakdown	↓
MLOC_75273.1	Cytochrome P450, putative, expressed	Role in general metabolism	↓

MLOC_75265.1	OsIAA27 - Auxin-responsive Aux/IAA gene family member, expressed	Regulation of Auxin production	↓
AK365257	Receptor-like kinase ARK1AS, putative, expressed	ATP, polysaccharide binding and protein kinase activity	↓
MLOC_75041.1	COBRA, putative, expressed	Key regulator of the orientation of cell expansion in the root	↓
MLOC_44152.1	Serine/threonine-protein kinase receptor precursor, putative, expressed	Precursor to receptors involved in protein phosphorylation	↓
MLOC_71416.1	BBTI12 - Bowman-Birk type bran trypsin inhibitor precursor, expressed	Stops endopeptidase activity	↓
MLOC_36591.3	Resistance protein LR10, putative	ADP binding protein	↓
MLOC_3978.1	OsSub3 - Putative Subtilisin homologue, expressed	Protease activity	↓
AK357656	OsPOP8 - Putative Prolyl Oligopeptidase homologue, expressed	Peptidase activity	↓
MLOC_39668.1	BBTI8 - Bowman-Birk type bran trypsin inhibitor precursor, expressed	Stops endopeptidase activity	↓
MLOC_56507.3	MLO domain containing protein, putative, expressed	Plant integral membrane proteins, Mlo proteins function as G-protein coupled receptors in plants	↓
MLOC_60267.2	Kelch repeat protein, putative, expressed	Protein degradation	↓
MLOC_78778.1	Cytokinin-N-glucosyltransferase 1, putative, expressed	glucose transferase	↓
AK365385	Hydrolase, alpha/beta fold family domain containing protein, expressed	Hydrogen removal from molecules, diverse role in plant metabolism	↓
MLOC_73656.2	BBTI13 - Bowman-Birk type bran trypsin inhibitor precursor, expressed	Stops endopeptidase activity	↓
MLOC_69485.1	S-locus-like receptor protein kinase, putative, expressed	ATP, polysaccharide binding and protein kinase activity	↓
AK363181	Cytochrome P450, putative, expressed	General role in metabolism	↑
AK370360	BBTI13 - Bowman-Birk type bran trypsin inhibitor precursor, expressed	Stops endopeptidase activity	↑

MLOC_70810.1	Cytochrome P450, putative, expressed	General role in metabolism	↑
MLOC_54362.3	Leaf senescence related protein, putative, expressed	Role in leaf senescence	↑
MLOC_71020.1	Jacalin-like lectin domain containing protein, expressed	Carbohydrate binding	↑
AK353701	Transferase family protein, putative, expressed	Transfer functional groups of molecules	↑
MLOC_79920.2	Slowmo homolog, putative	F box protein involved in plant development and auxin transport	↑
MLOC_41188.2	Growth regulator related protein, putative, expressed	Role in growth regulation	↑
MLOC_11916.3	OsSCP24 - Putative Serine Carboxypeptidase homologue, expressed	Protein maturation predominantly involved in seed filling	↑
MLOC_59286.1	Jacalin-like lectin domain containing protein, expressed	Carbohydrate binding	↑
MLOC_56250.1	Glycosyl hydrolases family 16, putative, expressed	Hydrolysis of glucose polymers	↑
AK356853	Ribosome-binding factor A, chloroplast precursor, putative, expressed	Plastid function in thylakoid membranes	↑
AK359892	Membrane protein, putative, expressed	Membrane protein	↑
MLOC_4447.2	DnaK family protein, putative, expressed	Molecular chaperone	↑
AK355829	Plant-specific domain TIGR01627 family protein, expressed	Secondary cell wall production, xylan production	↑
MLOC_37864.1	Plastocyanin-like domain containing protein, putative, expressed	Electron transfer in electron transport chain	↑
AK356722	Glycosyl hydrolase family 29, putative, expressed	Metabolism of various carbohydrates	↑
MLOC_7763.2	Phosphoethanolamine/phosphocholine phosphatase, putative, expressed	Maintenance of cellular phosphate homeostasis	↑
MLOC_58520.1	Cytochrome P450, putative, expressed	Role in general metabolism	↑
MLOC_2049.1	Cytokinin-O-glucosyltransferase 1, putative, expressed	Cell division and plant development	↑

AK366167	Cytochrome P450, putative, expressed	Role in general metabolism	↑
MLOC_73743.2	Cytochrome P450, putative, expressed	Role in general metabolism	↑
AK366176	Dehydrogenase E1 component domain containing protein, expressed	Catalyzes the overall conversion of pyruvate to acetyl-CoA and CO_2	↑
AK372803	Acyl-desaturase, chloroplast precursor, putative, expressed	Catalyzes desaturation of stearic to oleic acid in the stroma of chloroplasts	↑
MLOC_64351.2	AAA-type ATPase family protein, putative, expressed	ATP binding	↑
MLOC_18785.1	Gibberellin 20 oxidase 2, putative, expressed	Key oxidase enzyme in the biosynthesis of gibberellin	↑
MLOC_64714.1	C2 domain containing protein, putative, expressed	Transferase activity	↑
MLOC_44618.1	Purple acid phosphatase precursor, putative, expressed	Hydrolysis of phosphatase esters	↑
AK364355	Dehydrogenase E1 component domain containing protein, expressed	Catalyses the overall conversion of pyruvate to acetyl-CoA and CO_2	↑
Gene expression control			
AK362038	B3 DNA binding domain containing protein, expressed	Transcription factor	↓
MLOC_64636.1	AP2 domain containing protein, expressed	Transcription regulation	↓
MLOC_25297.1	trp repressor/replication initiator, putative, expressed	Regulation of transcription	↓
MLOC_69530.1	AP2 domain containing protein, expressed	DNA binding and transcription factor activity	↓
MLOC_36338.1	PPR repeat domain containing protein, putative, expressed	Regulation of gene expression at the RNA level	↓
MLOC_59073.1	Zinc finger, C3HC4 type domain containing protein, expressed	Bind DNA, RNA, protein and/or lipid substrates	↓
MLOC_5568.1	MYB family transcription factor, putative, expressed	Transcription factor	↓

MLOC_15681.2	No apical meristem protein, putative, expressed	DNA binding and transcription factor activity	↑
AK373398	OsMADS16 - MADS-box family gene with MIKCc type-box, expressed	DNA binding and transcription factor activity involved in plant development	↑
MLOC_78895.1	MYB family transcription factor, putative, expressed	Transcription factor	↑
MLOC_16981.1	MYB-like DNA-binding domain containing protein, putative, expressed	DNA binding and transcription factor	↑
MLOC_70077.2	EF hand family protein, putative, expressed	Proteins involved in transcription and translation, protein- and nucleic-acid-binding proteins and a large number of unknown proteins	↑
MLOC_5666.3	Zinc finger C-x8-C-x5-C-x3-H type family protein	mRNA splicing and metal binding	↑
AK250810.1	BEE 1, putative, expressed	Transcription factor	↑
MLOC_74184.1	MYB family transcription factor, putative, expressed	Transcription factor	↑
Unknown function			↑
MLOC_17458.1	No hits found	Unknown function	↓
MLOC_7244.1	No hits found	Unknown function	↓
MLOC_43425.2	Expressed protein	Unknown Function	↓
AK372631	Expressed protein	Unknown function	↓
MLOC_58164.2	Expressed protein	Unknown function	↓
MLOC_25269.1	No hits found	Unknown function	↓
MLOC_279.1	Expressed protein	Unknown function	↓
MLOC_45654.1	Hypothetical protein	Unknown function	↓
AK360714	Expressed protein	Unknown function	↓
MLOC_42173.1	Hypothetical protein	Unknown function	↓
MLOC_9555.1	No hits found	Unknown function	↓
MLOC_30862.1	No hits found	Unknown function	↓
MLOC_26013.2	No hits found	Unknown function	↓
MLOC_31997.1	No hits found	Unknown function	↓
AK357333	Conserved hypothetical protein	Unknown function	↑
AK370260	Membrane associated DUF588 domain containing protein, putative, expressed	Unknown function	↑
MLOC_17880.1	Expressed protein	Unknown function	↑
TA37439_4513	Expressed Protein	Unknown function	↑
AK374255	Expressed protein	Unknown function	↑
MLOC_80571.3	Expressed protein	Unknown function	↑
MLOC_75289.1	Expressed protein	Unknown function	↑

AK372024	Hypothetical protein	Unknown function	↑
TA30814_4513	Expressed protein	Unknown function	↑
MLOC_60871.1	Expressed protein	Unknown function	↑
MLOC_65531.1	Expressed protein	Unknown function	↑
MLOC_7807.1	Expressed protein	Unknown function	↑
MLOC_34983.1	expressed protein	Unknown function	↑
Genome rearrangement			
MLOC_38459.1	Retrotransposon protein, putative, unclassified	Genome rearrangement	↓
MLOC_44903.1	Retrotransposon protein, putative, Ty3-gypsy subclass	Genome rearrangement	↓
MLOC_30295.1	Retrotransposon protein, putative, unclassified, expressed	Genome rearrangement	↓
MLOC_31569.1	Retrotransposon protein, putative, unclassified	Genome rearrangement	↑
MLOC_29900.1	Retrotransposon protein, putative, LINE subclass	Genome rearrangement	↑
MLOC_23089.1	Retrotransposon protein, putative, unclassified	Genome rearrangement	↑
AK376450	Transferase family protein, putative, expressed	Genome rearrangement	↑

Appendix 4

Table A1 *– Model parameters of the logistic growth curve fitting using a nls model of biomas s and nitrogen accumulation of Proctor (P), Annabell (A), Chanson (C) and Krona (K) cultivars grown in isolation (A, C, K, P), intra-cultivar competition (AA, CC, KK, PP) and inter-cultivar competition with Tammi (T), (TA-A, TC-C, TK-K, TP-P). 95% confidence intervals are shown in brackets.*

Treatment	**Peak nitrogen accumulation rate timing (Days after planting)**	**Maximum accumulated shoot nitrogen (% dry mass)**
A	27.5 (26.0,28.5)	6.1 (5.55, 6.63)
AA	25.5 (25.0,26.0)	5.18 (4.09, 6.18)
TA-A	24.5 (24.0,25.0)	4.05 (3.50,4.50)
C	27.5 (26.5,28.0)	5.79 (4.99, 6.62)
CC	25 (23.5,26.0)	3.43 (2.88, 4.02)
TC-C	24.5 (24.0,25.5)	4.87 (3.92, 5.80)
K	26.5 (26.0,26.5)	5.79 (5.27, 6.35)
KK	24.5 (24.0,25.5)	4.15 (3.58, 4.84)
TK-K	24.5 (24.0,25.0)	4.16 (3.90, 4.42)
P	28 (27.0,30.0)	5.75 (5.26, 6.23)
PP	25.5 (24.5, 26.0)	4.35 (3.59, 5.04)
TP-P	24.5 (24.0, 25.5)	4.16 (3.80, 4.56)
	Peak biomass accumulation rate timing (Days after planting)	**Maximum accumulated biomass (mg)**
A	53.5 (49.5, 61.5)	1699.88 (1439.32, 2213.05)
AA	54.0 (50.0, 60.0)	1313.12 (1124.68, 1671.83)
TA-A	49.0 (46.0, 52.0)	947.75 (815.04, 1111.85)
TA-T	50.5 (47.0, 56.0)	1619.83 (1385.08, 1868.48)
C	64.0 (54.0, 88.0)	5499.00 (2015.74, 15278.94)
CC	53.5 (48.0, 64.5)	1473.3 (1093.15, 2672.23)
TC-C	53.5 (48.5, 62.0)	1283.88 (1049.80, 1737.84)
TC-T	50.5 (47.0, 56.0)	1636.39 (1407.42, 1891.19)
K	51.0 (47.0, 57.0)	1643.7 (1442.85, 1887.95)
KK	55.5 (47.5, 71.5)	1478.36 (1001.49, 3097.41)
TK-K	52.5 (45.5, 60.5)	1302 (961.93, 1831.10)
TK-T	46.5 (45.0, 48.5)	1615.98 (1519.52, 1708.95)
P	59.0 (54.0, 64.0)	1849.27 (1521.10, 2331.46)
PP	58.5 (54.0, 64.5)	1329.25 (1154.10, 1693.44)
TP-P	48.0 (47.0, 49.5)	689. 76 (683.09, 698.88)

Table A2 *- Bootstrapped confidence interval differences of timing of peak accumulation rate and maximum accumulated nitrogen and biomass of Proctor (P), Annabell (A), Chanson (C) and Krona (K) barley cultivars grown in isolation (A, C, K, P), inter-cultivar competition (AA, CC, KK, PP) and inter-cultivar competition with Tammi (T) (TA-A, TC-C, TK-K, TP-P). Asterisks indicate significant differences.*

Treatment	CI differences in timing of peak accumulation rate	CI differences in maximum accumulation
Nitrogen		
A vs AA	0.09, 0.59*	-0.17, 2.14
A vs TA-A	0.29, 0.76*	1.31, 2.78*
C vs CC	0.16, 0.81*	1.36, 3.33*
C vs TC-C	0.29, 0.79	-0.31, 2.20
K vs KK	0.19, 0.62*	0.75, 2.43*
K vs TK-K	0.26, 0.60	1.03, 2.23*
P vs PP	0.24, 0.91*	0.52, 2.33*
P vs TP-P	0.41, 1.03*	0.95, 2.17*
Biomass		
A vs AA	-1.53, 1.76	430.70, 1293.10*
A vs TA-A	-0.19, 2.76	-38.50, 975.50
C vs CC	-0.81, 6.78	126.59, 14111.20*
C vs TC-C	-0.56, 6.75*	652.31, 14110.10*
K vs KK	-4.14, 1.08*	-1579.00, 733.32
K vs TK-K	-2.08, 1.50	-206.37, 793.83
P vs PP	-1.35, 1.63	834.66, 1060.98*
P vs TP-P	1.16, 3.26*	0.42, 1060.88*

Table A3 *– Details of the C:N ANOVA Tukey test results.*

Comparison	diff	lwr	upr	P adj
C-A	13.971	-16.785	44.7273	0.50371
K-A	7.86765	-22.889	38.624	0.84398
P-A	-2.9653	-33.722	27.7911	0.98902
K-C	-6.1033	-36.86	24.653	0.91765
P-C	-16.936	-47.693	13.8201	0.35513
P-K	-10.833	-41.589	19.9234	0.68394

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Opinion

Temporal Dynamism of Resource Capture: A Missing Factor in Ecology?

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Temporal dynamism of plant resource capture, and its impacts on plant–plant interactions, can have important regulatory roles in multispecies communities. For example, by modifying resource acquisition timing, plants might reduce competition and promote their coexistence. However, despite the potential wide ecological relevance of this topic, short-term (within growing season) temporal dynamism has been overlooked. This is partially a consequence of historic reliance on measures made at single points in time. We propose that with current technological advances this is a golden opportunity to study within growing season temporal dynamism of resource capture by plants in highly informative ways. We set out here an agenda for future developments in this research field, and explore how new technologies can deliver this agenda.

What is Temporal Dynamism and Why Is It Important?

Understanding plant community composition and functioning are fundamental challenges in ecology. It is not yet fully understood why specific communities exist at particular points in space and time, why some communities are more diverse than others, and how diversity impacts on ecosystem function. In plant communities, many theories have been proposed to explain plant coexistence, including cyclical disturbance [1,2], different individual responses to species interactions [3], multiple limiting resources [4,5], intraspecific trait variation [6], and facilitative plant–plant interactions, particularly in extreme environments [7,8].

We argue that short-term (i.e., within growing season) **temporal dynamism** (see Glossary) in resource acquisition might be central to addressing these fundamental challenges. Temporal dynamism can be described as a form of **heterochrony** that is controlled by intrinsic gene expression but also influenced by external environmental factors such as climatic conditions [9]. However, apart from a few cases, **within growing season temporal dynamism** in resource acquisition is rarely considered as a topic in its own right, in part because it has historically proven hard to measure. This contrasts, for example, with our knowledge of other temporally dynamic processes such as plant phenology, about which much more is known.

