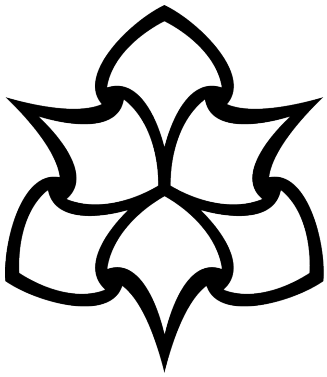
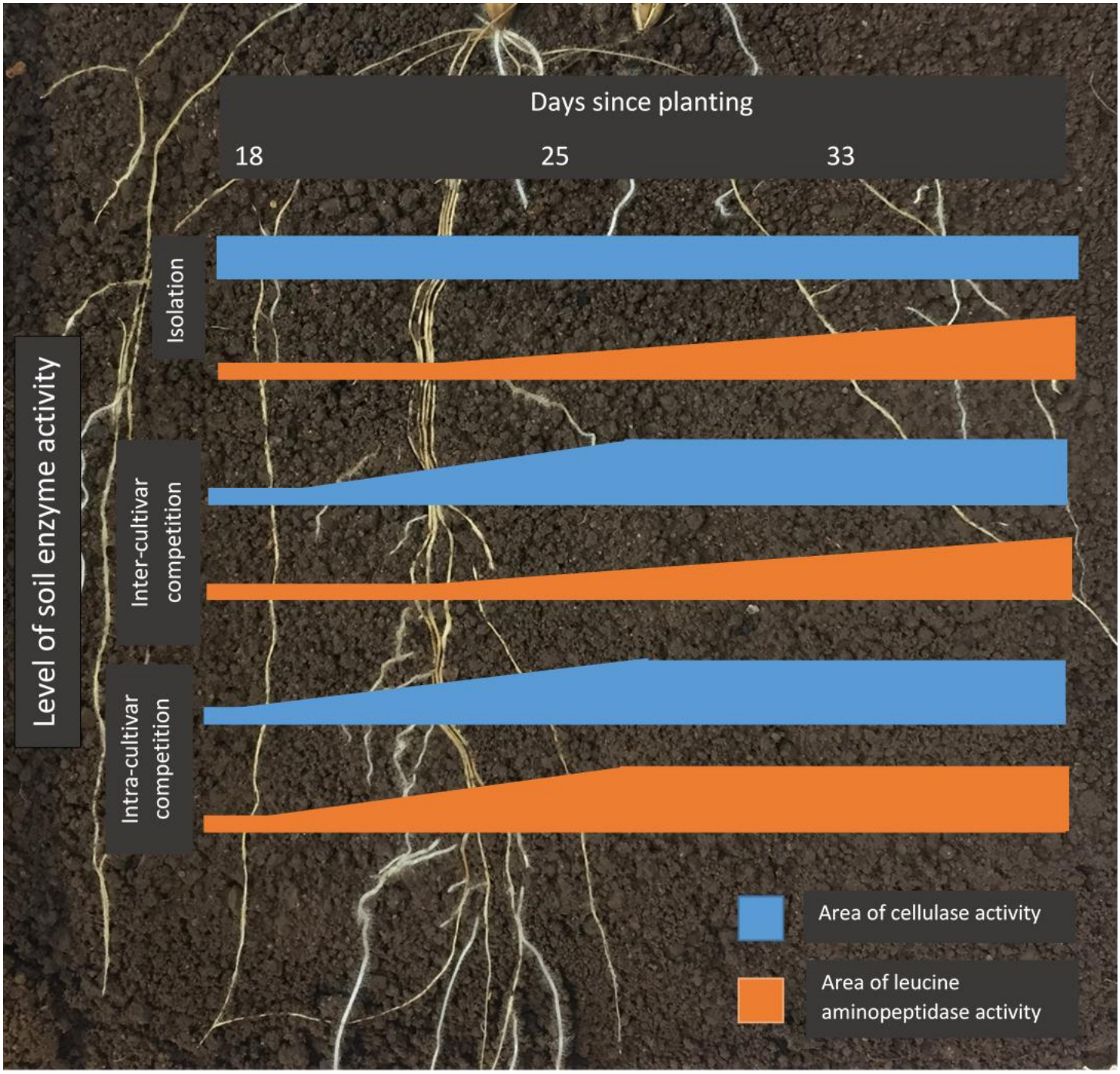


Graphical Abstract (for review)



Highlights

1. Soil enzyme activity was strongly associated with plant roots
2. Root axis activity was not temporally dynamic when in plant-plant competition
3. Root associated area was temporally dynamic in response to plant competition
4. Peak cellulase activity was delayed when in competition compared to isolation
5. Leucine aminopeptidase activity was delayed only in intra-cultivar competition

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

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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.

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 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

115 *2.1 - Soil characterisation*

116 Soil was collected from an agricultural field that had previously been cropped with spring
117 barley (*Hordeum* sp.) and had been subject to standard fertilisation conditions (500 kg of N
118 ha^{-1} yr^{-1} in the ratio of N 22 : P 4 : K 14) (Balruddery Farm, Invergowrie, Scotland, 56.4837°
119 N, 3.1314° W). The soil was then passed through a 3 mm sieve to homogenise the substrate.
120 The soil had an organic matter content (humus) of 6.2 % ± 0.3 % SEM (loss-on-ignition, n = 4)
121 and a mean pH (in water) of 5.7 ± 0.02 SEM (n = 4), a total inorganic nitrogen concentration
122 of 1.55 ± 0.46 mg g^{-1} (n = 4) and microbial C biomass (using a chloroform extraction) of 0.06
123 ± 0.002 SEM mg g^{-1} (n = 4). No fertilisation occurred during the experiment.

124

125 *2.2 - Rhizobox preparation*

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

137 The rhizoboxes were wrapped in foil to exclude light from the roots and placed at a
138 45° angle to encourage root growth over the soil surface. The rhizoboxes were kept in a
139 controlled environment cabinet (Jumo IMAGO 3000, Harlow, Essex, UK) at a constant 15°C,
140 65 % relative humidity and a 16/8 (day/night) (light intensity: 200 µmol m^{-2} s^{-1}) photoperiod
141 for the duration of the experiment to mimic local springtime conditions. Each rhizobox was
142 watered weekly with sufficient water to maintain soil moisture at field capacity and prevent
143 root desiccation.

144

145 *2.3 - Soil zymography*

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

155 Two fluorescently labelled substrates were selected for this study; 4-
156 methylumbellferyl ß-D-cellobioside, a substrate of cellulase which was imaged at 365 nm
157 (excitation at 365 nm, emission at 455 nm) and L-leucine-7-amido-methylcoumarin
158 hydrochloride, a substrate of leucine aminopeptidase that was imaged at 302 nm (excitation

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

171 Previous studies have incubated similar substrate soaked membranes for between
172 30 minutes and 3 hours (Giles et al., 2018; Spohn and Kuzyakov, 2014). Therefore, a
173 preliminary study was carried out which found that, for this system, an incubation of 1 hour
174 gave a good level of resolution and UV intensity when viewed (results not shown). Following
175 incubation (1 h), the membrane was placed onto a fresh 1 % agarose gel to minimise
176 bubbling of the membrane during imaging. The membrane and gel were then placed in an
177 UV imaging box (BioDoc-It^2 Imager, Analytik Jena, Upland, CA) and imaged at 365 nm (Spohn
178 and Kuzyakov, 2014). This was repeated for L-leucine-7-amido-methylcoumarin
179 hydrochloride, which was imaged at 302 nm (Ma et al., 2018). This order of substrate
180 sampling was maintained throughout the experimental period (Spohn and Kuzyakov, 2014).
181 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 - 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 - 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).

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.

2.5.1 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').

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 Gray 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).

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)

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).

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 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 Figure 2. Cellulase activity was not solely localised to the axis of sampled roots, and activity away from roots increased with time (Figure 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 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 5a). This shows a delay in the area of cellulase activity when Proctor was in competition compared to isolation. This is demonstrated in Figure 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

289 planted treatments showed a significant effect of treatment ($F_{(2,17)}$ = 4.72, P = 0.02), time
290 ($F_{(2,17)}$ = 44.98, P = <0.01) and interaction between treatment and time ($F_{(2,17)}$ = 12.88, P =
291 <0.01). Model details are in Supplementary Figure 1.