Phenological studies have shown the importance of the timing of key events in the structure and functioning of plant communities [10]. Therefore, similar important consequences for temporal dynamism in **resource capture** might reasonably be expected. For example, if different species **temporally segregate** the capture of common resources to avoid competition, increased complementarity can promote plant coexistence [11], with profound implications for fundamental processes such as biodiversity–ecosystem function relationships. Importantly, we propose that, owing to the wealth of new analytical approaches that are currently available, now is the time to address the historical oversight of within growing season temporal dynamism.

Highlights

Temporal dynamism has previously been studied in a range of specific habitats and generally over long timescales, but short-term within growing season temporal dynamics of resource capture and plant–plant interactions have so far been over looked.

Temporal dynamics have been overlooked due to reliance on traditional proxy methods to study plant–plant interactions such as biomass, and to measuring at only a single timepoint.

However, a suite of new non-destructive techniques are now available, including stable isotope-labelling systems, soil zymography, DNA and RNA technology, and X-ray computed tomography scanning of root growth to study the temporal dynamics of resource capture. These will allow us to identify and then understand the role of temporal dynamism in the structure and function of multispecies plant communities.

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Before considering these new opportunities, we examine previous studies of temporal dynamism, with a focus on resource capture. We discuss the limitations of, and lessons learned from, previous studies and how they can form the basis of a future research agenda. We then focus on new experimental approaches, considering how these can address current knowledge gaps, and discuss the wider relevance of this subject area to ecology.

Past Studies of Temporal Dynamism in Plant Communities

Previous research provides clear examples of how temporal dynamism of ecological processes can regulate the structure and functioning of plant communities. Arguably, one of the best-studied examples is plant–pollinator interaction dynamics. Pollinators vary the plant species visited interannually, which promotes coexistence in species-rich communities [12,13]. Other examples involve temporally dynamic resource capture; in arid environments, temporal dynamism has been found in the growth response of plants to erratic inputs of water [14], depending on both the timing of the water input in the growing season and the time since the previous water input [15]. In alpine systems, nutrient turnover is temporally dynamic, with mineralisation occurring throughout winter [16], and spring microbial turnover then providing nutrients to plants [17].

Such temporal dynamics are not only of academic interest – they can play a central role in regulating the impacts of key environmental change drivers. For example, one way non-native species can become invasive is by occupying a vacant niche [18]. Occupying a temporal niche left vacant by the native plant community could allow the invasive species to capture nutrients at a time of reduced competition. It may appear that in some cases invasive species take over a niche from native species. However, it is unclear whether invasive species establishment depends on the exploitation of a temporal niche gap. Although phenological differences between native and invasive species have been shown [16], the underlying role of within growing season temporal dynamism in nutrient capture has yet to be demonstrated (probably for the reasons we discuss below). A similar example is the phenology of hemiparasitic plants. The life cycle of many hemiparasites is shortened relative to its hosts, influencing nitrogen cycling with earlier leaf fall than the host community [19,20]. Early leaf fall provides an input of nitrogen to the host community when it becomes limited [21]. Here the rate of water and nitrogen uptake by *R. minor* parasitizing *Hordeum vulgare* (barley) has received attention [22], but the temporal dynamics of this interaction have yet to be explored.

These examples, only a selection from the many that could be listed, demonstrate the likely importance of temporal dynamism of resource capture by plants. Far fewer studies have sought to measure this process directly. An important example is the work by Trinder *et al*. which used a series of destructive harvests to examine the temporal dynamics of nitrogen capture and biomass accumulation of *Dactylis glomerata* (cock's foot) and *Plantago lanceolata* (ribwort plantain). Trinder *et al*. found that, in response to interspecific competition, both species shifted the timing of the maximum rate of biomass accumulation and nitrogen capture by up to 17 days [23]. The species diverged the timing of these resource capture processes in ways that possibly reduce direct competition. However, it is notable that this type of study, looking explicitly at the temporal dynamism of resource capture, is to the best of our knowledge extremely rare.

Why Does It Matter that Temporal Dynamism Has Been Overlooked?

Many of the fundamental processes and properties of terrestrial communities are governed by the outcome of plant–plant interactions [24]. However, despite a huge amount of work on plant–plant interactions, especially competition, there are still unanswered questions about the role of plant–plant interactions in governing plant community composition.

Glossary

Heterochrony: a change in the timing and rate of a developmental process within an organism compared to an ancestral species, including the onset and duration of flowering, leaf production, and internodal length [9].

Resource capture: the acquisition of resources, including nutrient, water, and light, by a plant. This is commonly expressed as a rate, namely units of resource capture over a period of time.

Soil zymography: non-destructive method to measure chitinase, cellulase, or nitrogen mineralisation hotspots at a fine spatial resolution in the soil. Useful for studying changes in the location and intensity of enzymatic activity over time.

Temporal dynamism: variation through time in the rate or effect of a particular process. For example, this could be variation in the per unit biomass capture by a plant of soil nutrients or water, or the extent to which neighbouring plants compete with each other (which might itself result from temporal dynamism in resource capture by individuals). Such temporal dynamism can be driven by external factors (changes through time in climate or resource availability) or intrinsic factors (e.g., plant developmental stage).

Temporal segregation: a shift in the timing of a process in response to a neighbouring individual. Commonly observed in animal feeding, it limits niche overlap and promotes coexistence. Some niche overlap is still to be expected, but direct resource competition is reduced.

Within growing season temporal dynamism: variation through time, but within a given growing season, in the rate or effect of a particular process. Such variation is distinct from interannual variation, which might be caused by factors such as variation in climate between growing seasons.

Box 1. Theory of Temporal Dynamism of Nutrient Capture

Plants do not uniformly take up nutrients throughout the growing season. Instead, nutrient capture is regulated based on the nutrient requirements and growth stage of the plant [56]. When plants are grown in isolation, nutrients are taken up at the optimum time (Figure I; panels A and B show two individuals grown in isolation). However, when plants are grown together the timing of nutrient capture might change, perhaps to minimise competition (panel C shows the two individuals grown together). This can then promote the coexistence of competing individuals [11], and might be an important factor in communities such as tropical rainforests and grasslands, with multiple species timing key processes differently to minimise competition (panel D shows a hypothetical multispecies community, with each line representing a different species).

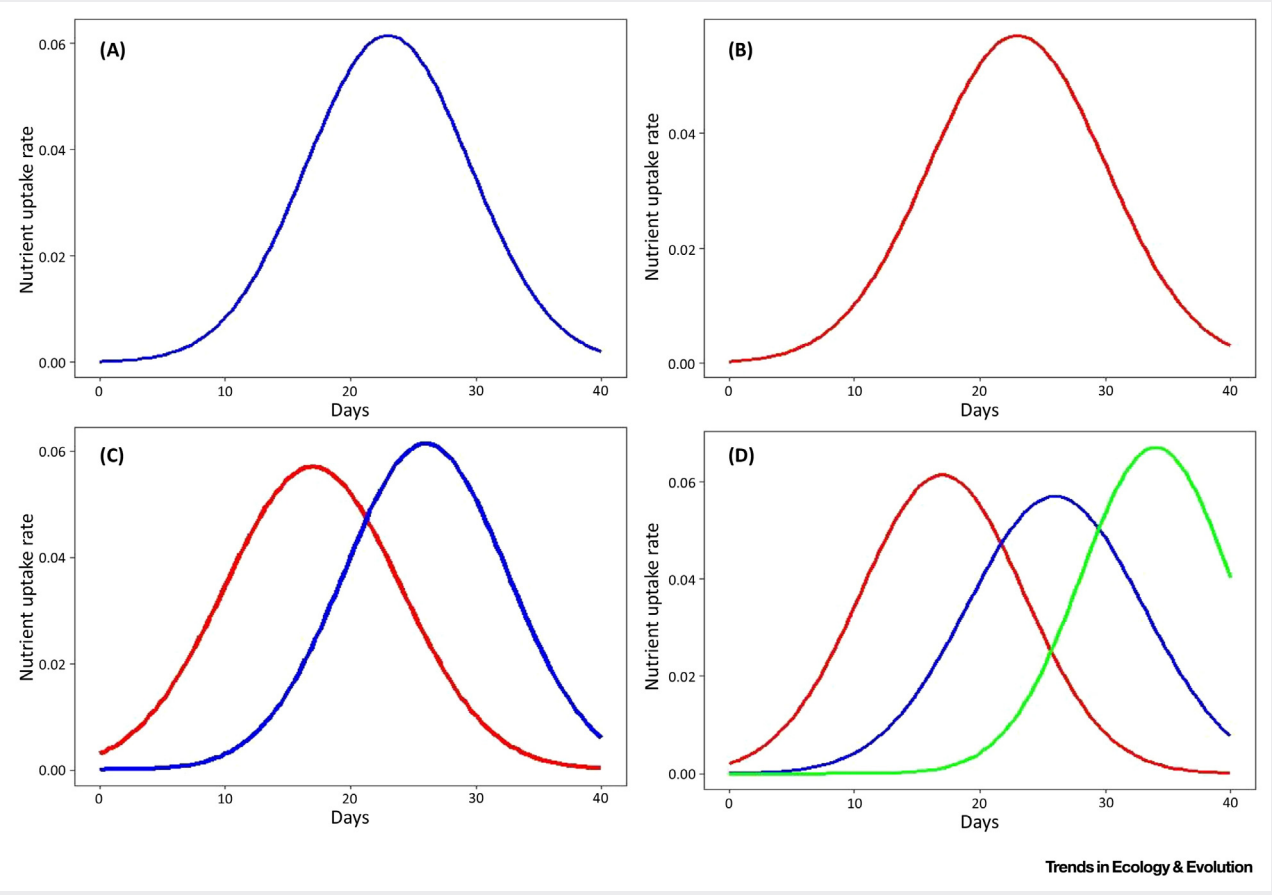

Figure I. Theoretical Role of Temporal Dynamism in Plant Coexistence. In isolation (A,B) plants take up nutrients in a specific profile over the growing season. By contrast, when grown together (C) the two plants offset the period of maximum nutrient capture to limit competition. In a multispecies community (D) this could lead to species occupying distinct temporal niches, leading to coexistence.

For example, our current understanding of the niches available within plant communities, which strongly regulate plant–plant interactions, cannot explain the level of observed coexistence [25]. A better understanding of short-term temporal dynamism in resource capture, and its consequences for plant–plant interactions, might help to explain this apparent paradox. Temporally dynamic resource-capture processes, and the temporal niche segregation which this could enable, could alter crucial plant–plant interactions so as to have a stabilising effect on communities. This would allow a higher diversity than would otherwise be the case to be supported [26], at potentially both a species [27] and genotypic level [28], with the community using a greater proportion of the available resources [29]. In this example, temporal dynamism in resource capture can be considered as an unmeasured trait (Box 1).

Why Has Temporal Dynamism in Resource Capture Been Overlooked?

Given the general importance of the temporal dynamism of ecological processes, and the likelihood that in many cases this is related also to temporally dynamic resource capture within a growing season, why have so few studies explicitly addressed this latter topic?

Plant ecology has traditionally relied on one final biomass measurement to assess the consequences of plant–plant interactions. Biomass is a relatively cheap and easy measure of plant response, making large-scale greenhouse and field studies possible [26]. However, there are some drawbacks to using single timepoint measurements of biomass to assess plant–plant interactions, and especially the short-term temporal dynamism of these processes. First, owing to the influence of other external environmental factors, the accumulation of biomass is rarely influenced by competition alone [23]. This makes it an unreliable direct measure of the outcome of competition. The use of only single harvesting to assess the outcome of plant–plant interactions is clearly inappropriate for measuring short-term temporal dynamism in resource capture. In addition, the precise timing of biomass harvest and measurement within a growing season can influence the perceived outcome of the plant–plant interaction because plants grow and develop at different times throughout the year [26]. The same criticisms can also be made of other common annual, single timepoint measurements, for example, flower production and seed set. To understand the role of temporal dynamism of resource capture in regulating community dynamics, repeated measures of resource capture are required. However, to take this step we need first to realise and accept the limitations of single timepoint studies, and move to more detailed studies of the competitive process itself.

Traditional approaches, for example plant biomass and tissue nutrient-content analysis, can be used to explore issues of temporal dynamism in plant–plant interactions. However, they need to be coupled to multiple harvesting points through time, as used by Trinder *et al*. to examine the temporal dynamics of resource capture in *Plantago lanceolata* and *Dactylis glomerata* [23]. Although the multiple-harvest approach is a valuable tool, it is destructive and requires large-scale and labour-intensive studies. The inclusion in a study of multiple harvests to track temporal dynamism of resource capture and plant–plant interactions through time increases the size and complexity of an experiment, and therefore reduces the complexity of the questions that can be asked [11,29]. In addition, multiple harvesting means that responses are averaged over many plants, potentially masking subtle dynamic individual-level responses in resource capture and growth. Non-destructive methods would instead allow the responses of an individual plant to be studied over time.

Such drivers of the historical oversight support a case for the use of innovative new technologies, particularly non-destructive and direct measures of resource capture, such that temporal dynamism of resource capture can be given the attention it deserves.

Setting and Addressing a New Research Agenda

From the above discussions, and consideration of well-known ecological concepts, a series of questions can be presented (see Outstanding Questions) in a clear research agenda. If addressed, this agenda could advance the study of temporal dynamism of resource capture. Importantly, this research agenda is not only of relevance to plant ecophysiologists or community ecologists. By influencing, for example, the temporal availability of resources to other groups such as soil organisms, pollinators, and herbivores, the study of temporal dynamism in plant resource capture will likely have wide-reaching consequences for ecological research.

As discussed, although temporal dynamism in resource capture can itself be detected using destructive harvesting techniques [23], new technological approaches will be necessary to look at the complex series of processes involved in the dynamics of plant nutrient capture and its role in community composition. Below, we provide examples of how these advances might enable some of the key questions of the research agenda to be addressed.

What Is the Interaction Between Temporal Dynamism of Resource Capture with Plant Physiology and Morphology?

The plasticity of plant root traits may facilitate the temporal dynamics of resource capture, while at the same time root physiology and morphology could be influenced by changes in the temporal dynamics of nutrient uptake. Therefore, the relationship between temporal dynamism of resource capture and root traits is a key topic because roots are the organs of nutrient uptake.

Microrhizotrons – small cameras inserted into the soil to record root foraging and fine root developing [30,31] – allow the study of root foraging activity. However, they are limited in not giving a view of the whole root system. Whole root system growth dynamics can be studied with automated root phenotyping facilities, using high-definition cameras to photograph root development of plants grown in Perspex boxes [32]. Changes in root morphology and foraging can then be related to the location of soil microbial activity (**soil zymography**, see below) and plant nutrient capture.

For a 3D view of root growth dynamics, X-ray computed tomography (CT) scanning can be used to visualise plant roots grown in soil. Root architectural development can then be related to resource capture. The development of specialist root-tracking software and facilities [33] will allow much larger and more complex experiments to be carried out on dynamic competition for soil resources between the roots of multiple individuals. This approach has already been used to study root growth in response to competition between *Populus tremuloides* (quaking aspen) and *Picea mariana* (black spruce) seedlings. Both species increased rooting depth and altered root architecture in response to a competitor [34], but this study did not simultaneously assess soil resource capture. By combining successive scanning of root growth and successive destructive harvesting to look at the temporal dynamics of nutrient uptake, the relationship between root growth and nutrient uptake can begin to be addressed.

Is Temporal Dynamism in Nutrient Capture Moderated in Response to Neighbours Simply by Overlapping Depletion Zones or by More Complex Signalling Pathways?

Traditionally plant competitive responses to a neighbour have been thought to occur when the zones of nutrient depletion in the soil overlap [35]. As the complexities of plant–plant communication are revealed [36], it is becoming clear that plant–plant competitive interactions might not occur solely based on nutrient availability. RNA sequencing, which enables us to examine the genes upregulated in specific circumstances in tissue samples, is one way to look at dynamic plant responses to the presence of a neighbour.