292 Leucine aminopeptidase activity occurred beyond the immediate rhizosphere (Figure
293 4). Mean root associated area activity at 33 days after planting ranged from 0.91 to 3.48
294 pmol mm^{-2} h^{-1}. When Proctor was grown in isolation and inter-cultivar competition, leucine
295 aminopeptidase root associated area steadily increased over time (Figure 5b). At 25 days,
296 the intra-cultivar competition root associated area was lower (31 %) than in isolation (48 %)
297 and inter-cultivar competition (52 %) (Figure 5b), indicating a delay in leucine
298 aminopeptidase activity in intra-cultivar competition compared to isolation and inter-
299 cultivar competition. This is demonstrated in Figure 4, with darker images in the intra-
300 cultivar competition treatment at 18 days after planting compared to the isolation and
301 inter-cultivar competition treatments. There was a significant effect of treatment ($F_{(2,17)}$ =
302 31.72, P = <0.01), time ($F_{(2,17)}$ = 30.36, P = <0.01) and a significant interaction between time
303 and treatment on the root associated percentage area of leucine aminopeptidase activity
304 ($F_{(2,17)}$ = 7.42, P = <0.01). Model details are in Supplementary Figure 1.

305

306 **4 - Discussion**

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

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

323

324 *4.1 - Root axis activity*

325 Both cellulase and leucine aminopeptidase mean root axis activity was much higher than the
326 whole sampled area, 3 - 4 times higher for leucine aminopeptidase and 4 - 6 times for
327 cellulase. This is most likely due to the influence of plant root exudates, which provide a
328 source of labile carbon, increase the rate of SOM mineralisation and, consequently, carbon
329 and nitrogen cycling in the rhizosphere compared to bulk soil (Bengtson et al., 2012;
330 Murphy et al., 2017). However, along root activity did not vary significantly over time for
331 either enzyme class. The area of root system sampled was in the zone of maturation, a zone
332 associated with a stable rate of nutrient uptake (Giles et al., 2018). We hypothesised that
333 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 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 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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597

Figure 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.*

Figure 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 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 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 *– 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.