Studies in *Arabidopsis thaliana* have identified that common stress-response pathways such as jasmonate production are activated in response to a competitor [37]. Detection of the upregulation of stress-associated genes can indicate when a target plant detects the presence of a neighbour, whether the response differs depending on the identity of the neighbour, and the length of time between neighbour detection and any form of additional physiological response by the target plant (e.g., priming of soil microbes; see below).

A key question is whether upregulation of gene expression occurs before the nutrient-depletion zones of neighbouring plants overlap. Such an effect would indicate that responses to neighbouring plants are more complex than simply a response to the overlap of soil depletion zones as a consequence of developing root systems. The question of whether plants start responding to neighbours and to the threat of potential competition long before they come into close physical contact can then be addressed. This approach, therefore, provides a unique opportunity to understand temporal dynamism and competition at a molecular level, and to determine how temporal dynamism of resource capture is moderated in response to competition through a cascade of molecular responses in the target plant.

How Does the Activity of the Soil Microbial Community Influence Temporal Dynamism in Resource Capture?

Throughout the year, soil microbial communities mineralise and immobilise nutrients from soil organic matter (SOM), driving nutrient cycles that mobilise organic nutrient stocks into plant-available forms during the growing season [38,39]. Plants can influence these processes through the rhizodeposition of labile carbon and amino acids to influence microbial process rates (rhizosphere priming effects, RPE [40,41]), with rhizodeposition varying with plant development, species, and genotype [42–44].

One method to examine the influence of plants on the dynamics of SOM mineralisation is to study the timing of rhizosphere priming effects for plants in competition versus isolated plants. Stable-isotope labelling ($^{15}N/^{13}C$) can allow plant impacts on soil nutrient cycles to be quantified [45]. This can be done non-destructively and dynamically through isotopic partitioning of soil CO_2 efflux into plant and SOM-derived components [46], or tracing ^{15}N fluxes (derived from labelled organic matter) in soil solution [47–49]. This approach allows the timing and magnitude of soil community priming to be measured over time, and compared relative to other temporally dynamic measurements including RNA expression (see above) and resource capture (Figure 1).

Further information about specific soil microbial activities can then be provided through soil zymography, allowing the location and intensity of enzyme activity in soil to be quantified over time [50]. This methodology has already been used to identify 'hot moments' when microbial activity is higher than background levels [51]. Such 'moments' can be occasional or occur periodically with events such as spring growth and autumn leaf fall [52]. Using these techniques, it can be assessed, for example, whether periods of greater microbial activity precede plant nutrient capture or whether they are themselves dependent on priming activities by the plant.

How Are the Temporal Dynamics of Soil Microbial Community Composition Influenced by Plant Temporal Dynamics?

A crucial factor regulating the functional capacity of soil communities to mediate nutrient cycling is their composition. The soil community is known to be temporally dynamic seasonally and with plant developmental stage [24]. Shi *et al*. used a 16S ribosomal RNA approach to produce a network representation of microbial diversity over two growing seasons, comparing bulk and rhizosphere soil (Figure 2) [53]. The decreasing cost, increasing throughput capacity, and analysis speed of genomics creates an opportunity to study temporal dynamism in the soil community over the growing season [54]. When compositional studies are combined with studies of soil microbial activity (e.g., using metatranscriptomics), it can be assessed how changes in the dynamism of plant resource capture are associated with either short-term (i.e., more activity-based) or long-term (i.e., more community-composition based) changes in the soil community.

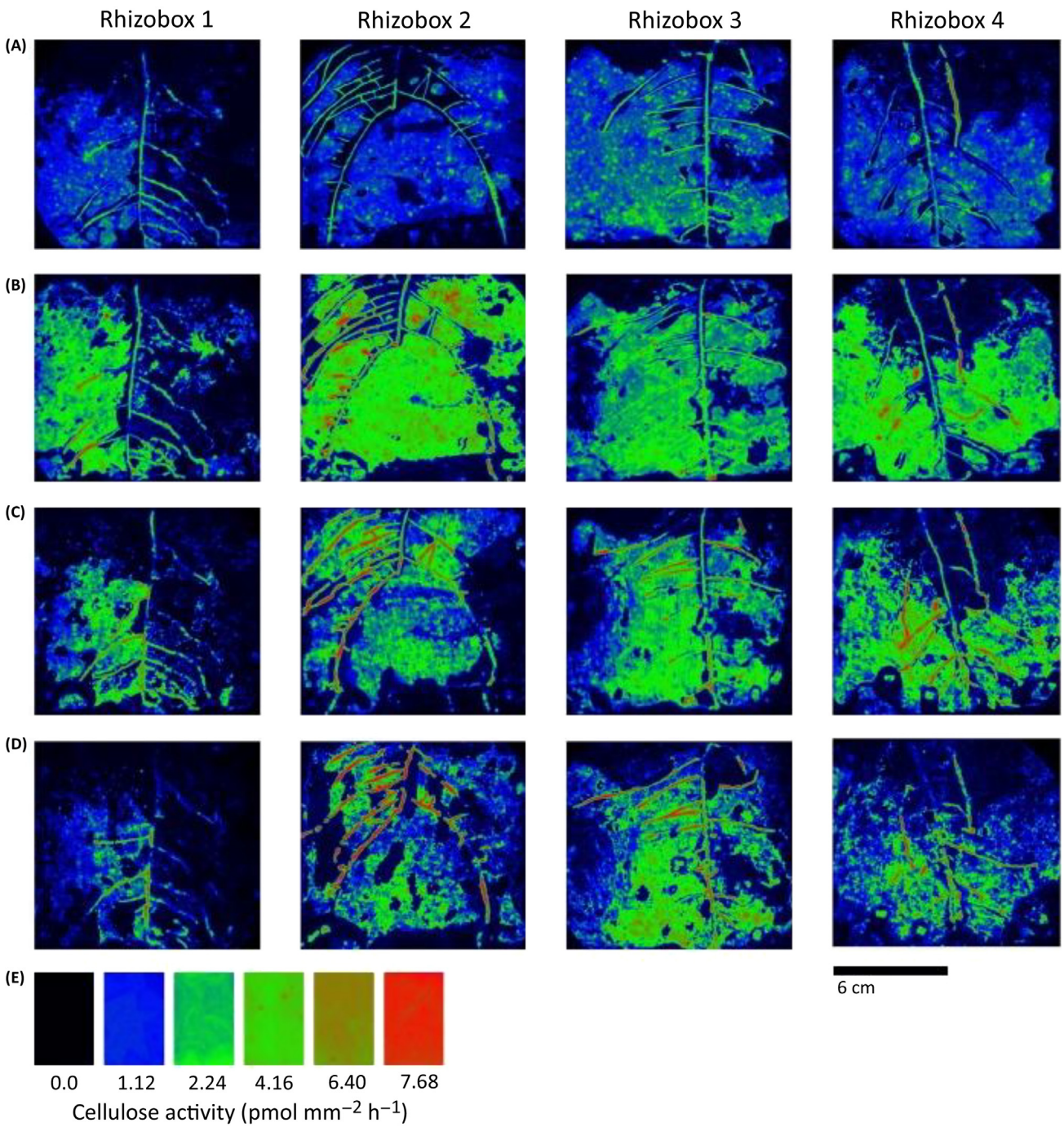

Figure 1. The Potential Role of Soil Zymography in Studying Temporal Dynamism in Soil Community Activity. The potential role that soil zymography analysis can play in studying the temporal dynamics of soil functions. The cellulase activity surrounding roots of *Lupinus polyphyllus* (large-leaved lupin) was analysed 18 days after sowing (A), and 10 days (B), 20 days (C), and 30 days (D) after cutting shoots. Adapted, with permission, from [50].

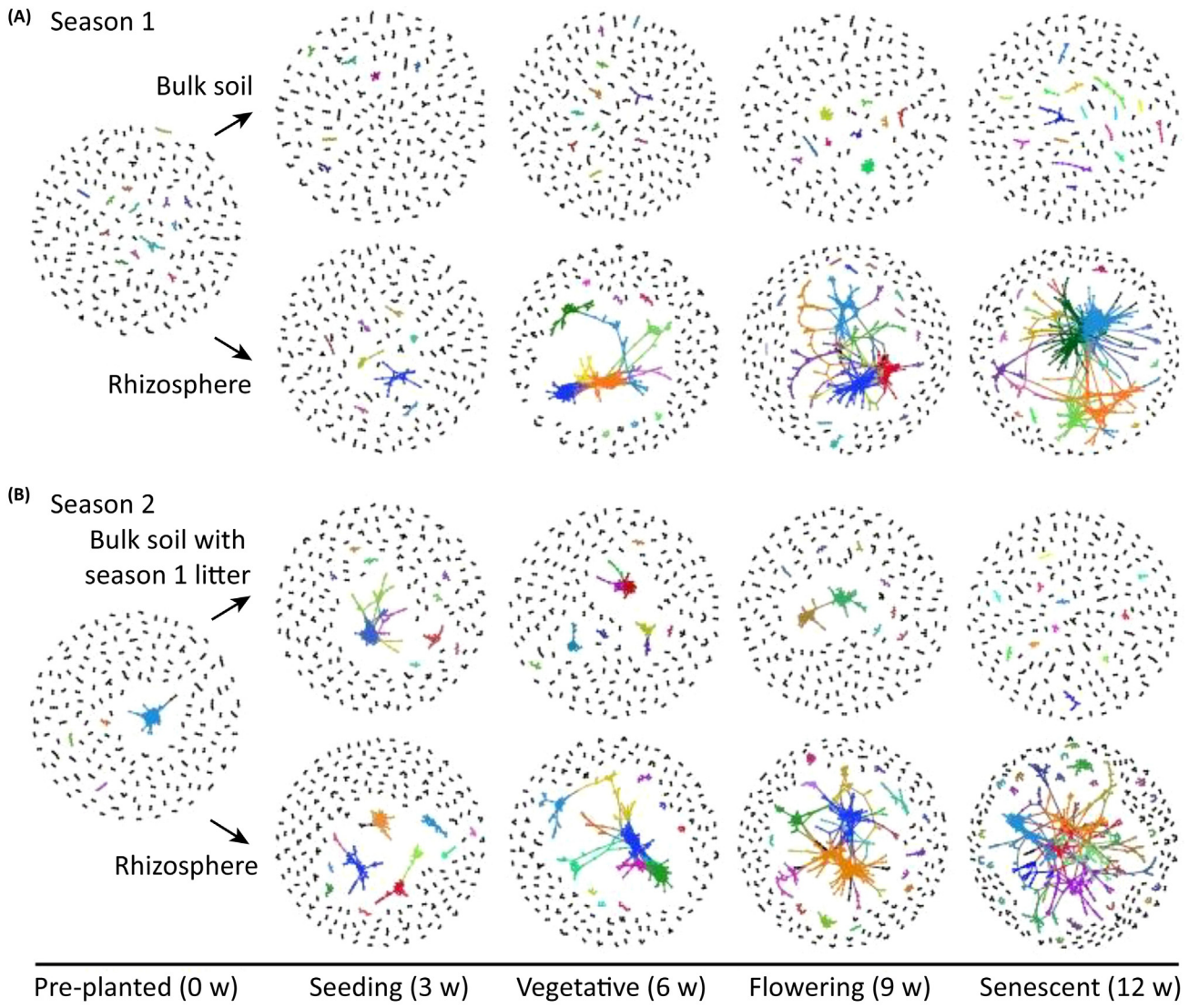

Figure 2. Temporal Dynamics of the Plant-Associated Soil Community. The figure shows the potential role of soil community characterisation and network analysis in studying the temporal dynamics of the soil community associated with resource capture. (A,B) Differences in the rhizosphere and bulk soil community of *Avena fatua* were compared over two growing seasons. Samples were taken every 3 weeks (w) for two seasons. Shi *et al*. looked at the difference in the diversity and level of interconnection between bulk and rhizosphere soil. Nodes represent operational taxonomic units (OTUs), and lines represent the linkages between them. The rhizosphere soil becomes more interconnected but less diverse over time because the plant exerted a selection pressure on the soil community. Adapted, with permission, from [53].

What Is the Future Strategy To Study Temporal Dynamism?

Temporal dynamism is an overlooked factor in ecology and could be a vital central mechanism by which plants coexist in complex communities. Although studying temporal dynamism of resource capture will not be straightforward, the potential benefit to the understanding of ecosystem functioning is likely to be considerable. There is now an ideal opportunity to understand the within growing season temporal dynamics of resource capture as part of broader ecological system dynamics.

To understand the role of temporal dynamism of resource capture in plant coexistence it needs to be understood how plants coordinate temporally dynamic responses, the intermediary role of the soil microbial community, and the consequences at the individual plant and plant community level. Therefore, to study these distinct but interconnected processes, an integrated approach is required [55]. From the examples we have discussed above it is clear that a vast amount of knowledge can be gained about temporal dynamism in resource capture from using these cutting-edge technologies. Once the fundamental questions about temporal dynamism of resource capture have been addressed, the wider community-level consequences can then be considered, building upon these fundamental studies.

The ultimate goal of this research should be to integrate temporal dynamism as a factor into existing models, to define new niche space, and aid the explanation of coexistence in complex communities. Only then can the question of whether temporal dynamism in resource capture leads to coexistence of neighbouring plants can begin to be addressed. This approach can then be applied to other temporally dynamic processes, answering other fundamental questions about ecosystem functioning.

Outstanding Questions

A Research Agenda for Temporal Dynamism in Plant Resource Capture

The following are key research questions which set out a clear research agenda for linking the issue of temporal dynamism in resource capture to central aspects of plant ecophysiology, plant community ecology, and community ecology more widely. We have ordered them such that they run from studies which might be conducted on individual plants to studies with increasing complexity in terms of biotic interactions – initially interactions with other plants, then with soil organisms, then with other elements of the wider community (for example pollinators and herbivores).

(i) What is the interaction of temporal dynamism of resource capture with plant physiology and morphology?

(ii) Is temporal dynamism in phenology matched by patterns of temporal dynamism in nutrient uptake?

(iii) Is temporal dynamism in nutrient uptake moderated in response to neighbours simply by overlapping depletion zones or by more complex signalling pathways?

(iv) How do interactions with soil organisms influence temporal dynamism in resource capture?

(v) Is temporal dynamism in resource capture widespread, or is it associated with particular plant strategy types?

(vi) Does temporal dynamism in resource capture lead to a reduction in competition, and contribute to plant coexistence and the development of multispecies plant communities?

(vii) What are the wider consequences of temporal dynamism for community structure and function at other trophic levels?

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ORIGINAL RESEARCH
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Cultivar Differences and Impact of Plant-Plant Competition on Temporal Patterns of Nitrogen and Biomass Accumulation

Emily Jane Schofield[1,2], Jennifer K. Rowntree[2], Eric Paterson[1], Mark J. Brewer[3], Elizabeth A. C. Price[2], Francis Q. Brearley[2] and Rob W. Brooker[1]*

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Current niche models cannot explain multi-species plant coexistence in complex ecosystems. One overlooked explanatory factor is within-growing season temporal dynamism of resource capture by plants. However, the timing and rate of resource capture are themselves likely to be mediated by plant-plant competition. This study used Barley (*Hordeum* sp.) as a model species to examine the impacts of intra-specific competition, specifically inter- and intra-cultivar competition on the temporal dynamics of resource capture. Nitrogen and biomass accumulation of an early and late cultivar grown in isolation, inter- or intra- cultivar competition were investigated using sequential harvests. We did not find changes in the temporal dynamics of biomass accumulation in response to competition. However, peak nitrogen accumulation rate was significantly delayed for the late cultivar by 14.5 days and advanced in the early cultivar by 0.5 days when in intra-cultivar competition; there were no significant changes when in inter-cultivar competition. This may suggest a form of kin recognition as the target plants appeared to identify their neighbors and only responded temporally to intra-cultivar competition. The Relative Intensity Index found competition occurred in both the intra- and inter- cultivar mixtures, but a positive Land Equivalence Ratio value indicated complementarity in the inter-cultivar mixtures compared to intra-cultivar mixtures. The reason for this is unclear but may be due to the timing of the final harvest and may not be representative of the relationship between the competing plants. This study demonstrates neighbor-identity-specific changes in temporal dynamism in nutrient uptake. This contributes to our fundamental understanding of plant nutrient dynamics and plant-plant competition whilst having relevance to sustainable agriculture. Improved understanding of within-growing season temporal dynamism would also improve our understanding of coexistence in complex plant communities.

Keywords: *Hordeum* sp. (Barley), nitrogen, nutrient uptake, peak accumulation rate, plant-plant competition, plant community coexistence, temporal dynamism

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INTRODUCTION

Niche differentiation is suggested to lead to coexistence of plants by reducing competition, either for a specific form of a resource or simultaneous demand for the same resource (Silvertown, 2004). However, in complex plant communities such as rain forests and grasslands there are seemingly insufficient niches to explain coexistence of the many species

present. Plants seem to occupy the same niche dimensions but without it leading to competitive exclusion (Clark, 2010).

One factor which is often not included in niche models is time, more specifically the temporal dynamism of key developmental and physiological processes such as resource capture (Schofield et al., 2018). Competition can be influenced by temporally dynamic physiological processes (Poorter et al., 2013), such as flowering (Kipling and Warren, 2014) and nutrient uptake (Jaeger et al., 1999). Differences in the temporal dynamics of nutrient capture could reduce temporal niche overlap, reducing competition for resources. This could result in increased complementarity and promote coexistence (Ashton et al., 2010).

As well as temporal dynamism influencing competition, competition can influence the temporal dynamics of resource capture, although the extent to which these processes affect each other is unclear. As there are many aspects of temporal dynamism in plant communities that are not fully understood, temporal dynamism in resource capture may be currently unsuitable as an indicator of plant-plant competition. However, a change in the temporal dynamics of resource capture may be a wider consequence of competition or a mechanism by which plants avoid direct competition for resources. Trinder et al. (2012) found a change in the temporal dynamics of nitrogen and biomass accumulation in response to inter-specific plant-plant competition. But the impact of competition on temporal dynamism in resource capture, and how this could influence coexistence in plant communities, remains largely unexplored (Schofield et al., 2018).

There is in particular a lack of information on the relationship between temporal dynamism and intra-specific competition, and how the degree of relatedness of competitors might influence temporal dynamism. The genetic distance between competing individuals can influence the functional plasticity of an individual response to competition (Murphy et al., 2017), including biomass allocation and root morphology (Semchenko et al., 2017). Differential competitive responses have been demonstrated between closely related individuals (Murphy et al., 2017), including in a number of crop species (Dudley and File, 2007). The use of two cultivars in this study allows a tight control of the relatedness of individuals, which in turn allows us to address how diversity regulates interactions and ultimately functions in a range of systems [not least for the development of sustainable agricultural practice (Schöb et al., 2018)]. In this sense, crop species are ideal model systems for undertaking such studies.

Here, we conducted a pot experiment with Barley (*Hordeum vulgare*) as a model species, using an early and a late cultivar. Barley is a suitable model in this case as its nutrient uptake has been studied in detail to optimize the timing of fertilizer application in agriculture (Nielsen and Jensen, 1986), allowing us to address fundamental ecological questions of plant coexistence, as well as investigating a topic of relevance for agricultural practices.

It is expected that early and late cultivars of barley will have different temporal dynamics of nitrogen uptake and biomass accumulation, in a similar way to two species or genotypes in a natural system. The two cultivars in this study have been bred for different uses and therefore will have differing combinations of traits. Tammi has been bred for an early lifecycle (Nitcher et al., 2013), whereas Proctor was bred for malting quality (Hornsey, 2003). The nitrogen uptake and biomass accumulation dynamics are predicted to be altered by plant-plant competition, and this will be more pronounced in intra-cultivar compared to inter-cultivar competition as the individuals will more completely occupy the same niche space.

This study aimed to understand: (1) whether early and late cultivars of barley exhibit temporal dynamics in nitrogen uptake and biomass, (2) how plant-plant competition changes the temporal dynamics of nitrogen and biomass accumulation in early and late barley cultivars, (3) how any temporally dynamic response differs with inter- and intra- cultivar competition, and ultimately (4) how this impacts on niche complementarity.

MATERIALS AND METHODS

Temporal Patterns of Nitrogen and Biomass Accumulation

A pot-based competition study was used to investigate temporal dynamism in nitrogen uptake, using barley (*Hordeum* sp.) as a model species. An early (Tammi: T) and late (Proctor: P) cultivar of barley (sourced from The James Hutton Institute, Dundee, United Kingdom) were chosen as they have similar height and limited tillering, enabling the study to focus on phenological rather than physiological differences. Each cultivar was grown in pots either in isolation, or with another individual of either the same or other cultivar (i.e., T, P, TT, PP, and TP).

Soil Characteristics

Soil was sourced from an agricultural field (Balruddery Farm, Invergowrie, United Kingdom) that had previously contained spring barley (*Hordeum* sp.) and had been subject to standard management for barley production (including fertilizer addition at a rate of 500 kg of 22N-4P-14 K ha^{-1} $year^{-1}$). The soil had an organic matter content of 6.2 ± 0.3% SEM (n = 4), a mean pH (in water) of 5.5 ± 0.02 SEM (n = 4), a total organic nitrogen concentration of 0.078 ± 0.024 mg l^{-1}, mean NH_4 concentration of 0.008 ± 0.006 mg l^{-1} and mean NO_3 concentration of 0.078 ± 0.024 mg l^{-1} (n = 4) and microbial biomass of 0.06 ± 0.002 SEM mg g^{-1} (n = 4) [analyzed by Konelab Aqua 20 Discrete Analyser (Thermo Fisher Scientific, Waltham, MA, United States)]. Before use, the soil was passed through a 6 mm sieve. No fertilization of the soil occurred during the experiment.

Setup and Growing Conditions

Seeds of both cultivars were pre-germinated in the dark on damp paper towels and planted into cylindrical 2 L pots (diameter 152 mm, height 135 mm) with five replicate pots of each of the five treatments for each planned harvest (11 harvests in total), giving a total of 275 pots. The pots were randomized to account for potential positional effects and grown in controlled

environment rooms (Conviron, Isleham, United Kingdom) at a constant 15°C with an 8/16 (day/night) hour photoperiod (irradiance of 100–150 μmol m^{-2} s^{-1}) and 65% relative humidity, to mimic local spring-time conditions. The pots were watered twice weekly and the soil was kept moist to avoid competition for water. Mesh screens [45 cm × 16 cm, mesh size 0.08 mm (Harrod Horticulture, Lowestoft, United Kingdom)] were inserted in those pots containing two plants to separate the plants above ground, and ensure competitive interactions only occurred below ground. Foliage was relatively upright without support and the presence of a screen – although important in ensuring above-ground competition was minimized – was unlikely to have resulted in differences in shoot development in pots with two plants compared to one.

Sequential Harvesting

Five randomly selected pots of each treatment were harvested every 5 days until ear formation (when grain begins to form) was observed on the early Tammi cultivar (60 days). During this period both cultivars produced flag leaves, the stage prior to grain production, when most nitrogen has already been absorbed (Spink et al., 2015). This covered the period most likely to contain the peak nitrogen and biomass accumulation rate for both cultivars, the focus of this study. The plants were then removed from the pots, the roots washed, and individual shoot and root material separated. The root and shoot material of each plant were dried at 30°C until a stable weight was reached and weighed. Milled shoot samples were analyzed for carbon and nitrogen concentration (Flash EA 1112 Series, Thermo Fisher Scientific, Bremen, Germany).

Data Analysis

Temporal Patterns of Nitrogen and Biomass Accumulation

To analyze temporal changes in biomass and nitrogen accumulation, the rate of each was modeled with logistic growth curves using non-linear least squares (nls) models (R Core Team, 2015). A cumulative time series data set of biomass accumulation was bootstrapped using resampling with replacement 1000 times to estimate variability and confidence intervals. A logistic growth curve was used as the nls model and this was fitted to each of the bootstrapped data sets to produce a set of logistic instantaneous uptake rate curves for each treatment, as well as sets of modeled maximum accumulation values. This was then repeated for the nitrogen accumulation data set. A non-linear model was used as the growth dynamics of plants with determinate growth such as barley (Yin et al., 2003) are mostly sigmoidal, making a linear growth model unsuitable (Robinson et al., 2010). Therefore, the use of the non-linear least squares model with bootstrapping is a robust method to examine the temporal dynamism of resource capture of annual species and to properly account for uncertainty. Significant differences between the timing of peak accumulation and final maximum accumulation between treatments were determined from the difference in bootstrapped 95% confidence intervals of the model outputs (**Supplementary R Code 1**).

Shoot C:N

C:N ratio at the final harvest (65 days after planting) was analyzed using an ANOVA test from the MASS package *in R* (R Statistical Software, R Core Team, 2015) as the residuals were normally distributed, with treatment as the fixed factor and C:N as the response variable (**Supplementary R Code 2**). A Tukey *post hoc* test was carried out to compare the individual treatment groups.

Neighbor Effects

The effect of a neighboring plant on a target plant's biomass was quantified using the Relative Intensity Index (RII; Eq. 1), an index that accounts for both competitive and facilitative interactions between neighboring plants (Díaz-Sierra et al., 2017). RII was calculated using the final harvest biomass data. For each cultivar, RII was calculated separately for plants grown in intra- and inter-specific competition. The mean total biomass of each cultivar grown in isolation was used for the *Isolation* value, and the individual RII value was then calculated for each plant of that cultivar experiencing competition.

$$RII = \frac{(Competition - Isolation)}{(Competition + Isolation)} \quad (1)$$

Competition = Biomass of plant when in competition, *Isolation* = Mean biomass of plant in isolation.

The land equivalent ratio (LER; Eq. 2) was used to determine if the inter-cultivar mixture (TP) overyielded when compared to intra-cultivar competition (TT or PP) (Mead and Willey, 1980). The mean LER value was calculated by randomly pairing inter- and intra- cultivar competition treatments using a random number generator. A LER value was calculated for each pairing, from which a mean and SEM was calculated. A mean LER value above 1 indicates that inter-cultivar pairings produced more biomass than to intra-cultivar combinations. As the residuals were normally distributed, the LER and RII values were compared between competition treatments using an ANOVA test as above, with treatment as the fixed factor and either LER or RII as the response variable (**Supplementary R Code 2**).

$$LER = \frac{Tammi\ mixture\ biomass}{Tammi\ own\ cultivar\ \ biomass} + \frac{Proctor\ mixture\ biomass}{Proctor\ own\ cultivar\ biomass} \quad (2)$$

Tammi mixture biomass = Tammi biomass when in competition with Proctor, *Tammi own cultivar biomass* = Tammi biomass of the focal plant when in competition with another Tammi. *Proctor mixture biomass* = Proctor yield when in competition with Tammi, *Proctor own cultivar biomass* = Proctor biomass when in competition with another Proctor.

RESULTS

Nitrogen (**Figure 1A**) and biomass (**Figure 1B**) accumulation were temporally distinct for both cultivars. The peak rate of nitrogen accumulation occurred between 17.5 and 19.0 days after planting for Tammi and 19.5–35.0 days for Proctor. The peak rate

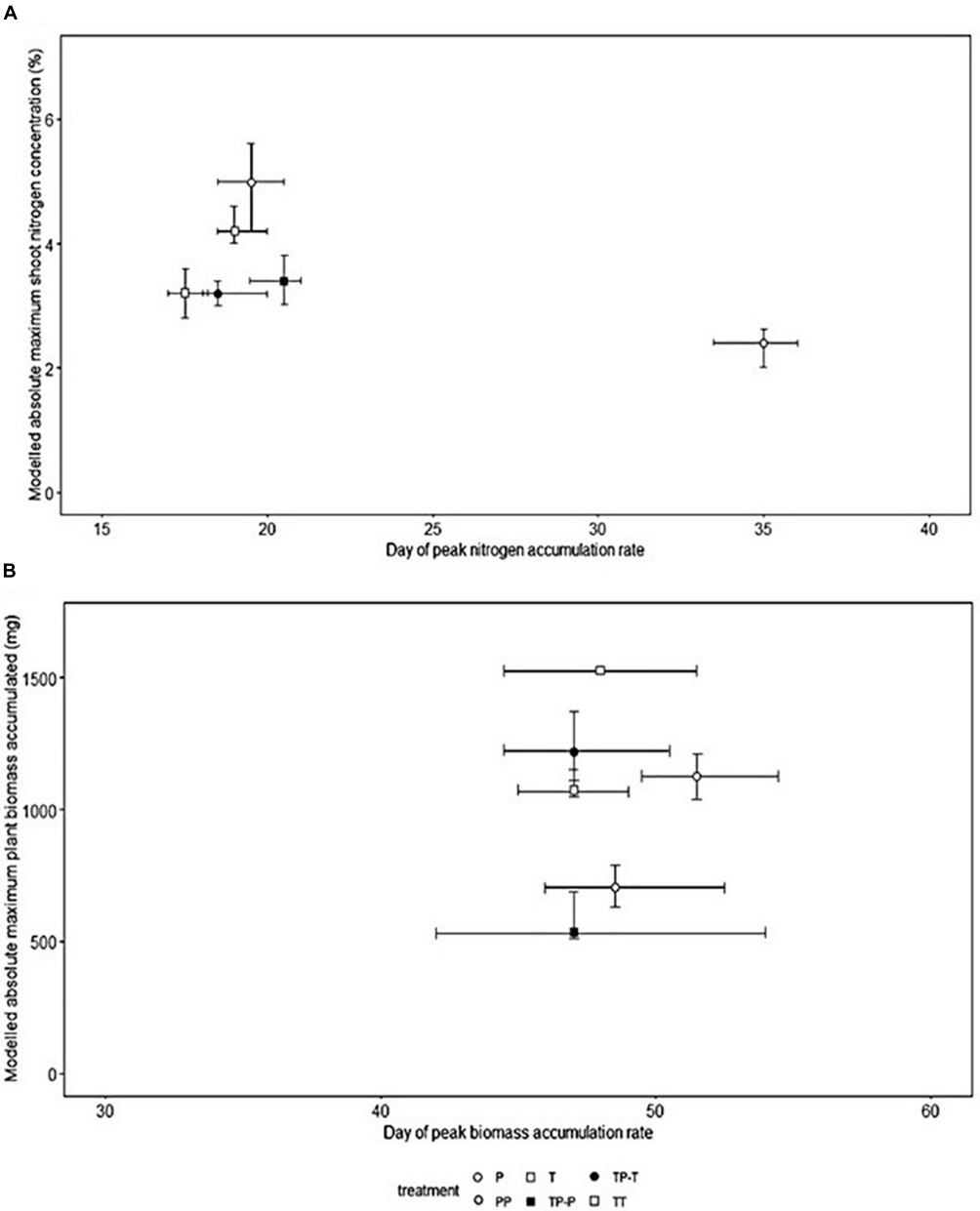

FIGURE 1 | Timing of peak nitrogen **(A)** and biomass **(B)** accumulation rate, the shoot nitrogen concentration and absolute maximum accumulated total biomass at the end of the experiment in barley (*Hordeum* sp.). Bootstrapped modeled accumulation derived from non-linear least squares model (T, Tammi; P, Proctor; TP-T, Tammi in competition with Proctor; TP-P, Proctor in competition with Tammi; TT, Tammi own cultivar competition; PP, Proctor own cultivar competition). Error bars represent the 95% confidence intervals derived from the non-linear least squares model.

of biomass accumulation occurred between 47 and 48 days after planting for Tammi and 47.0–51.5 days for Proctor (Model details in **Supplementary Table 1**).

Temporal Dynamics of Nitrogen Uptake

Nitrogen uptake for both cultivars followed similar temporal dynamics, increasing until 45 days after planting, then plateauing (**Figures 2A,B**). There was no significant change in the timing of peak nitrogen uptake rate in response to inter-cultivar competition for either cultivar. However, both cultivars showed a significant shift in peak accumulation rate in response to intra-cultivar competition (**Figure 1A**). Tammi demonstrated an advance in peak uptake rate by 0.5 days and Proctor a delay of 14.5 days (**Supplementary Table 2**).