Figure 1

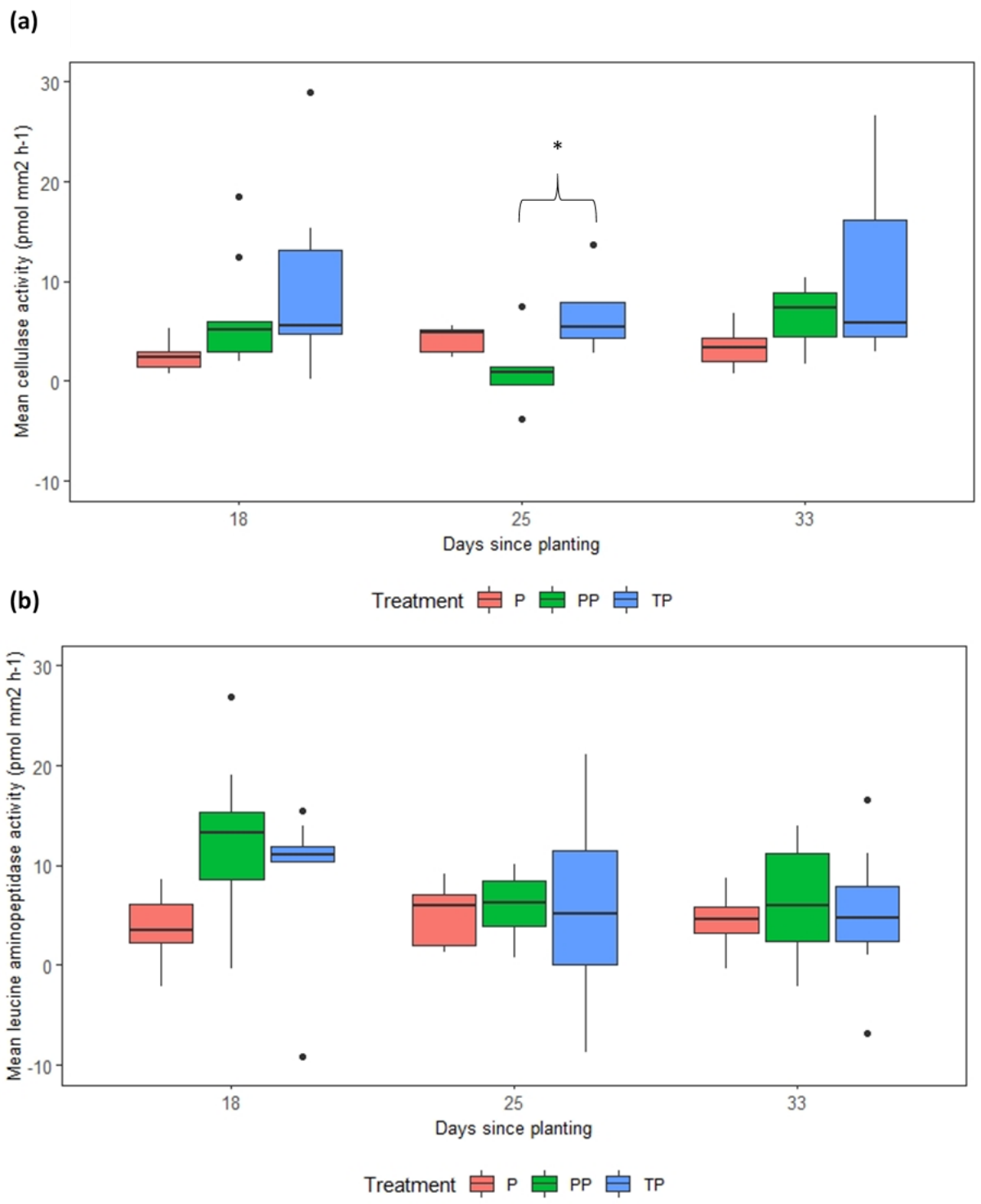

(a)
Mean cellulase activity (pmol mm2 h-1)
30
20
10
0
-10
*
18
25
33
Days since planting
Treatment
P
PP
TP
(b)
Mean leucine aminopeptidase activity (pmol mm2 h-1)
30
20
10
0
-10
18
25
33
Days since planting
Treatment
P
PP
TP