Maximum Accumulated Shoot Nitrogen

Proctor's absolute maximum shoot nitrogen concentration was significantly lower when in competition with Tammi

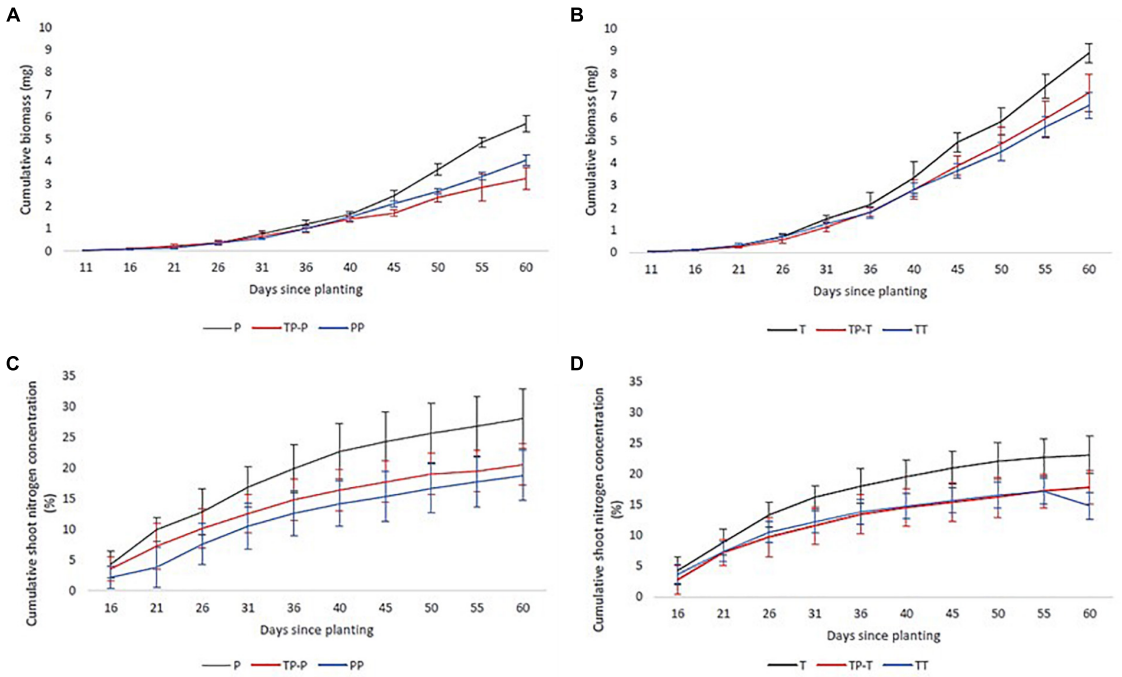

FIGURE 2 | Mean cumulative nitrogen **(C,D)** and biomass **(A,B)** accumulation of Tammi and Proctor barley cultivars over time. Pots contained Proctor in isolation (P), in competition with Tammi (TP) and in competition with another Proctor (PP), Tammi in isolation (T), in competition with Proctor (TP) and another Tammi (TT). Error bars are two times the SEM.

or Proctor compared to isolation (**Figure 1A**). Inter-cultivar competition caused a significantly lower maximum shoot nitrogen concentration compared to intra-cultivar competition for Proctor but not Tammi. Intra-cultivar competition caused a significantly lower maximum shoot nitrogen concentration for Tammi but not Proctor (**Supplementary Table 3**).

Temporal Dynamics of Biomass Accumulation

Biomass accumulation increased throughout the growing period with a lag period until 31 days after planting and then rapidly increased during the remainder of the experiment (**Figures 2C,D**). In response to competition, Tammi did not exhibit a shift in peak biomass accumulation rate, with peak accumulation rate always occurring 47–48 days after planting. Proctor biomass accumulation rate peaked between 48 and 51.5 days after planting (**Figure 1B**); although there was a trend toward an earlier peak in biomass accumulation when in competition there were no significant differences between treatments (**Supplementary Table 2**).

Maximum Accumulated Total Plant Biomass

For both Tammi and Proctor, absolute maximum accumulated biomass was significantly lower when in competition compared to isolation (**Figure 1B**). However, neither cultivar demonstrated a significant difference between intra- and inter- cultivar competition in maximum accumulated biomass (**Supplementary Table 3**).

Shoot C:N

Proctor in isolation had a C:N ratio of about half that of Tammi in isolation throughout the experiment, i.e., more nitrogen relative to carbon. However, for neither cultivar were there significant differences in C:N ratio between plants in isolation compared to plants in competition at the end of the experiment [Proctor ($F_{(2,17)} = 1.44$, $P = 0.26$); Tammi ($F_{(2,17)} = 2.74$, $P = 0.09$)] (details in **Supplementary Table 4**).

Neighbor Effects

The significantly negative RII of final biomass indicated competitive interactions for both cultivars irrespective of whether they were in inter- or intra- cultivar mixtures. RII values also showed that Tammi and Proctor experienced a greater intensity of competition when in inter-cultivar compared to intra-cultivar competition (**Figure 3**). Proctor in intra-cultivar competition experienced the lowest intensity of competition; however, there was no significant difference between the competition treatments [$F_{(3,26)} = 2.86$, $P = 0.06$].

The LER value for Tammi and Proctor in competition was 2.05 (±0.35 SE), indicating that the inter-cultivar mixture had a greater total biomass (root and shoot) than would be expected from the intra-cultivar mixtures.

DISCUSSION

This experiment aimed to detect and quantify temporal dynamism in nitrogen uptake and biomass accumulation in two

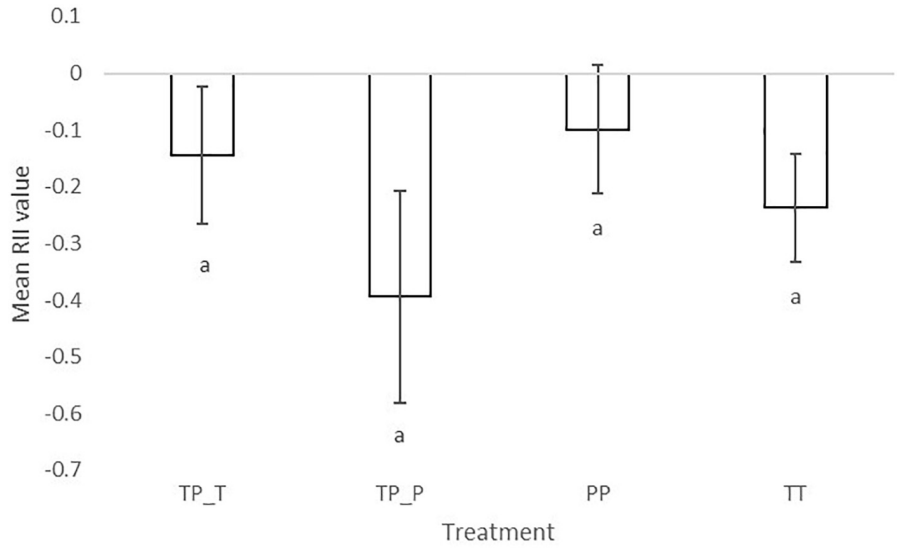

FIGURE 3 | Mean relative intensity index of barley (*Hordeum* sp.) Tammi and Proctor cultivars in inter- and intra-cultivar competition. The more negative the result the greater competition the plant experienced. TP-T, Tammi in inter-cultivar competition; TP-P, Proctor in inter-cultivar competition; TT, Tammi in intra-cultivar competition; PP, Proctor in intra-cultivar competition. Error bars are two times the SEM. Letters indicate significant differences from a Tukey *post hoc* test.

barley cultivars and determine responses to inter- and intra-cultivar competition.

We found that competition significantly reduced maximum accumulated biomass and shoot nitrogen in both cultivars. Neither intra- or inter-cultivar competition impacted the timing of peak biomass accumulation in either cultivar. However, intra-cultivar competition significantly delayed peak nitrogen accumulation rate by 14.5 days in Proctor and advanced it in Tammi by 0.5 days. Relative Intensity Index values indicated that both cultivars experienced competition, with no significant difference in intensity between intra- and inter-cultivar competition. However, a positive LER value indicated that the inter-cultivar mixture overyielded when compared to the intra-cultivar mixtures.

Shifts in the Timing of Biomass Accumulation in Response to Competition

Neither of the cultivars in this study significantly altered the temporal dynamics of peak biomass accumulation in response to a competitor. The mismatch between biomass and nitrogen accumulation dynamics in response to competition indicates biomass may not effectively measure the temporal dynamics of within-growing season resource capture, an issue previously raised by Trinder et al. (2012).

Shifts in the Timing of Nitrogen Accumulation in Response to Competition

Tammi and Proctor only demonstrated significant changes in temporal dynamism of nitrogen accumulation when in intra-cultivar competition. Tammi advanced peak accumulation rate by 0.5 days and Proctor delayed it by 14.5 days. As this only occurred in intra-cultivar competition, it suggests that this is more complex than a competition avoidance response based on a source-sink (soil – plant) relationship. If this was a simple source-sink relationship, for example, based on soil nitrogen availability (Dordas, 2009), the inter- and intra-cultivar responses to competition should be identical. However, a response to only intra-cultivar competition suggests a kin recognition mechanism.

Kin recognition has been suggested as a mechanism by which plants alter functional traits when in competition with closely related individuals (Sousa-Nunes and Somers, 2010). It has been found to most commonly be mediated belowground through root exudates (Biedrzycki et al., 2010; Bais, 2015). This may mediate specific responses depending on the identity of a competing plant, as found in this study.

The results of this study contrast with those of a temporal dynamism study by Trinder et al. (2012) which examined the influence of interspecific competition on the temporal dynamics of nitrogen uptake and biomass accumulation using *Dactylis glomerata* and *Plantago lanceolata,* two perennial grassland species. *D. glomerata* was the later of the two species, and *P. lanceolata* the earlier species. They found a 7 days delay for *D. glomerata* and a 5 days advancement for *P. lanceolata* in maximum biomass accumulation rate in competition compared to plants in isolation, with a similar pattern of divergence for peak nitrogen accumulation rate. We did not find these trends between two cultivars, with no significant shifts in peak biomass accumulation rate and a significant delay in peak nitrogen accumulation rate only when Proctor was in own cultivar competition.

In our study Proctor was the less competitive of the two cultivars, as it experienced a greater decrease in nitrogen and biomass accumulation when in competition compared to Tammi.

This contrasts with the Trinder et al. (2012) study which found that *D. glomerata* took up the most nitrogen and it could be argued was therefore the most competitive, despite being the later species for peak nitrogen and biomass accumulation rate. Therefore, it should not be assumed that the earlier species or cultivar is automatically the most competitive.

Trinder et al. (2012) also found that competition reduced the period between peak nitrogen and biomass accumulation rate compared to plants in isolation, from 10 to 1 days for *D. glomerata*, and from fourteen to 3 days for *P. lanceolata*. We also found this effect, but only when Proctor was in competition, which caused a shortening of the period between peak rate of nitrogen uptake and biomass accumulation by 18.5 days in intra-cultivar competition and 5.5 days when in inter-cultivar competition. However, the reason for this response is unclear. It could be a phenological change in response to competition, a pattern previously observed in cases of abiotic stress (Kazan and Lyons, 2016) and pathogen attack (Korves and Bergelson, 2003).

Temporal Segregation of Nitrogen and Biomass Accumulation

The processes of nitrogen and biomass accumulation were temporally distinct for both cultivars. The peak rate of nitrogen accumulation was 29.0–29.5 days before peak biomass accumulation for Tammi and 16.5–27.5 days for Proctor (**Figure 1**). The gap between peak nitrogen and biomass accumulation was less variable for Tammi compared to Proctor. Tammi was specifically bred for an early phenotype (Nitcher et al., 2013), whereas Proctor was bred for malting quality (Hornsey, 2003). This selection pressure for phenology in Tammi may go some way to explaining the lack of variability in the gap between peak nitrogen and biomass accumulation in response to competition. Future studies could investigate whether similar response patterns are found in the genotypes of wild species or in wild species with contrasting phenologies.

Barley has been found to have temporally distinct nitrogen and biomass accumulation with a 23–24 day gap between peak nitrogen and biomass accumulation in field studies (Malhi et al., 2006). The gap between the peak nitrogen and biomass accumulation rate was shortened when Proctor was in competition, indicating the impact of plant-plant competition on the temporal dynamics of nitrogen accumulation. The greatest reduction in the gap between peak nitrogen and biomass accumulation rate occurred when Proctor was in intra-cultivar competition. This was also the treatment with the lowest absolute shoot nitrogen concentration, suggesting delaying peak rate of nitrogen accumulation for this cultivar is a response to intra-cultivar competition.

Impact of Competition on Final Nitrogen and Biomass Accumulation

Competition significantly reduced the final maximum nitrogen concentration and biomass that both Proctor and Tammi were able to accumulate in intra- or inter-cultivar competition. A Proctor competitor caused a significant decrease in Tammi maximum biomass accumulation and nitrogen shoot concentration, despite not achieving the greatest biomass above or below ground. This suggests that another factor influenced the rate of nitrogen uptake. Signaling through root volatile compounds or root exudates has been found in a number of species including legumes and grasses (Pierik et al., 2013) and may be acting here. Plant root exudates select for a specific microbial community (Shi et al., 2016) and have been found to affect the rate of microbial soil organic matter turnover (Mergel et al., 1998). Therefore, plants may influence the timing of soil microbial community activity in order to reduce direct competition for resources. However, as we are only starting to understand the role of short term-temporal dynamism in plant interactions (Schofield et al., 2018) it is not surprising that further studies are required to determine the role of the root exudates in neighbor recognition and temporally dynamic responses, and why this response is greater for intra- compared to inter-specific competition.

Shoot C:N in Response to Identity of a Competing Individual

The two cultivars differed in their C:N ratio by the end of the experiment. This is likely due to the earlier cultivar Tammi being more advanced developmentally than Proctor. By the end of the experiment, Tammi had begun grain production, whereas Proctor had produced a flag leaf, the stage before grain formation. However, there was no significant increase in C:N in either cultivar in response to competition. Due to selective breeding for a specific seed C:N (grain nitrogen content) with known mapped genes (Cai et al., 2013) it is unlikely that C:N is highly plastic in barley, making it a poor measure of competition in this case.

Is Greater Complementarity Achieved?

The negative RII indicated both cultivars experienced competition when grown with a neighboring plant, but no significant difference depending on the identity of the competitor. This contrasts with the positive LER value which indicated overyielding of the two cultivars when grown in inter-cultivar competition compared to intra-cultivar competition. The reason for this is unclear and may be due to the timing of the final harvest, before both cultivars had set seed. This highlights the difficulty of using multiple metrics to measure the outcome of competition, especially as the measurements were only taken at the end of the experiment, i.e., at a single timepoint. Therefore, single timepoint competition indices should be used with caution when examining the consequences of temporal dynamism of resource capture.

There is a need to understand the extent to which a species or genotype is temporally dynamic and the factors that lead to temporal dynamism in resource capture. This will allow temporal dynamism in resource capture to be included in models of coexistence, furthering our understanding of coexistence in complex plant communities.

CONCLUSION

This study demonstrates how a previously overlooked factor in plant community coexistence, within-growing season temporal

dynamism of resource capture, can be measured through successive harvesting and the novel application of commonly used statistical approaches. Only peak nitrogen accumulation rate was temporally dynamic in response to competition, not biomass peak accumulation rate or shoot C:N. Therefore, we suggest that to understand the temporal dynamics of resource capture within a growing season, direct measures of mineral resources accumulated (e.g., nitrogen uptake) are important to understand the mechanisms of temporally dynamic responses to competition. By measuring shoot nitrogen accumulation rate over time, intra-cultivar competition was found to advance peak nitrogen accumulation rate in Tammi and delay it in Proctor. This suggests that temporally dynamic nitrogen uptake responses are greater in intra-cultivar competition and may be due to kin recognition. This may be mediated through root exudates and the soil microbial community, an area that requires further investigation and extension to semi-natural and natural ecosystems. Ultimately understanding the role of temporal dynamism in plant communities will lead to improved niche models of coexistence in plant communities.

DATA AVAILABILITY

The datasets generated for this study can be found in the Dryad Digital Repository.

AUTHOR CONTRIBUTIONS

ES, RB, JR, EP, FQB, and EACP conceived the experimental design. ES collected the data. RB, MB, JR, and ES analyzed the data. All authors wrote and/or edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00215/full#supplementary-material

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Plant-plant competition influences temporal dynamism of soil microbial enzyme activity

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A B S T R A C T

Root-derived compounds can change rates of soil organic matter decomposition (rhizosphere priming effects) through microbial production of extracellular enzymes. Such soil priming can be affected by plant identity and soil nutrient status. However, the effect of plant-plant competition on the temporal dynamics of soil organic matter turnover processes is not well understood. This study used zymography to detect the spatial and temporal pattern of cellulase and leucine aminopeptidase activity, two enzyme classes involved in soil organic matter turnover. The effect of plant-plant competition on enzyme activity was examined using barley (*Hordeum vulgare*) plants grown in i) isolation, ii) intra- and iii) inter-cultivar competition. The enzyme activities of leucine aminopeptidase and cellulase were measured from portions of the root system at 18, 25 and 33 days after planting, both along the root axis and in the root associated area with detectable enzyme activity. The activities of cellulase and leucine aminopeptidase were both strongly associated with plant roots, and increased over time. An increase in the area of cellulase activity around roots was delayed when plants were in competition compared to in isolation. A similar response was found for leucine aminopeptidase activity, but only when in intra-cultivar competition, and not when in inter-cultivar competition. Therefore, plant-plant competition had a differential effect on enzyme classes, which was potentially mediated through root exudate composition. This study demonstrates the influence of plant-plant competition on soil microbial activity and provides a potential mechanism by which temporal dynamism in plant resource capture can be mediated.

1. Introduction

One of the key processes governing plant nutrient acquisition is mineralisation of soil organic matter (SOM) mediated by microbial communities, a process that can be significantly influenced by plant roots (rhizosphere priming effects: Murphy et al., 2017). Plant root exudates contain large quantities of labile carbon, and increase carbon availability to the soil microbial community (Garcia-Pausas and Paterson, 2011; Kuzyakov et al., 2000). Addition of carbon causes an increase in the carbon to nitrogen to phosphorus ratio (C:N:P), leading to nutrient "mining" by the soil microbial community to restore the stoichiometry of these resources (Paterson, 2003), driven by extracellular enzyme production (Penton and Newman, 2007). These rhizosphere priming effects eventually lead to plant nutrient acquisition through turnover of the soil microbial community (Hodge et al., 2000).

The breakdown of organic matter in the soil is driven by enzyme activity, the majority (90–95%) of which is derived from the soil microbial community (Xu et al., 2014), with some directly from plant roots (Spohn and Kuzyakov, 2013). Enzymatic activity is temporally dynamic, changing in response to the prevailing environmental conditions and associated plant community activity throughout the growing season (Bardgett et al., 2005). The temporal dynamics of soil processes vary with abiotic conditions such as temperature (Steinweg et al., 2012) and nutrient availability (Mbuthia et al., 2015). Therefore, using enzyme activity as a measure of a range of soil microbial community activities and the influence of different factors on these processes, including plant-plant interactions, through time.

As a focus for assessing temporal dynamism in soil enzyme activity, and the impact on this of plant-plant interactions, this study chose two catabolic enzyme classes involved in SOM breakdown and nitrogen cycling, cellulase (EC number: 3.2.1.4) and leucine aminopeptidase (EC number 3.4.1.1). Both the spatial and temporal dynamics of catabolic enzymes, including cellulase and leucine aminopeptidase can be examined using zymography. This method uses fluorescently labelled substrates to measure extracellular enzyme activity in soil. The area and intensity of fluorescence can be calibrated and used for spatial quantification of enzyme activity (Spohn and Kuzyakov, 2014). As this method is non-destructive, it allows a range of enzymes to be studied spatially and temporally (Giles et al., 2018), making it ideal to explore the impact of plant-plant competition on the temporal dynamics of soil enzyme activity.

The intensity of competition between plants for nutrients can vary

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spatiotemporally (Caffaro et al., 2013); this can alter the temporal dynamics of nitrogen accumulation (Schofield et al., 2019) when plants are in competition compared to isolation, with potential consequences for the temporal dynamics of soil microbial community enzyme activity. The temporal dynamics of nitrogen and biomass accumulation have been studied in barley (*Hordeum vulgare*) (Schofield et al., 2019). A delay in peak nitrogen uptake was found when the Proctor cultivar was grown in intra-cultivar competition but not inter-cultivar competition. This response may be due to a change in the temporal dynamics of root associated soil enzyme activity influencing nutrient availability for plants. Therefore, to explore whether such changes in the timing of soil processes do occur, Proctor was chosen as the focal cultivar of this study.

Two main approaches for analysing zymography images have emerged in the last decade. Spohn and Kuzyakov (2014) measured the root associated area of cellulase activity as a percentage of the total sampled area (root associated area) when assessing the activity of cellulases, chitinases and phosphatases in the presence of living and dead *Lupinus polyphyllus* roots. Alternatively, Giles et al. (2018) took a root-centric approach, measuring phosphatase activity along *Hordeum vulgare* root axis (root axis). The Spohn and Kuzyakov (2014) method takes a subsection of the greyscale values, excluding the lightest and darkest pixels; in contrast Giles et al. (2018) used the total pixel range. The Spohn and Kuzyakov (2014) method excludes pixels that are extremely bright, which may skew the total dataset. However, by focussing on the extent of activity in terms of area instead of intensity of activity along the root axis, a relatively small proportion of the soil volume, subtle temporal dynamics of enzyme activity may be more easily detected.

This study aimed to determine the influence of plant-plant competition on the soil microbial community while keeping other environmental factors constant. We also took the opportunity to use both approaches for analysing zymography images. Our aim was to determine the effect of plant-plant competition on the temporal activity dynamics of the two enzyme classes, outside of the zone of most intense competition. Plant root architecture can demonstrate a compensatory response to plant-plant competition (Caffaro et al., 2013). It is expected that enzyme activity surrounding plant roots will show similar trends to root architecture, with increased enzyme activity surrounding roots outside the zone of most intense competition when the plants are in competition compared to isolation. As competition can be less intense between more closely related individual plants, due to changes in the temporal dynamics of resource capture, it is expected that interactions between more closely related individuals will promote less intense enzyme activity than inter-cultivar competition.

2. Materials and methods

2.1. Soil characterisation

Soil was collected from an agricultural field that had previously been cropped with spring barley (*Hordeum* sp.) and had been subject to standard fertilisation conditions (500 kg of N ha^{-1} yr^{-1} in the ratio of N 22: P 4: K 14) (Balruddery Farm, Invergowrie, Scotland, 56.4837° N, 3.1314° W). The soil was then passed through a 3 mm sieve to homogenise the substrate. The soil had an organic matter content (humus) of 6.2% ± 0.3% SEM (loss-on-ignition, n = 4) and a mean pH (in water) of 5.7 ± 0.02 SEM (n = 4), a total inorganic nitrogen concentration of 1.55 ± 0.46 mg g^{-1} (n = 4) and microbial C biomass (using a chloroform extraction) of 0.06 ± 0.002 SEM mg g^{-1} (n = 4). No fertilisation occurred during the experiment.

2.2. Rhizobox preparation

Rhizoboxes (150 mm × 150 mm x 10 mm Perspex boxes with a removable side for access to roots) were packed to a bulk density of 1.26 g cm^{-3}, ensuring the soil was level with the edge of each box. Seeds of Proctor and Tammi barley (*Hordeum* sp.) cultivars were pre-germinated on damp tissue paper in the dark at room temperature for two days before planting. Three replicates of each treatment: Proctor alone (P), Proctor in intra-cultivar competition (PP) and Proctor in inter-cultivar competition with Tammi (TP) were planted, as well as a bare soil control, giving 12 rhizoboxes in total. In the planted treatments, the germinated seeds were placed on the surface of the soil, ensuring contact between the emerging roots and soil surface, and then the side of the box was replaced and secured. In the planted treatments containing two plants, the germinated seeds were placed 2.5 cm apart to ensure no aboveground interaction between the two plants.

The rhizoboxes were wrapped in foil to exclude light from the roots and placed at a 45° angle to encourage root growth over the soil surface. The rhizoboxes were kept in a controlled environment cabinet (Jumo IMAGO 3000, Harlow, Essex, UK) at a constant 15 °C, 65% relative humidity and a 16/8 (day/night) (light intensity: 200 µmol m^{-2} s^{-1}) photoperiod for the duration of the experiment to mimic local spring-time conditions. Each rhizobox was watered weekly with sufficient water to maintain soil moisture at field capacity and prevent root desiccation.

2.3. Soil zymography

Enzyme activity was measured three times at weekly intervals between 18 and 39 days after planting. This is the period prior to peak barley nitrogen accumulation rate found in our previous study (Schofield et al., 2019). Areas away from the competition zone between the two plants were visually identified and labelled on the rhizobox rim to ensure measurements of soil enzyme activity occurred at a consistent location throughout the study. These were roots of the focal individual that consistently did not encounter roots of the other individual within the system. This setup was used to indicate whether a compensatory or systemic response to plant-plant competition could be detected in soil enzyme activity.

Two fluorescently labelled substrates were selected for this study; 4-methylumbellferyl ß-D-cellobioside, a substrate of cellulase which was imaged at 365 nm (excitation at 365 nm, emission at 455 nm) and L-leucine-7-amido-methylcoumarin hydrochloride, a substrate of leucine aminopeptidase that was imaged at 302 nm (excitation at 327 nm, emission at 349 nm) (Sigma-Aldrich, Reading, UK). Both substrates were diluted to a 6 mM concentration, the concentration used in previous studies using methylumbellferyl ß-D-cellobioside (Spohn and Kuzyakov, 2014) and the optimum concentration found during preliminary experiments (results not shown). A 47 mm diameter polyamide membrane (Whatman, GE Healthcare, Buckinghamshire, UK) was soaked in 300 µl of 6 mM of 4-methylumbellferyl ß-D-cellobioside or L-leucine-7-amido-methylcoumarin hydrochloride. On sampling days, the side of each rhizobox was removed and a 1% agarose (Invitrogen, Carlsbad, CA, USA) gel of 1 mm thickness was placed on the soil surface to protect the membrane from soil particles which could adhere to it and disrupt the final image, whilst allowing the diffusion of extracellular enzymes (Spohn and Kuzyakov, 2014). The membrane was then placed on top of the gel and the foil was replaced over the top to exclude light and minimise moisture loss during enzyme assays.

Previous studies have incubated similar substrate soaked membranes for between 30 min and 3 h (Giles et al., 2018; Spohn and Kuzyakov, 2014). Therefore, a preliminary study was carried out which found that, for this system, an incubation of 1 h gave a good level of resolution and UV intensity when viewed (results not shown). Following incubation (1 h), the membrane was placed onto a fresh 1% agarose gel to minimise bubbling of the membrane during imaging. The membrane and gel were then placed in an UV imaging box (BioDoc-It^2 Imager, Analytik Jena, Upland, CA) and imaged at 365 nm (Spohn and Kuzyakov, 2014). This was repeated for L-leucine-7-amido-methylcoumarin hydrochloride, which was imaged at 302 nm (Ma et al., 2018). This order of substrate sampling was maintained throughout the experimental period (Spohn

and Kuzyakov, 2014). The sampled area was marked on the rim of each rhizobox to ensure that the same area was sampled each time for both enzymes. After sampling, the rhizobox was watered and replaced in the controlled environment chamber.

2.4. Calibration curves

Known dilutions of 4-Methylumbelliferone (the fluorescent tag of 4-methylumbellferyl ß-D-cellobioside) and 7-Amino-4-methylcoumarin (the fluorescent tag of L-leucine-7-amido-methylcoumarin hydrochloride) (1, 2, 4, 6 mM) were prepared and used to soak membranes, using the same procedure as the experiment (Giles et al., 2018). The membranes were then imaged using the same method and settings as the samples. The images were used to calculate the substrate concentration per mm^2 and provide the calibration curve values from the sample images. This also informed the range of 8 bit greyscale values (the integer brightness value per pixel between 0 and 255) sampled in the percentage area analysis (Spohn and Kuzyakov, 2014).

2.5. Root growth measurements

The roots of each rhizobox were photographed weekly from 4 to 39 days after planting using an iPhone 6 (8 - megapixel iSight camera with 1.5 µm pixels, Apple Inc). The root architecture photographs were then analysed using the SmartRoot plugin (Lobet et al., 2011) of the ImageJ software (Schneider et al., 2012). The roots of each plant were manually traced and labelled using the Trace tool. This was used to measure total root length over time. Dry root biomass was also recorded at the end of the experiment by drying roots at 100 °C for 24 h.

The effect of time and treatment on the measured root architecture parameters were assessed using a Generalised Least Squares model using the nlme package in R (R statistical software, R Core Team, 2016). Time and treatment were included as fixed factors as well as the interaction between treatment and time. A covariate of rhizobox number and treatment was included to account for autocorrelation caused by the repeated measures in this study. This was followed by an ANOVA test (MASS package, R statistical software, R Core Team, 2016).

2.6. Enzyme image analysis

The intensity and location of enzyme activity was analysed using two approaches: root axis activity (Giles et al., 2018) and root associated area (Spohn and Kuzyakov, 2014). These two approaches differ in that the root axis activity records soil enzyme activity only along the root itself, whereas the root associated area measures soil enzyme activity in the surrounding rhizosphere as well. By comparing these two approaches the most appropriate image analysis method to study the temporal dynamics in root associated soil microbial activity can be determined. Root associated area was defined as the percentage of the total sampled area with greyscale values above a threshold defined by the calibration curves that indicated enzyme activity.

2.6.1. Root axis enzyme activity

For this approach, root axis image analysis technique developed by Giles et al. (2018) was used. Proctor roots contained within the sample area were tracked using the segmented line tool in the Fiji image analysis software (Schindelin et al., 2012). The RProfile plugin developed by Giles et al. (2018) was then used to extract a profile of greyscale values along the sampled root. The nodes of the segmented line placed along the root were then centralised and placed evenly along the sampled root to refine the data using the Python script developed by Giles et al. (2018). The mean greyscale value was calculated for each root (subsequently referred to as 'root axis activity').

2.6.2. Root associated area analysis

To measure the root associated area of enzyme activity, the approach developed by Spohn and Kuzyakov (2014) was used. Each image was first converted into an 8-bit greyscale image. The range of 80–170 Gy values was extracted from each image (informed by the calibration curves) then split into 10 Gray value increments, and the area of each increment measured using Image J Software (Schneider et al., 2012). This was then expressed as a percentage of the total membrane area (subsequently referred to root associated area). The percentage root associated area was then compared between treatments. The mean enzyme activity rate was the most common enzyme activity rate, i.e. the rate with the greatest percentage cover of the total sampled area.

2.7. Statistical analysis

The effect of time and treatment on the root axis activity and root associated area were each assessed using a Generalised Least Squares model, accounting for repeated measures with an autocorrelation term, using the nlme package (Pinheiro et al., 2016) in R (R Core Team, 2015). This was followed by an ANOVA test for significant differences using the MASS package (Venables and Ripley, 2002) in R (R Core Team, 2015). The interaction between treatment and time was included as a fixed factor, to detect differences between treatments in enzyme activity temporal dynamics, with an autocorrelation term for treatment and rhizobox number.

3. Results

3.1. Total root growth

Total root length increased over time for all treatments (Table 1). There was a significant effect of treatment ($F_{(2,52)} = 5.45$, $P = <0.01$) and time ($F_{(4,52)} = 45.04$, $P = <0.01$) on total root length but no significant interaction between treatment and time ($F_{(8,52)} = 1.27$, $P = 0.28$). There was no significant difference in total root biomass between the different treatments at 33 days ($F_{(2,10)} = 0.78$, $P = 0.48$).

3.2. Root axis activity

Mean cellulase root axis activity at 33 days after planting ranged between 1.4 and 11.8 pmol mm^{-2} h^{-1} and leucine aminopeptidase between 4.5 and 6.3 pmol mm^{-2} h^{-1} (Fig. 1). For cellulase activity there was a significant effect of treatment ($F_{(2,42)} = 5.03$, $P = 0.01$) but no significant effect of time ($F_{(2,42)} = 0.51$, $P = 0.60$) or interaction between treatment and time ($F_{(4,42)} = 0.94$, $P = 0.45$). However, there was no significant effect of time ($F_{(2,63)} = 2.92$, $P = 0.06$), treatment ($F_{(2,63)} = 2.74$, $P = 0.07$) or the interaction between the two factors ($F_{(4,63)} = 1.02$, $P = 0.40$) for leucine aminopeptidase activity.

3.3. Root associated area

The activity of both enzyme groups was highest nearest to the sampled roots, indicated by the brighter areas, and decreased with distance from them. The consistent sampling position is shown for each pot in Fig. 2. Cellulase activity was not solely localised to the axis of sampled roots, and activity away from roots increased with time (Fig. 3), with a mean root associated area activity of 0.57–2.10 pmol mm^{-2} h^{-1} 33 days

Table 1
Mean total root length and biomass at 33 days after planting of Proctor barley plants in isolation (P), intra-cultivar competition (PP) and inter-cultivar competition (TP) (n = 3). Values in the brackets are the standard error of the mean (SEM).

Treatment	Total root length (mm)	Root biomass (g)
P	158 (±23.2)	0.036 (±0.004)
PP	138 (±15.5)	0.191 (±0.004)
TP	153 (±42.4)	0.042 (±0.007)

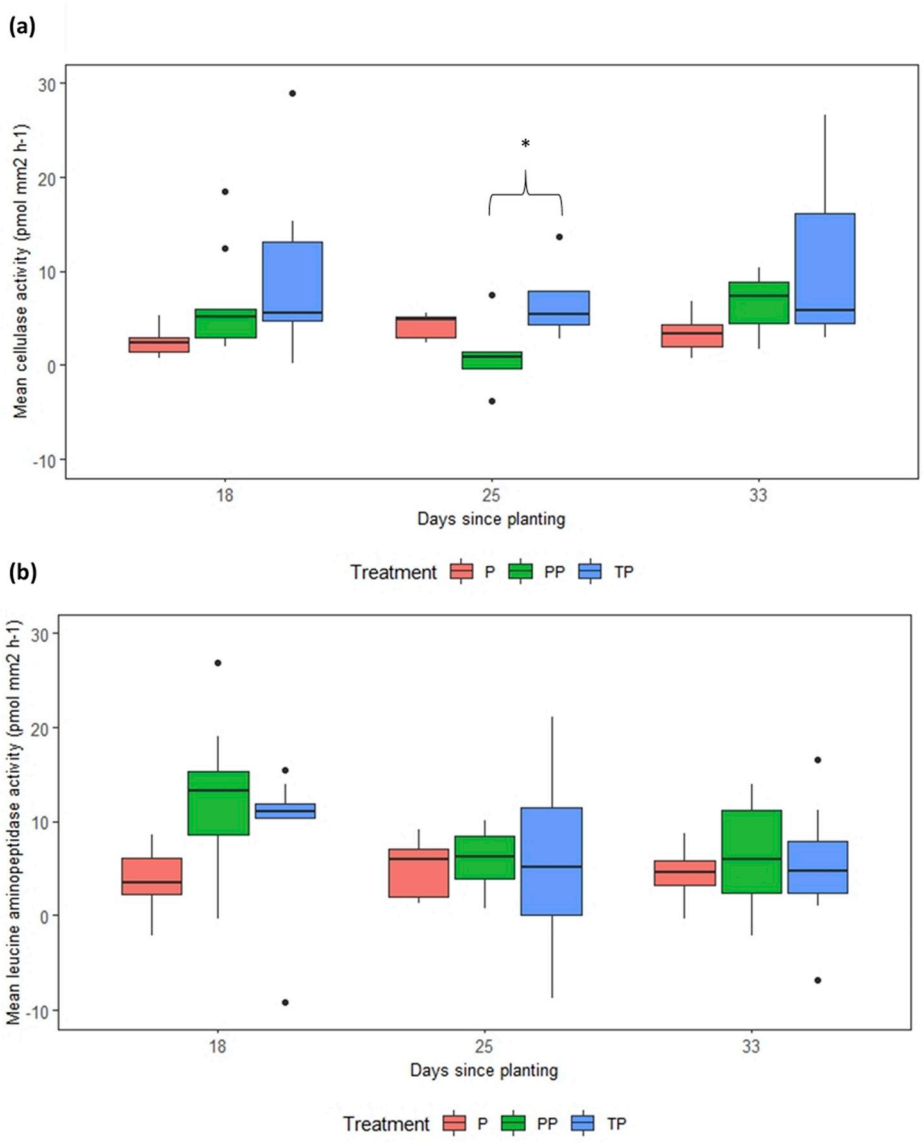

Fig. 1. Mean cellulase and leucine aminopeptidase activity (pmol mm^{-2} h^{-1}) along the root axis of Proctor roots grown in isolation (P), intra- (PP) and inter- (TP) cultivar competition (n = 12). A = Mean root axis cellulase activity, B = Mean root axis leucine aminopeptidase. Boxplot shows the median, first and third quartiles and whiskers the maximum and minimum values. Significant differences (P = <0.05) denoted by asterisk.

after planting. When Proctor was grown in isolation, the root associated area of cellulase activity was relatively constant (53–58%) (Fig. 5a). However, when Proctor was in inter- or intra-cultivar competition the initial percentage area was low (11% in intra-cultivar competition and 13% in inter-cultivar competition) but then rapidly increased to 25 days before stabilising at a similar percentage as Proctor in isolation (47% in intra-cultivar competition and 58% in inter-cultivar competition) (Fig. 5a). This shows a delay in the area of cellulase activity when Proctor was in competition compared to isolation. This is demonstrated in Fig. 3, with darker images in the competition treatments at 18 days after planting compared to the isolation treatment. The root associated area in which cellulase activity occurred in the planted treatments showed a significant effect of treatment ($F_{(2,17)} = 4.72$, $P = 0.02$), time ($F_{(2,17)} = 44.98$, $P = <0.01$) and interaction between treatment and time ($F_{(2,17)} = 12.88$, $P = <0.01$). Model details are in Supplementary Fig. 1.

Leucine aminopeptidase activity occurred beyond the immediate rhizosphere (Fig. 4). Mean root associated area activity at 33 days after planting ranged from 0.91 to 3.48 pmol mm^{-2} h^{-1}. When Proctor was grown in isolation and inter-cultivar competition, leucine aminopeptidase root associated area steadily increased over time (Fig. 5b). At 25 days, the intra-cultivar competition root associated area was lower (31%) than in isolation (48%) and inter-cultivar competition (52%) (Fig. 5b), indicating a delay in leucine aminopeptidase activity in intra-cultivar competition compared to isolation and inter-cultivar competition. This is demonstrated in Fig. 4, with darker images in the intra-cultivar competition treatment at 18 days after planting compared to the isolation and inter-cultivar competition treatments. There was a significant effect of treatment ($F_{(2,17)} = 31.72$, $P = <0.01$), time ($F_{(2,17)} = 30.36$, $P = <0.01$) and a significant interaction between time and treatment on the root associated percentage area of leucine aminopeptidase activity ($F_{(2,17)} = 7.42$, $P = <0.01$). Model details are in Supplementary Fig. 1.

4. Discussion

This experiment aimed to determine the effect of plant-plant competition in barley on the temporal dynamics of nutrient cycling by measuring activity of cellulase and leucine aminopeptidase, two enzyme classes associated with nutrient turnover, specifically of carbon and nitrogen. Root axis activity for both enzyme classes was not significantly

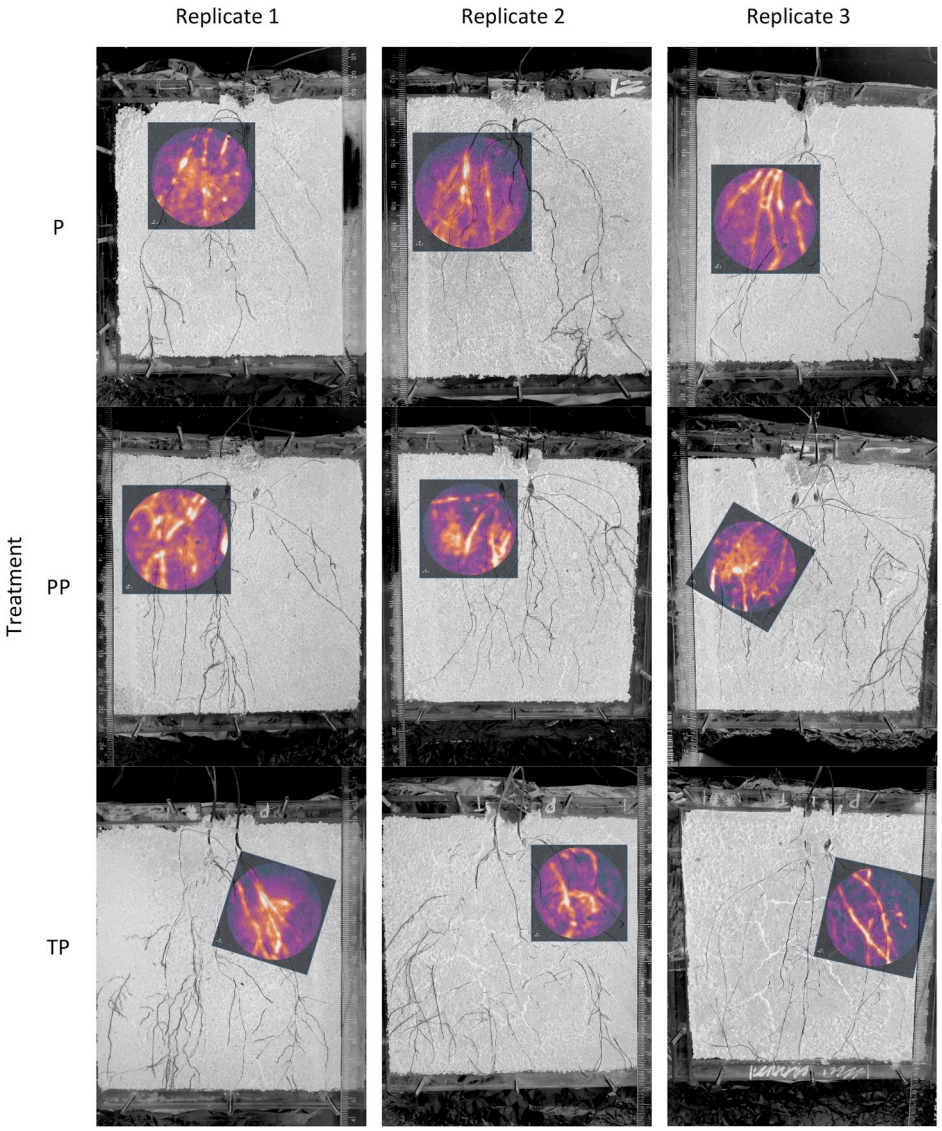

Fig. 2. Images of the sampled rhizoboxes, showing the consistent sampling location used in this study and the relationship between root presence and soil enzyme activity.

temporally dynamic (the interaction between time and treatment) when the focal plant (Proctor cultivar of barley) was in intra- and inter-cultivar competition compared to isolation. However, using the Spohn and Kuzyakov (2014) root associated area approach, cellulase activity was found to be delayed when in intra- and inter-cultivar competition compared to isolation (significant interaction between treatment and time). In contrast, leucine aminopeptidase root associated area was delayed when in intra-competition, but not inter-cultivar competition compared to isolation (significant interaction between treatment and time). This demonstrates that the temporal dynamics of soil enzyme activity were influenced by plant-plant competition independent of other environmental factors, that plant-plant competition did not have a uniform effect on different classes of soil enzymes, and that the observed effects are also dependent on the method of measurement.

4.1. Root axis activity

Both cellulase and leucine aminopeptidase mean root axis activity was much higher than the whole sampled area, 3–4 times higher for leucine aminopeptidase and 4–6 times for cellulase. This is most likely due to the influence of plant root exudates, which provide a source of labile carbon, increase the rate of SOM mineralisation and, consequently, carbon and nitrogen cycling in the rhizosphere compared to bulk soil (Bengtson et al., 2012; Murphy et al., 2017). However, along root activity did not vary significantly over time for either enzyme class. The area of root system sampled was in the zone of maturation, a zone associated with a stable rate of nutrient uptake (Giles et al., 2018). We hypothesised that plant-plant competition would have changed the temporal dynamics of root axis enzymatic activity, but it seems the inherent stability of this root zone was greater than the influence of plant-plant competition. Other root zones are associated with uptake of specific nutrients, for example the apical root zone is associated with iron absorption and the elongation zone with sulphur uptake (Walker et al., 2003). Therefore, depending on the root zone sampled and nutrient studied, there will likely be differing patterns of enzyme activity.

There is the potential for some enzyme activity to be produced by the plants themselves: up to 10% (Xu et al., 2014). Plant-derived leucine aminopeptidases genes have been detected in the plant genome, and found to have a role in protein turnover (Bartling and Weiler, 1992). Plants also have cellulases, but these are used for remodelling of cell walls and are not thought to be strong enough for large scale

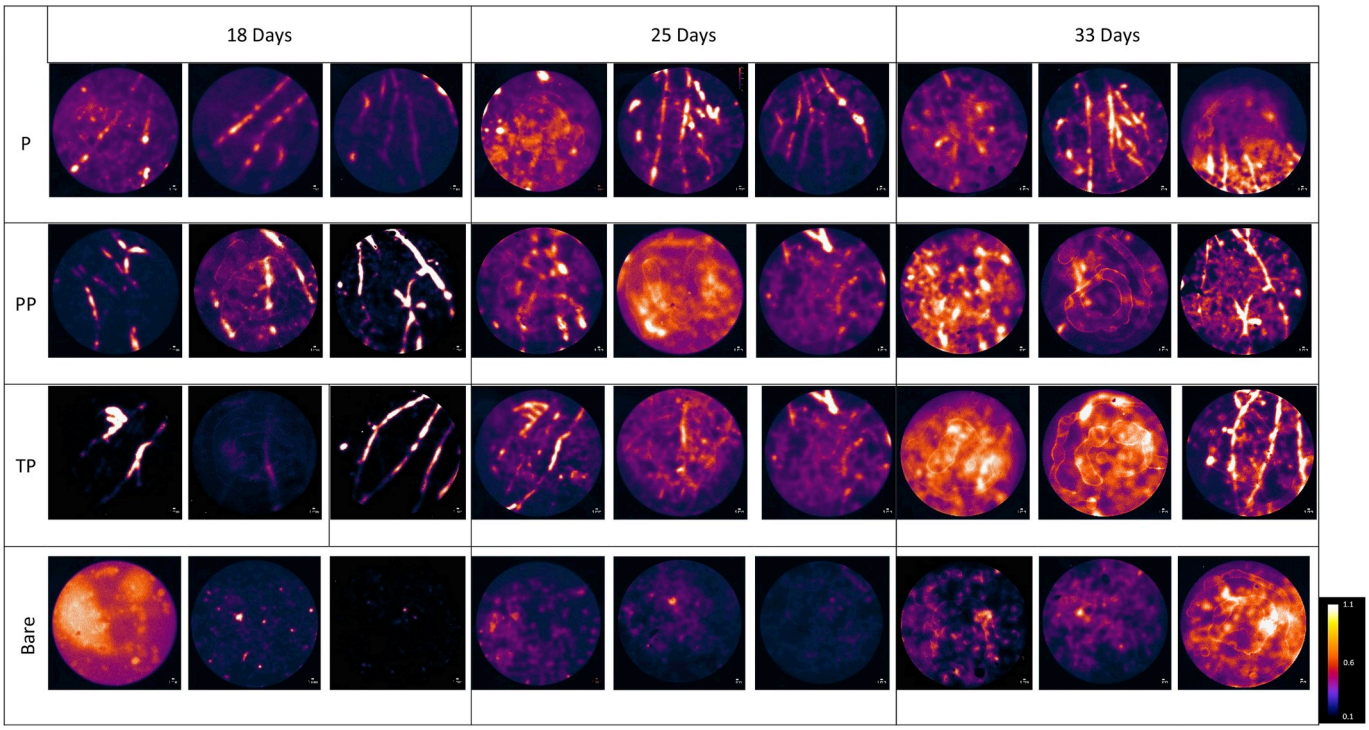

Fig. 3. Soil zymography images showing (pmol mm^{-2} h^{-1}) cellulase activity around Proctor roots sampled from plants grown in isolation and competition as well as a bare soil control (n = 3). A. = Bare soil control, B. = Proctor, C. = Proctor and Proctor, D. = Proctor and Tammi.

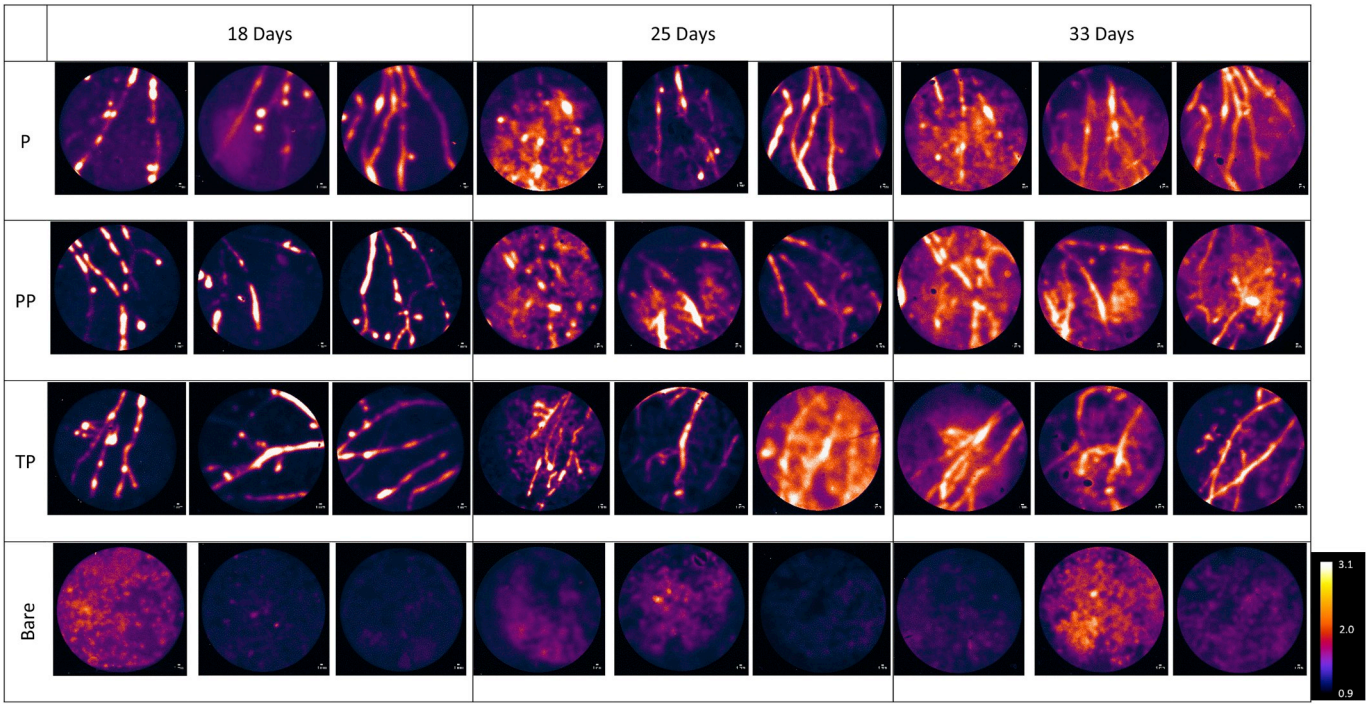

Fig. 4. Soil zymography images showing (pmol mm^{-2} h^{-1}) leucine aminopeptidase activity around Proctor roots sampled from plants grown in isolation and competition as well as a bare soil control (n = 3). A. = Bare soil control, B. = Proctor, C. = Proctor and Proctor, D. = Proctor and Tammi.

degradation of cellulose (Hayashi et al., 2005). Therefore, due to their intra-cellular roles, it is unlikely that plant-derived enzymes contributed to the enzyme activity outside of the plant roots detected in this study.

4.2. Root associated area

Cellulase and leucine aminopeptidase root associated area were not solely confined to the root axis, with increased activity across the sampled areas, including background soil activity. Cellulase root associated area was temporally dynamic, with a delay in peak enzyme

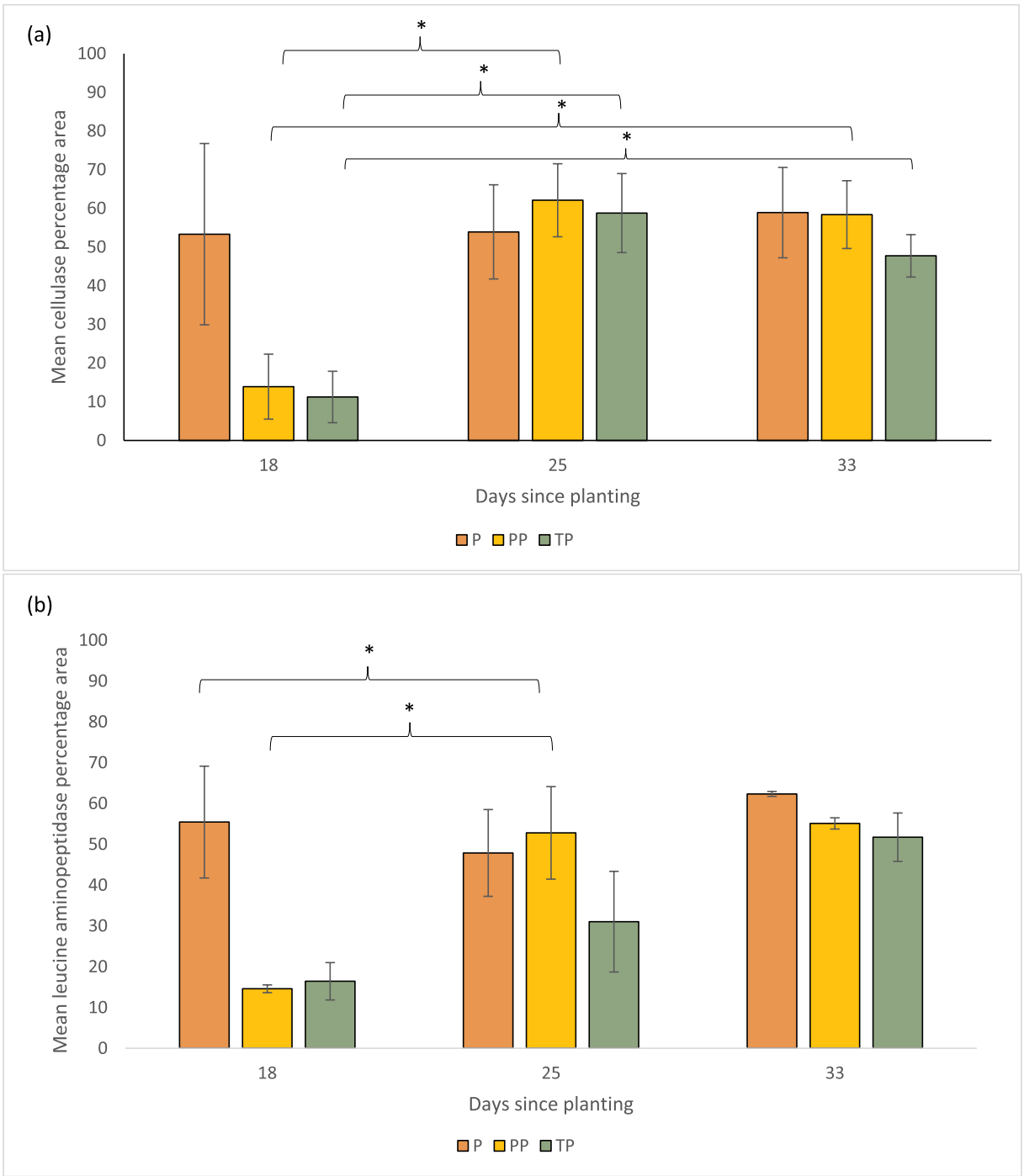

Fig. 5. The mean percentage of sampled areas in which the activity of cellulase and leucine aminopeptidase were recorded (n = 12). Cellulase activity (a) and leucine aminopeptidase (b) activity were sampled surrounding Proctor roots outside the competition zone of plants grown in isolation, intra-cultivar competition and inter-cultivar competition. Significant differences (P = <0.05) denoted by asterisks.

activity (i.e. when the largest percentage area of membrane was recording either cellulase or leucine aminopeptidase activity) when in competition compared to isolation. The zymography assay measured total cellulase activity of multiple microbial functional groups and did not differentiate between exo- and endo-glucanase activities. Exo-glucanases break glucose from the end of cellulase polymers, whilst endo-glucanases break bonds within the cellulose chains (Pappan et al., 2011). There may have been differing dynamics if endo- and exo-glucanase activity were examined separately.

Leucine aminopeptidase root associated area also demonstrated a delay in activity but only when Proctor was in intra-cultivar competition. This delay in leucine aminopeptidase root associated area when in intra-cultivar competition echoes a similar trend to the delay of 14.5 days in Proctor peak above-ground nitrogen accumulation rate found in a previous study (Schofield et al., 2019). The mechanism that links these two observations is not clear. Proctor plants may have delayed peak root exudate production when in intra-cultivar competition, influencing microbial activity to limit competition between the two plants. However, there may also be further mechanisms, for example involving plant-microbe signalling, already known to be important in recruitment of microbial symbionts and plant growth promoting rhizobacteria (Chagas et al., 2018; Labuschagne et al., 2018).

As the same area was sampled consistently over the experiment, the sampled area became increasingly far from the root tip, a known hotspot of soil microbial community enzyme activity. This may have influenced the activity of the two enzyme classes. Phosphatase activity has previously been found to vary with distance from the root tip (Giles et al., 2018), which may have influenced the results presented. However, there was no significant difference in root biomass or total root length between any of the treatments (Table 1), indicating that the relative sampling position remained consistent across treatments in this study. One benefit of sampling in the mature root zone is that it allows comparisons among treatments as the sampled areas were all a similar distance from the root tip at each time point. The zone of maturation is a region of the root with less exudation compared to the zone of elongation (Badri and Vivanco, 2009), but with root hairs that provide greater surface area for nutrient absorption (Gilroy and Jones, 2000). There may have also been an influence of root branching which occurred in some of the sampled areas due to plant foraging for nutrients (Forde, 2014). This hypothesis requires further sampling of a greater proportion of the root system for a high resolution of spatiotemporal trends in microbial enzyme activity with root branching.

4.3. What role could root exudates have in the temporal dynamics of enzyme activity?

The different patterns of soil enzyme activity associated with the three treatments may have been driven by differences in root exudation, with changes in root exudate composition then affecting microbial activity. Plants select for a specific microbial community through root exudates (Hu et al., 2018; Shi et al., 2011). Therefore, root exudates may do more than simply increase the rate of nitrogen mineralisation (Mergel et al., 1998), and may also influence the timing of mineralisation by influencing soil microbial community composition.

Root exudation quality and quantity is known to change over time (van Dam and Bouwmeester, 2016) with root exudates increasing the carbon to nitrogen ratio in the rhizosphere, regulating mining of SOM by the soil microbial community (Chaparro et al., 2012; Meier et al., 2017). Exudates also act as a form of signalling between plants (van Dam and Bouwmeester, 2016), eliciting a change in root architecture (Caffaro et al., 2013), branching (Forde, 2014) and biomass allocation (Schmid et al., 2015). Therefore, the observed delay in soil enzyme activity could be regulated by temporally dynamic root exudation. Root branching would have also increased the total root area within the measurement areas, potentially increasing the total exudates available to the soil microbial community and promoting greater enzymatic activity. Consequently, the active control of root exudates instead of root biomass or surface area alone may be an important part of the mechanism behind the observed shifts in soil microbial community activity. This is an exciting avenue for future research.

4.4. Temporal dynamics of enzyme activity in response to plant-plant competition

The soil enzyme classes in this study demonstrated different temporal patterns in activity in response to changes in plant-plant competition. Relative to the isolated-plant control, the temporal dynamics of cellulase root associated area were influenced by both intra- and inter-cultivar competition, whereas leucine aminopeptidase dynamics were only significantly influenced by intra-cultivar competition.

The influence of plant-plant competition on the temporal dynamics of root associated enzyme area occurred beyond the immediate zone surrounding the root. This contrasts with the results of Ma et al. (2018), who found a strong localisation of leucine aminopeptidase and cellulase activity close to plant roots across the whole root system. Furthermore, they found that the root associated area did not increase over time around lentil roots (*Lens culinaris*) and only began to increase around Lupin (*Lupinus albus*) roots eight weeks into the study (Ma et al., 2018). This is much later than the barley in our study, where sampling occurred in the first month of growth, the period prior to peak nitrogen accumulation rate in these barley cultivars (Schofield et al., 2019). This is likely to be a period of soil microbial community priming to mine for nitrogen within soil organic matter and may account for the differences between Ma et al.'s and our study. In our study the extent of the rhizosphere and therefore activity of leucine aminopeptidase and cellulase may have increased over time, as labile carbon in root exudates diffused away from roots and the zone of nutrient depletion surrounding roots enlarged.

Our study does however have its limitations. The rhizobox system is a very artificial setup with roots growing in a single plane, which would influence root growth and development. This does not account for the 3D nature of root growth and interactions with the soil particles and the soil microbial community. More complex interactions and temporally dynamic responses may be occurring in a 3D system through localised changes in the soil microbial community. Therefore, development of the zymography method in order to sample 3D root systems is a natural avenue for future research.

The temporal dynamics of enzyme activity are likely to be strongly influenced by environmental conditions including temperature (Steinweg et al., 2012), soil moisture (Barros et al., 1995) and soil nutrient concentration (Mbuthia et al., 2015). This study demonstrates that the temporal dynamics of the two groups of enzymes, both involved in nutrient turnover, were affected differently by plant-plant competition when grown in constant environmental conditions. This could be due to the composition of root exudates and concentration of secondary metabolites that selected for a soil microbial community with specific functions (Hu et al., 2018; Shi et al., 2016). Plants could have therefore regulated soil microbial community activity through the differing sensitivity of microbial taxa to root exudates (Shi et al., 2011; Zhang et al., 2017).

5. Conclusions

Root axis activity of leucine aminopeptidase and cellulase was not temporally dynamic in response to plant-plant competition. Plant-plant competition influenced the root associated area of the two enzymes in this study differently. The extent of root associated cellulase area was delayed by inter- and intra-cultivar competition, whilst leucine aminopeptidase root associated area was only delayed by intra-cultivar competition. This may have been mediated through root exudates selecting for specific microbial functions. Therefore, conclusions concerning the temporal dynamics of nutrient cycling are likely to be dependent on the enzyme class being studied and method of image analysis used. Changes in these temporal dynamics may have been mediated through changes in the quantity and composition of root exudates by plants in competition, leading to a delay in peak soil enzyme activity. The extent of plant root influence was found to increase over time as exudates diffused away from roots, an important factor in studies of the soil microbial community activity. This study therefore demonstrates the close link between the temporal dynamics of plant and microbial resource capture and the influence each process has on the other.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2019.107615.

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