Figure 2

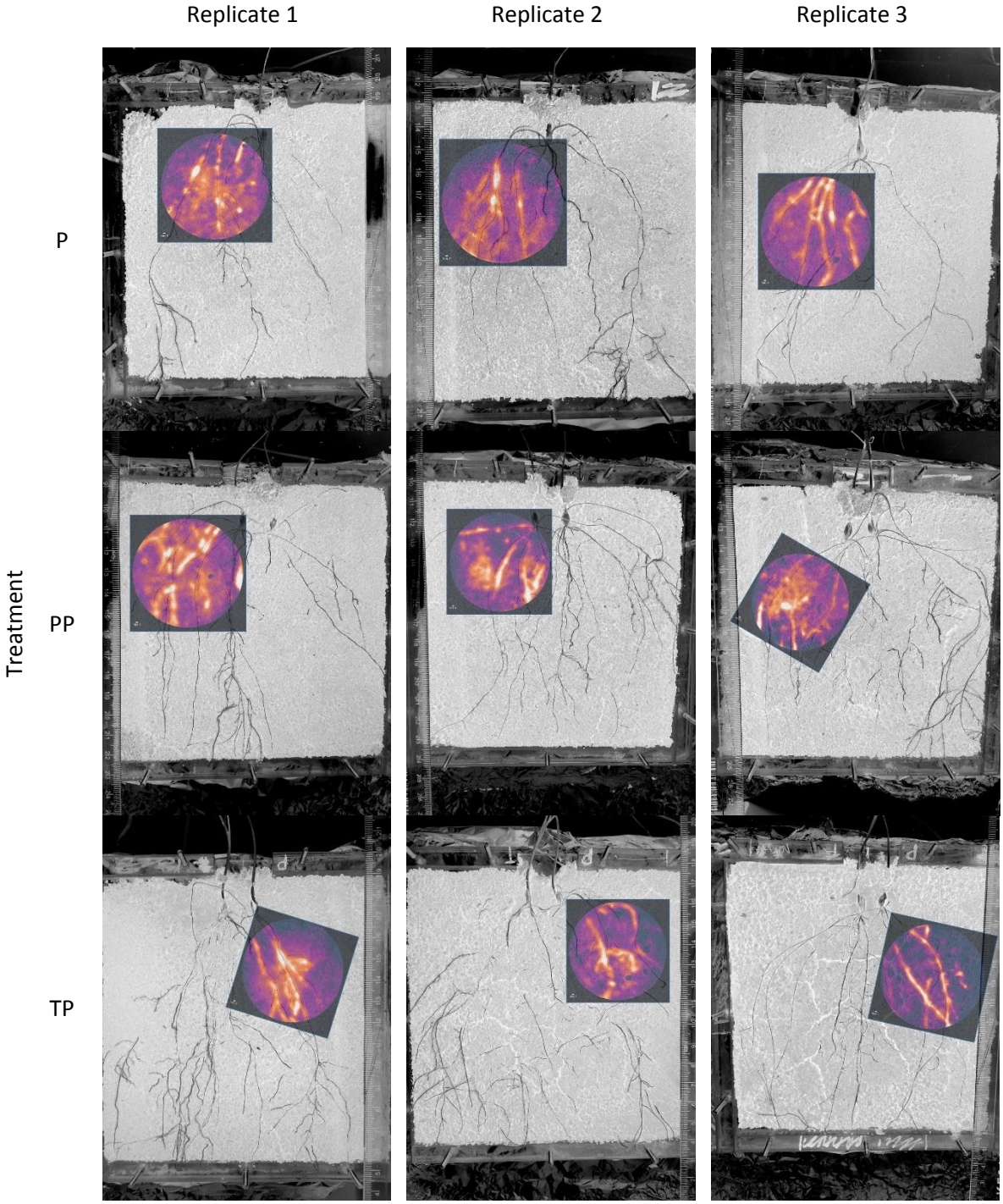


Figure 3

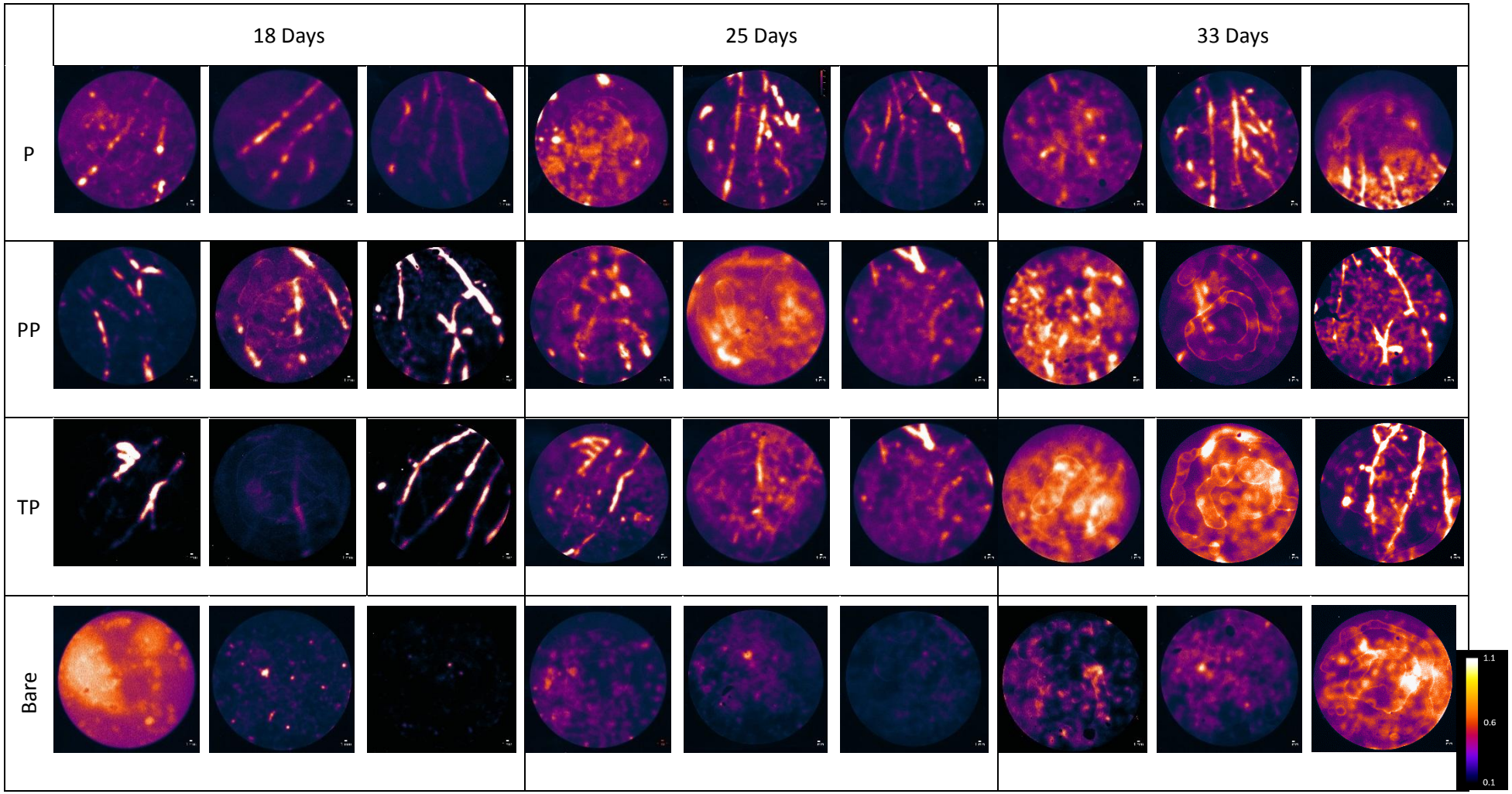


Figure 4

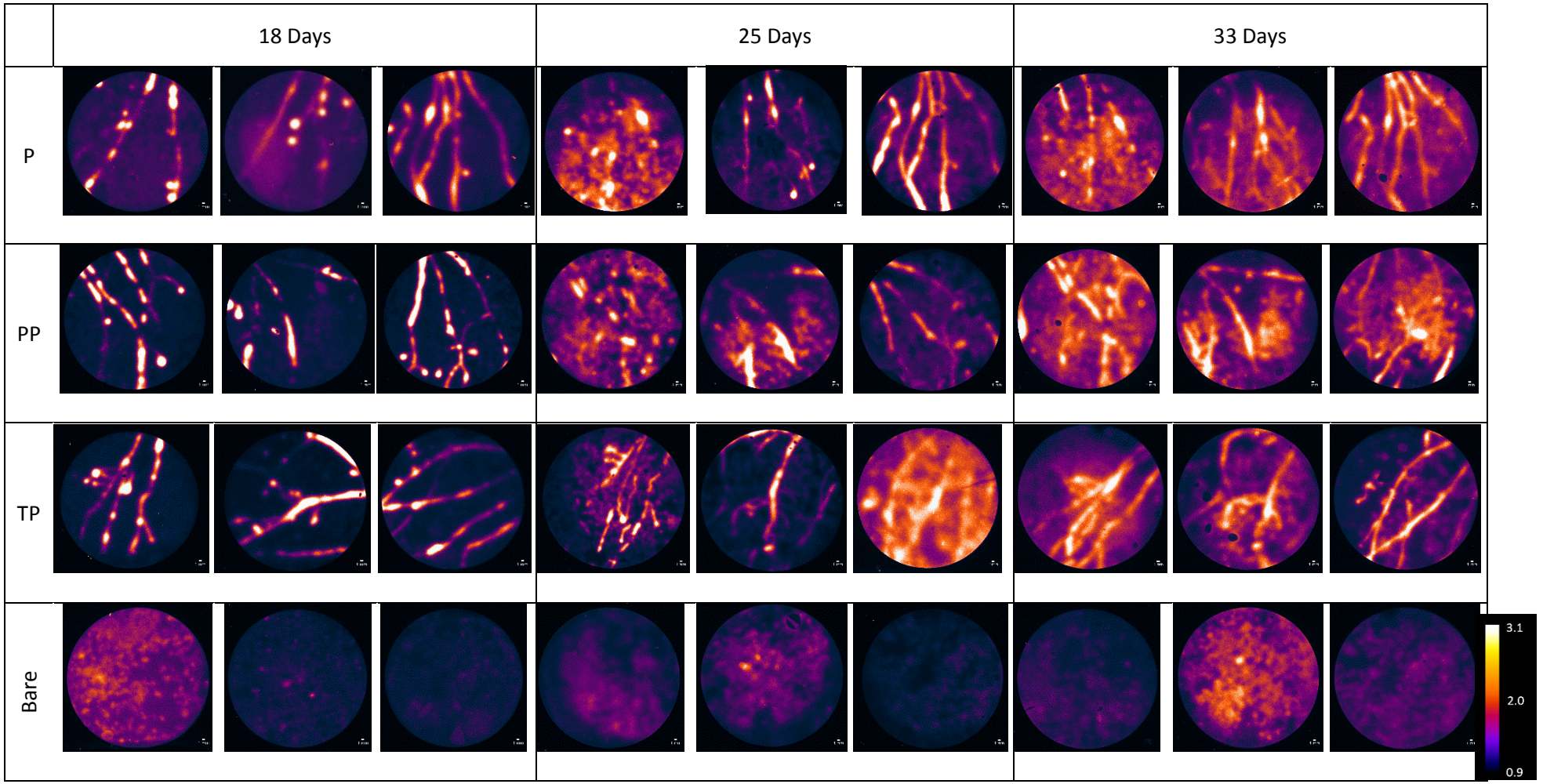
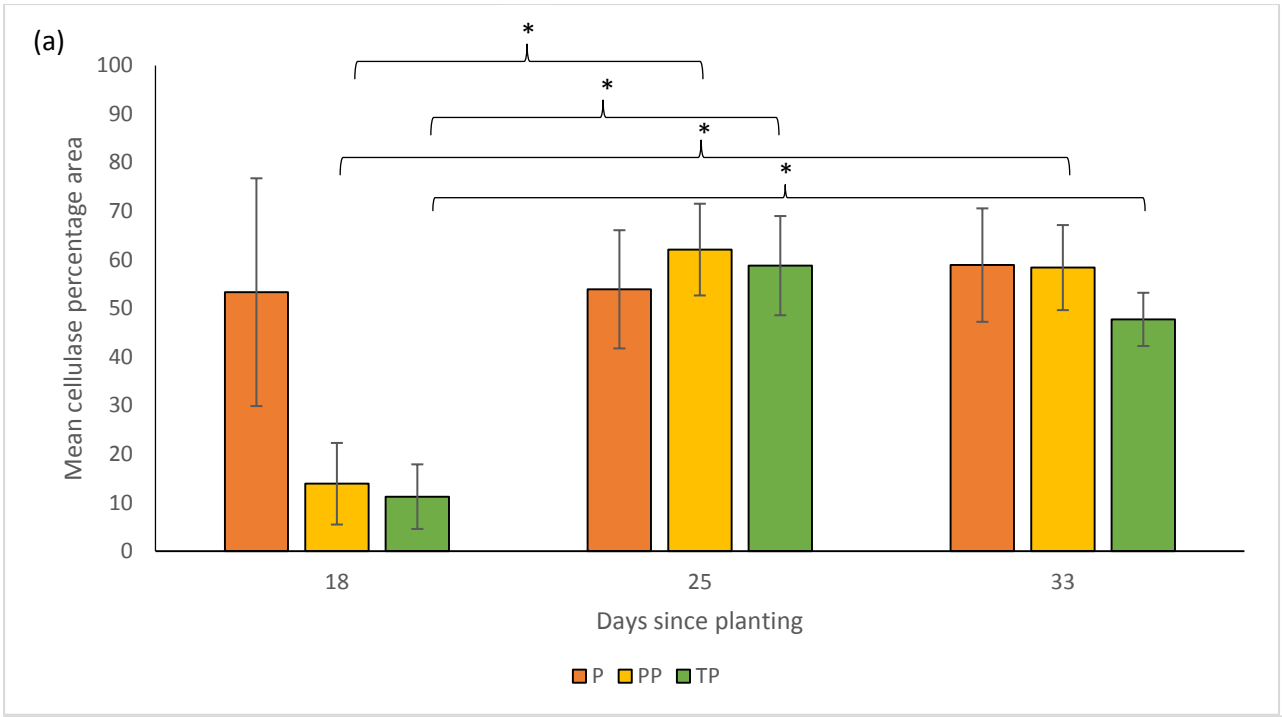
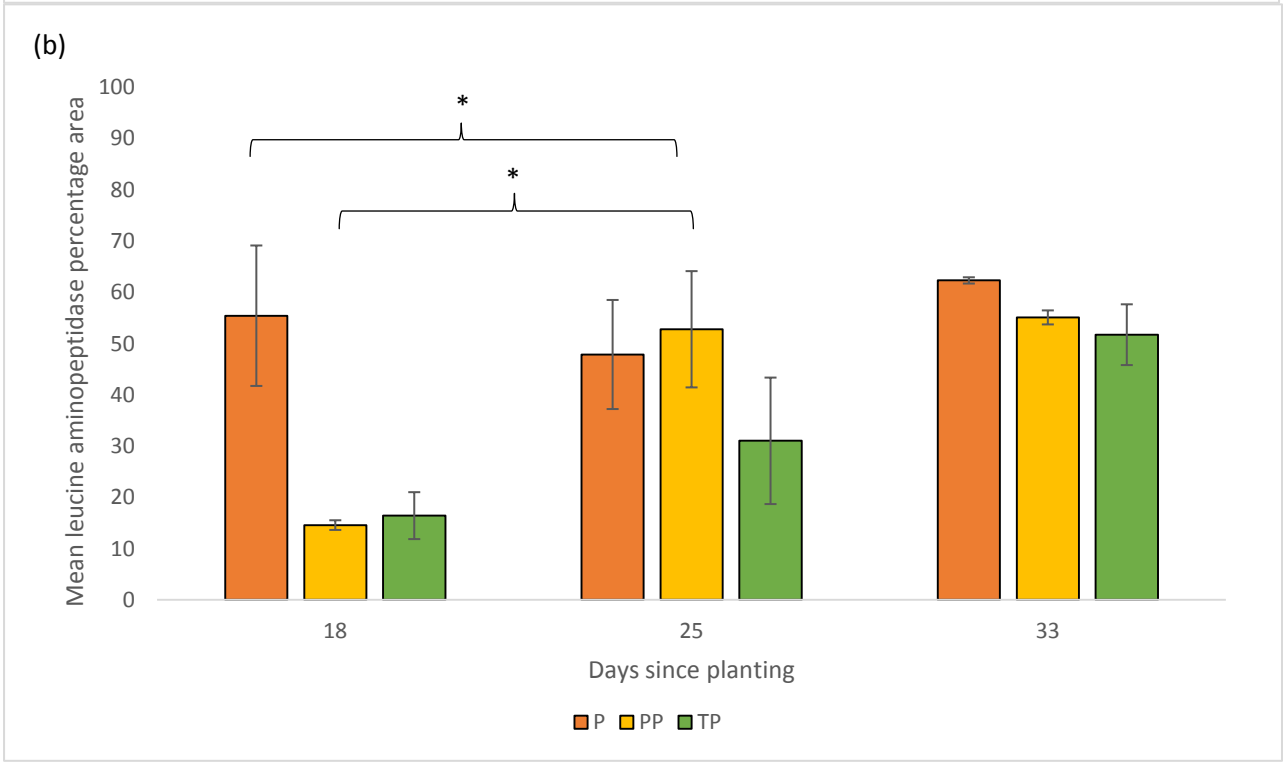


Figure 5


(a)
Mean cellulase percentage area
100
90
80
70
60
50
40
30
20
10
0
*
*
*
*
18
25
33
Days since planting
P
PP
TP


(b)
Mean leucine aminopeptidase percentage area
100
90
80
70
60
50
40
30
20
10
0
*
*
18
25
33
Days since planting
P
PP
TP

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: