COMBINED EFFECTS OF ALTERED N:P STOICHOMETRY AND TREES ON MEDITERRANEAN SAVANNA ROOT DYNAMICS

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Abstract.

Mediterranean grasslands are highly seasonal and co-limited by water and nutrients. In such systems little is known about root dynamics which may depend on plant habit and environment as well seasonal water shortages and site fertility. This latter

5 factor is affected by the presence of scattered trees and site management including grazing, as well as chronic nitrogen deposition, which may lead to N:P imbalance.

In this study we combined observations from minirhizotrons collected in a Mediterranean tree-grass ecosystem (Spanish *Dehesa*), with root measurements from direct soil cores and ingrowth cores, and above-ground biomass to investigate seasonal root dynamics and root:shoot ratios. We investigated responses to soil fertility, using a nutrient manipulation (N / NP additions)

10 and microhabitats effects between open pasture and under tree canopy locations. Root dynamics over time were compared with indices of above-ground growth drawn from proximal remote sensing (Normalised Difference Vegetation Index and Green Chromatic Coordinate derived from near-infrared enabled digital repeat photography).

Results show distinct differences in root dynamics and biomass between treatments and microhabitats. Root biomass was higher with N additions, but not with NP additions in early spring, but by the end of the growing season root biomass had

15 increased with NP in open pastures but not higher than N alone. In contrast, root length density (RLD) in pastures responded stronger to the NP than N only treatment, while beneath trees RLD tended to be higher with only N. Even though root biomass increased, root:shoot ratio decreased under nutrient treatments. We interpret these differences as a shift in community structure and/or root traits under changing stoichiometry and altered nutrient limitations. The timing of maximum root cover, as assessed by the minirhizotrons, did not match with above-ground phenology indicators at the site as root growth was low during autumn

20 despite the 'greening up' of the ecosystem.

In other periods, roots responded quickly to rain events on the scale of days, matching changes in above-ground indices. Our results highlight the need for high resolution sampling to increase understanding of root dynamics in such systems, linkage with shifts in community structure and traits, and targeting of appropriate periods of the year for in-depth campaigns.

1 INTRODUCTION

Terrestrial semi-arid ecosystems are important determinants of the interannual variability in the global land C sink (Ahlstrom et al., 2015) and switch from net C sources to C sinks through the year due to plant phenology (Randerson et al., 1997; Richardson et al., 2013). Large regions in this climate zone are characterized by managed agro-silvopastoral 'savanna' systems, such as

5 the Iberian ('*Dehesa*' and '*Montado*'). These are 20-40 % *Quercus ilex.* Ballota (Desf.) and *Quercus suber* (L.) canopy cover with seasonally variable intercanopy grassland mediated by livestock grazing (Moreno and Pulido, 2009). Regions around the Mediterranean basin are particularly vulnerable to climate change (Giorgi and Lionello, 2008; Sillmann et al., 2013) due to increasing aridity and rising temperatures (IPCC, 2014; Peñuelas et al., 2018). Savanna regions are also particularly badly represented in predictive models (Beringer et al., 2011) due to their complex structure, which is a result of a combination of low

10 water and nutrient availability limiting conversion to other cover types (Eagleson and Segarra, 1985) as well as management practices including tree thinning and grazing (Moreno and Pulido, 2009). *Dehesa* systems are a man-made conversion from oak forest (Joffre et al., 1999) and while water limits in summer, other periods of the year are wet enough for nutrient limitation to dominate. Hence the slow shifting of nutrient limitations in future scenarios due to chronic N inputs (from deposition and management) resulting in ongoing N:P imbalances (Peñuelas et al., 2013) is of particular interest but must be considered within

15 the context of both major structural, micrometorological and soil fertility-associated differences between microsites (Moreno et al., 2013) and the impact of summer drought.

Most ecological attention is focused on above-ground organs, particularly in phenological studies (Radville et al., 2016)), despite the fact that below-ground systems are the main source of carbon dioxide emissions to the atmosphere (Schlesinger

20 and Andrews, 2000), contain 2/3 of the world's carbon (C) stocks (Batjes, 1996) and are integral to uptake of both water and nutrients. In grassland (and by extension, grass-dominated systems such as savannas), below-ground systems are also the site of most competition between individuals (Mokany et al., 2006), the major short-term sink for recently fixed C due to high ratios of roots to shoots (Hui and Jackson, 2006; Mokany et al., 2006), the main source of litter (Casals et al., 2010), and the main contributor to long-term soil C stocks (Rasse et al., 2005)). In most ecosystems, root biomass changes substantially

25 throughout the year, although understanding drivers of phenological change is limited, especially when using quantitative metrics (Radville et al., 2016). This includes considerable uncertainty about both global change factors (Norby and Jackson, 2000); elevated atmospheric CO_2 (Arnone et al., 2000) , increasing temperatures (Bland and Kaspar, 1992; Fitter et al., 1999), precipitation changes, N availability (Dukes et al., 2005) and local fertility (Maestre et al., 2011), other soil properties such as compaction (e.g. Shierlaw and Alston, 1984), as well as species-specific (Eissenstat and Caldwell, 1988) determinants. In

30 many cases, root growth is also desynchronized from production of shoots (e.g. Steinaker and Wilson, 2008; McCormack et al., 2014; Blume-Werry et al., 2017). Hence phenology of above-ground biomass may not necessarily translate to seasonal patterns below ground under both natural and altered experimental conditions.

Typical of Mediterranean regions, grass phenology centres around a summer dormancy, with a dry down in late Spring and a 'green up' in autumn following the onset of rains. In the more 'continental' Mediterranean, cool winters lead to an additional temperature-driven winter dormancy (Thompson, 2005; Milla et al., 2010). The main growing season is in spring for oaks and pasture (Orshan, 1989; Oliveira et al., 1994) before an arid summer with senescence of annual pasture species. Roots in such

5   systems are highly spatially segregated (Moreno et al., 2005) between herbaceous plants, which dominate the upper 30 cm of soil and trees, with deep roots which can access water sources (Castro-Díez et al., 2005) through the dry summer. Due to the relatively low canopy cover, microsites may differ substantially in soil properties due to the influence of recalcitrant oak litter and nutrient transport from deeper soil layers under trees (Gallardo, 2003). Typically, trees also have less tightly coupled above- and below- ground phenology than grasses (Steinaker and Wilson, 2008; Steinaker et al., 2010) due different abilities

10 to store carbohydrates and nutrients over time. A 2015 meta analysis (Abramoff and Finzi, 2015) of root and shoot phenology found that in Mediterranean systems (a very coarse definition including both forests and deserts across only 4 studies), peak root growth tended to lag behind peak shoot growth by over a month on average. Overall shoots were produced in a peak of the main (spring) growing season while root production continued through the year. However, there are very few quantitative comparisons of root and shoot phenology in Mediterranean ecosystems, fewer investigating global change factors, and to our

15 knowledge none in mixed-canopy oak savannas where both microsite factors and extremes of temperature and water availability may promote desynchronization above- and below- ground.

One reason for the relative lack of information on fine root growth patterns in many systems is the difficulty of sampling an opaque, vertically distributed environment usually only accessible from above. Root biomass typically varies spatially due

20 to resource environment heterogeneity (Hodge, 2004) and in diverse systems, small scale patchiness is increased by individual species with different root habits. While root biomass is a direct measure of C stocks, it is also relatively inconsistent in response to global change factors (Arnone et al., 2000) and other physical attributes (such as spatial distribution of root systems, or traits such as root length density (RLD)) may be more relevant for explaining ecosystem functions. However, all field methods to measure roots have significant downsides (Mancuso, 2011) ; biomass methods require destructive sampling as

25 roots must be extracted from the soil and cleaned, while visual methods such as minirhizotrons require both pre-installation of observatories, consideration of artefacts and require lengthy and somewhat subjective post-processing.

At our site, a nutrient (N, NP addition) experiment located in a typical Spanish oak-grass dehesa, (described in El-Madany et al. (2018)), we used a combination of minirhizotrons (providing strict repeatability and information on root phenology), and

30 soil 'ingrowth' cores (measuring root production in root-free soil analogous to recolonization around minirhizotrons) and direct soil coring (providing direct biomass ground truth). These latter methods were direct measures but highly labor intensive and impractical in drought periods. We sampled mostly the herbaceous layer roots, which dominated root biomass in the shallow soil, although these were also likely to be the pool most variable on a seasonal and interannual basis. We hypothesized that 1) fine root biomass followed an annual cycle, developing through a growing season which begins in autumn, and declining with

35 summer drydown the following calendar year, and 2) nutrient addition of N and NP would alter overall root production and

3) nutrient responses to the treatments would be less under trees due to higher intrinsic microsite fertility. If standard resource limitation theory (Bloom et al., 1985) was applicable, outside of periods of water limitation, these nutrient additions should decrease overall root biomass relative to shoot biomass (Fichtner and Schulze, 1992; Shipley and Meziane, 2002) as additions of N should relieve the expected site-level N limitation and shift the system towards a P limited state which would be further

5 relieved by the successive P addition treatment. Considering interacting root-shoot phenology, we also investigated the null hypothesis that root phenology was closely synchronized with shoot phenology, and indexes of above-ground growth (such as Phenocam imagery (hereafter, phenocam)-derived GCC (green colour coordinate) would pair with observed root development if roots were produced at a similar rate between observations as development above-ground.

2 METHODS

10 ### 2.1 Study site, treatments and Above-Ground Phenology

We worked at Majadas del Tiétar, a long-term experimental site in Extremadura, Spain (central location: 39°56'25.12" N, 5°46'28.70"W) from December 2016 until May 2018. The mean annual temperature is 16°C, mean annual precipitation is ˜650 mm, mostly falling between October and April with a typical Mediterranean climate of long, hot, dry summers and mild, wet winters. The soils are classified as Abruptic Luvisols with a sandy upper layer (˜ 9% clay, 11% silt, 80% sand), and a clay layer

15 between 30 and 60 cm (Perez-Priego et al., 2017). The site is a typical Spanish 'Dehesa' with a low density of oak trees (Quercus ilex (L.)) at ˜ 20 trees ha-1. The herbaceous layer, dominated by a seasonally changing mixture of species (Perez-Priego et al., 2015) is grazed by cows (<0.3 cows ha^{-1}) during productive seasons but during dry periods are pastured elsewhere. In this study, cows were absent from May 2017 – December 2017. The site is an eddy covariance (EC)-instrumented FLUXNET site ('ES-Lma') since 2004, and since 2014 has also operated as a stoichiometry manipulation experiment, three 15 m eddy

20 covariance tower installations (El-Madany et al., 2018) and additions of N (as Ca-ammonium nitrate fertilizer), and N and P (as ammonium nitrate and triple superphosphate fertilizer) to one EC footprint each in growing seasons 2014/2015 (100 kg N ha^{-1}, 50 kg P ha^{-1}) and 2015/2016 (20 kg N ha^{-1}, 10 kg P ha^{-1}). There are thus three tower treatment footprints (control; the original FLIUXNET site with no nutrient additions, N; the nitrogen addition only site, and NP; the nitrogen and phosphorus addition site (El-Madany et al., 2018)). The three tower footprints are instrumented with a variety of standardized instruments

25 including phenocams (Luo et al., in review.) using standard Phenocam Network methodology (Sonnentag et al., 2012) and tower-mounted SRS Decagon (NDVI) sensors, as well as seasonal direct measurements of plant biomass and traits.

2.2 Root Observatories

The 36 minirhizotron observatories (1 m x 10 cm (l x d), 3mm plexiglass) used in this study were installed in May 2016, 8

30 months before the first measurement. We used 12 observatories in each of 'control', 'N' and 'NP' nutrient treatments, arranged in sets of 4 around individual trees, randomly selected from suitable candidates. Two minirhizotrons (MRs) per tree were in-

stalled in 'tree covered' (TC) locations (halfway between the stem and canopy edge), and two observatories in 'open pasture' (OP), at least three times the canopy radius from the stem and no closer to any other tree. Each set of 4 observatories was installed on a roughly north-south axis to minimize daily variation in solar heating and all observatories were parallel to the stem. We placed a small bag of silica gel on a piece of high-friction plastic weave within each observatory in order to reduce

5 condensation on the inside of the tube. Despite a tight fit at installation, at first sampling (December 2016), winter soil swelling in wetter microsites had moved some observatories, which we immediately repaired with additional stabilization equipment, resulting in no movement in further campaigns. We excluded these observatories from the first sample, but included them from March 2017 onwards as processed images were not different to other sites. Similarly, two observatories (one in the N, one in the NP treatment) were damaged while in the field during the study. These observatories were carefully removed and replaced

10 with a spare tube which was imaged from the subsequent campaign.

2.3 Minirhizotron Method / Sampling Protocol

We used a custom built minirhizotron camera in our observatories modified from other designs (e.g. Amato et al., 2012). This was a visible light camera (FCB-MA 130-FG, JenCam GmbH, Germany) using a 45° mirror to view the outer tube surface

15 with an adjustable handle allowing movement both on the long axis and in defined rotational positions. Images were collected using VREO OneView software in .bmp format at 4192 x 3104 pixel resolution and consisted of an image of the mirror surface. The camera was lit with two rows of LEDs on either side of the mirror. We trimmed images to remove image overlap which also removed areas of poor illumination, resulting in a 'window' of observation 5.25 x 4.14 cm per image. We sampled all observatories on six dates spanning December 2016 to March 2018, corresponding roughly to bimonthly measurements in

20 sequential phenology phases of winter, early spring, late spring, late summer drought, winter, and early spring. Each time, we calibrated the camera against a grid of known dimensions fixed to an unburied observatory and collected a complete rotational profile of images (8 total) every 5 cm along the minirhizotron (complete coverage) with image midpoints 0 to 23 cm vertical depth and three additional depths (28, 38 and 44 cm), resulting in 2880 images per sampling date. In December 2016 we only measured two depths (10 and 20 cm), while in May 2017, September 2017, and March 2018, we imaged the entire observatory.

25 We also took a second set of images at all depth positions from the 'control' treatment alone in mid-May 2017, 10 days after the complete survey in early May. This corresponded to a few days after a major rain event, allowing an observation of the short-term response in fine roots to increased water availability.

2.4 Image Analysis

30 We rescaled each trimmed image to a standard 1123 x 1434 pixel resolution. After manually filtering images for quality (removing those with image artifacts or ambiguity in cover), a 10 x 13 grid was superimposed over each image, producing 130 squares. Each square was marked as either 'roots present' or 'roots absent' following the criteria that a visually unambiguous, apparently living (i.e. with clearly defined form and no obvious structural breaks) root, crossing at least half the square long

was present. The proportion of squares with roots per image was calculated (Root Cover Index, RCI). This required 0-2 minutes per image and had significant time advantages over comparative methods which focus on markup of all roots in the image. We validated this method against a standard methodology using the open source minirhizotron interpretation software Rootfly 2.02 (Birchfield and Wells, 2011) to mark up all roots in the images, using 100 randomly selected images from each of the March

5 2017, May 2017, September 2017 datasets for calibration.

2.5 Direct Soil Measurements

We collected ancillary measurements of root biomass from two non-concurrent methods, sampling within 1m of the minirhizotron tubes; soil cores (Dec 2016, March 2017) and two rounds of ingrowth cores installed in December 2016 (removed May

10 2017, December 2017) and December 2017 (removed March 2018, May 2018). Ingrowth cores were installed by removing a 13 cm, 4.5 cm wide soil column with an auger, homogenizing the soil and removing the live roots and replacing the soil inside a metal 13 cm core with three large root windows. Two cores were recovered from each location at each date using a soil auger (i.e. n = 72). Soil cores were 30 cm deep in December 2016 (n = 36, one per minirhizotron) but reduced to 20 cm depth (n = 108, 3 per minirhizotron) on subsequent samplings but in this manuscript we only consider the top 13 cm, to pair with

15 root ingrowth cores. The December 2016 Experiment was amended with a separate amount of replacement amount of litter designed to equalize the total root litter in each core to a previously observed seasonal site mean. This was part of a separate isotope labelling experiment not detailed in this paper (Nair et al in prep.). Root decomposition rates in dehesas are typically very high (Casals et al., 2010) and when combined with a relatively coarse threshold for sieving we judged that these data were acceptable to use along with unamended cores as most root litter added would be fragmented by the time of sampling. In all

20 cases, roots were extracted from soil samples by passing through a 2mm mesh, and picking through the remaining material for intact roots. The extracted material was cleaned in distilled water and dried at 40°C until weight loss ceased. The final weight was recorded as root biomass.

2.6 Comparisons with site-level and above-ground measurements

25 Additional to the minirhizotron measurements collected in this study, we compared root profiles both a) over time and b) with depth to site-level instrumentation. These were i) harvests of above-ground understory biomass made in each treatment area, dried to determine dry weight on 23 March 2017 and 25 May 2017, used to calculate root:shoot ratios (henceforth, RSR) and ii) phenocam derived GCC products (Luo et al. in prep.) and grass NDVI measured from the towers. In both cases these above-ground measurements were not directly paired with root measurements, so RSR and phenological synchronicity is examined

30 on the treatment-level.

2.7 Statistical Analyses

All statistical analyses were conducted in R 3.50 (R Core Team, 2018,). We performed a series of (OLS) linear and generalized-linear regressions, aiming to explain i) relationship between image RCI with image RLD, volume per area or root number and this fast markup and ii) use observed differences between treatments (control, N, NP) and locations TC (tree canopy) and OP

5 (open pasture) to explain root dynamics (both from minirhizotrons and direct soil cores) in this system.

   For converting between our mark-up methodology and conventional root properties, we tested different empirical models (i.e. linear, 2-6 degree polynomial, exponential) and chose the best model for further analyses using BIC model selection, bearing in mind that the granular RCI measurement would always fall within a range of 0-100 so polynomial models with inflection points outside this range could be valid for conversion. This model formulation selected with the BIC was then

10 cross-validated against the dataset using k-fold cross validation (k=10), that was repeated 500 times. Data was transformed where appropriate to meet assumptions of normality (Shapiro-Wilks test) and reduce the clumping of low observations.

   We used mixed effects models to understand the effect of nutrient and habitat on both minirhizotron observations (predicted RLD) and soil measurements (root biomass). In these, we assumed that individual minirhizotrons and sampling dates were crossed random effects but did not implement a time series correlation structure due to the large interval between observations

15 and the rapid turnover of species (Fernández-Moya et al., 2011) throughout the year. We tested for appropriateness of including interaction effects between treatment and location by comparing models with and with-out interaction terms, checked residuals for normality, and report P values for these models using Satterthwaite approximation of degrees of freedom. (Satterthwaite, 1946). We also performed simple linear model comparisons within individual sampling dates, reducing the models to their most parsimonious form via Akaike Information Criterion (AIC)

20 ## 3   RESULTS

3.1   Validation of Minirhizotron Markup

We found a good correlation of our cover-based markup at our site against all Rootfly-derived indices RLD (Figure 1), volume per area, and root number were well predicted using the fast cover markup with the best predictive models (RLD: 2nd order polynomial, k-fold validation $R^2 = 0.77$, volume per area: 3rd order polynomial, k-fold validation = R^2 0.81, root number: 2nd

25 order polynomial, k-fold validation $R^2 = 0.71$)). Particularly high root density caused saturation in RCI but not in Rootfly, but as this affected only a small number of images in the dataset and residuals were otherwise normally distributed , we judged this as acceptable. The residuals for RLD were quite dispersed (NRMSE = 0.96) but applied we judged this sufficient for predicting across a large image dataset. We converted treatment mean root covers to RLD using this observed relationship in all further analyses.

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3.2 Depth and seasonal profile from minirhizotrons

RLD from the minirhizotrons decreased with depth at all periods of the year (Figure 2), with the deepest soil (< 40cm) having a mean RLD of 0.09 ± 0.03 (S.E.) mm mm^{-2} in the most abundant period (May 2017, in contrast to maximum above-ground biomass and peak shallow RLD in March) and 0.03 ± 0.00 in the least abundant period (December). The seasonal cycle of root

5   production, peaking in March, was most evident in the shallow soil (Figure 3 where maximum RLD at was 0.55 ± 0.03 mm mm^{-2} in OP, and 0.36 ± 0.03 mm mm^{-2} in TC. Following March, root biomass declined in September (mean 0.05 ± 0.01 mm mm^{-2} in OP, 0.03 ± 0.00 mm mm^{-2} TC), and stayed relatively constant until December (OP: 0.08 ± 0.02 mm mm^{-2}, TC: 0.05 ± 0.00 mm mm^{-2}). In general, the minirhizotron images contained less roots in TC than in OP locations (Figure 3).

10   ### 3.3 (Seasonal) treatment differences from minirhizotrons

Differences between the nutrient treatments were smaller than variation between locations or within time but evident in some periods of the growing season. Minirhizotron-calculated RLD tended to be higher in all measured depths in the NP treatment during the growing season, peaking in the March 2017 sample (Figure 2). Taking the cumulative RLD in the top 13 cm of soil (corresponding to the depth of our ingrowth cores and containing the majority of roots, we compared the treatment x location

15   effect on RLD. Both location ($P < 0.005$) and NP treatment ($P < 0.05$) had significant effects on the RLD calculated from the minirhizotrons over the experiments, but the N treatment did not differ from control ($P = 0.40$). This difference tended to be greatest during the spring as low average RLDs outside the main growing season limited the potential for variation. When separated into individual sampling dates, this nutrient-driven difference in Spring was stronger in 2017 than 2018 (Table 1).

3.4 (Seasonal) differences from direct soil measurements

20   The two methods of direct soil measurement (soil cores and ingrowth cores) produced similar results. While these were less frequent than the minirhizotron measurements, they also indicated seasonal changes in roots (Figure 4). Root biomass in December 2016 in the top 13 cm (canopy median 2020 kg ha^{-2}, grassland median 1140 kg ha^{-2}) was substantially lower than in the following March (TC median 6390 kg ha^{-2}, OP median 5670 kg ha^{-2}). At this low point, there were no significant treatment effects but a difference between locations ($P < 0.05$). As the ecosystem developed into the spring growing period there

25   was a difference in these absolute stocks in March as following transformation to fit the assumptions of linear models, the N treatment had significantly more ($P < 0.05$) roots than the NP and control (although NP tended to be higher than control cores). This treatment difference was strong enough that the most parsimonious model did not include a location effect unlike all other comparisons.

From the ingrowth cores, we also found significant effects. The most parsimonious model for recovery in May found an effect

30   of both nutrient treatments, where both nutrient amended treatments increased over control ($P < 0.05$) and also significantly less production in OP compared to TC ($P < 0.001$) but no interaction. For the year-round ingrowth cores the treatment effect was lost in December but the highly significant ($P < 0.001$) location effect remained. Likewise, in 2018, the most parsimonious

models showed that in March (where an interaction term remained in the model), the N treatment had significantly more roots (P < 0.001) and differed between locations (N-location interaction, P < 0.01). In May, both location (P < 0.01) and both nutrients (N, P < 0.05; NP, P < 0.05) had significant effects. Post-hoc Tukey HSD groupings for linear models for all individual dates for direct soil measurements are shown in Figure 3.

3.5   Short-Term Rain Pulse Responses and Comparison with High Resolution Site Measurements

The short-term minirhizotron measurements in May 2017, separated by 7 days around a rain pulse (1 day pre-pulse, 6 days post-pulse) showed a clear proliferation of roots following the pulse (Figure 5). This increase was significant (P < 0.001) in both OP and TC locations and evident in all soil depths measured with the minirhizotron. Similar short-term responses were

10   evident in NDVI and GCC during this period (Figure 6). The relatively sparse distribution of minirhizotron campaigns means we were unable to diagnose similar responses to other rain events although from these site-level above-ground indices the May event was the largest shift against overall trend in the year. From comparison from site indices it is also notable that while the minirhizotron root-cover time series correlates well with both NDVI and GCC in respect to the March peak and decline into the summer dry period, root cover was not in sync with either of these indices in the autumn growing season where root cover

15   was low in 2016 and remained low at the December 2017 sampling, but had recovered in both March 2017 and 2018 (Figure 5). This indicates that the majority of root growth was in the period of December to March unsampled in either year by the minirhizotron campaigns and after the apparent 'green up' of the ecosystem from near-surface remote sensing.

3.6   Comparison with Above-Ground Biomass

For the two campaigns where above-ground biomass data was available for the vegetative layer, even only using the top 13 cm

20   of soil indicated that RSR leaned heavily towards roots (in control treatments in March, these were 20:1 and 15:1 in OP and TC respectively, while in May these were 21:1 and 22:1). Nutrient treatments showed a typically lower ratio in the N compared to NP treatment in March but not in grassland in May and generally, root:shoot were higher in N than control but equal or lowerin NP than control (Table 2). Regardless of this potential change in root:shoot ratios, the absolute difference between amount of roots and shoots was substantially larger than any changes induced by nutrients.

25 ## 4   DISCUSSION

Measurements of roots must always be carefully interpreted (Mancuso, 2011) to assess ecological meaning as all procedures are both affected by methodological biases and logistically intensive to perform. Notably, we found a different directional effect of habitat type between open pasture and under canopy locations which could possibly be due to methodological limitations. Using our direct methods, we treated all root biomass remaining within the sieve as roots, while the visual MR method allowed

30   roots to be ignored if broken or clearly dead. On the other hand, artefacts due to MR presence are particularly acute close to installation (Joslin and Wolfe, 1999). MR equilibrium time can range from a few months (see Johnson et al., 2001) to years

(Mueller et al., 2018; Strand et al., 2018) and there is little consensus towards an appropriate time to leave observatories before good data is collected. This is especially true in manipulation experiments but measurements during an 'stabilization' phase after installation can be assumed to have similar biases between treatments and thus relative differences in this period used to understand effects on root dynamics, if not absolute equilibrium root presence (Burke and Raynal, 1994). Slow-growing

5   species may also take considerably longer than this time to equilibrate (Strand et al., 2018) but most *Q.ilex* roots probably reached deeper soil layers (Moreno et al., 2005), causing both of our methods to mostly sample herbaceous layer roots. In any case, the 8 months before first measurements included summer drought and almost total annual mortality of the herbaceous layer, followed by autumn rewetting which we expected to have stronger effects than installation on root presence around minirhizotrons. Hence we expected time since installation to be unlikely to have impacted the observed trends.

Similarly, another potential source of errors in our study is the effect of low replication, particularly acute in systems such as *Dehesas*, which are highly diverse and heterogeneous (Moreno, 2008). Below-ground systems additionally cannot be seen before sampling and representative locations may often be assigned based on above-ground properties. We used 6 replicates per treatment-vegetation combination for MRs (36 in total), informed by installation and sampling effort (2 days to fully sample

15   and also lengthy post-processing). This level of replication was similar to other multifactorial field experiments using MRs (Ziter and Macdougall, 2013; Arndal et al., 2017; Mueller et al., 2018) and sites were relatively consistent between measurements. Additionally, with the notable exception of the reversed location effect between MR and soil cores, our treatment trends tended to be consistent between methods (Figure 3, Figure 4). We hence treat the differences shown by different methods as being both real and ecologically relevant for the rest of this discussion.

4.1   Treatment and Location Effects on Root Measurements Between Methods

The nutrient treatments used at each site were designed to induce a N:P stoichiometric imbalance with N addition and reduce it by restoring N:P ratios when adding P alongside N (El-Madany et al., 2018). Differences in N and NP treatments together can be interpreted as N effects and differences in N treatments alone can be interpreted as P deficiency, while NP effects without

25   corresponding N effects co-limitation of both nutrients together. We expected these to differ between habitat types at our site. Tree-grass systems, combining short-term and long-term optimality in vegetation habit (Eagleson and Segarra, 1985)), usually occur in areas with major seasonal variation in water availability, leading to major variation in soil and understory properties between microhabitats (Moreno et al., 2013). These typically include altered soil water storage (Joffre and Rambal, 1993), and water stress (Joffre et al., 1987) as trees increase shading, allowing reduced transpiration beneath the tree, while extending

30   their root systems to obtain water from both UC and OP locations (Cubera and Moreno, 2007). Additionally, litter input and waste from animals congregating beneath trees result in higher SOC (Howlett et al., 2011), N (Gallardo et al., 2000) and sometimes P (Gallardo, 2003; Rolo et al., 2013) concentrations beneath trees and in many cases lead to higher herbaceous layer above-ground biomass (Moreno, 2008). In March, this was also observed in our study, alongside an increase in root biomass in soil cores beneath trees. Nevertheless, (above-ground) production is frequently higher in open pasture than under canopies

(Fernández-Moya et al., 2011; Rivest et al., 2011), as we found in May (Table 3). This inversion of directional effect, which is probably the due the combination of sustained grazing pressure through the season and increasing water demands into the summer dry down (Perez-Priego et al., 2017), highlights the importance of seasonal observations in this system.

The same difference was not found from minirhizotrons, where we consistently observed greater RLD in open pasture throughout the year. The major alteration by tree canopies on environmental properties also affects herbaceous layer diversity (López-Carrasco et al., 2015) and community vegetation composition (Marañon, 1986), which affects plant trait distributions. RLD and specific root length are important belowground plant traits linked to plants ability to explore soil and acquire resources (Fort et al., 2014) and may be potentially important to competitive success in herbaceous layer communities. Our MR observations indicated a significant increase in RLD in the NP treatment, present in the growing season of both years but strongest during the spring of 2017. However, the direction of the habitat effect difference differed between RLD calculated from minirhizotrons and root biomass measured from cores. This was not a methodological effect as the RCI method fit well with RLD across most of the range of data (figure 1), albeit with a polynomial fit due to high root density within some individual squares. We note that this correlation was a calibration for our site only, and that MR observations may notably not correlate with biomass differences between treatments as other root traits and properties such as root length may be affected in dissimilar ways by the nutrient and habitat treatments. This is especially important for the uppermost soil layer, where fine roots disappear progressively with soil dryness but remaining roots must grow in diameter to support developing water harvesting architecture in deeper layers. Rapid processing of roots from soil cores does not allow architectural properties to be easily examined, as roots are often fragmented during sieving and breaking up soil clusters whereas MR measurements, while invasive, do not alter the position or distribution of roots once they have colonized the area around the tube. Excluding this possibility of miscategorised root biomass in soil cores at different periods of the year (i.e. more root litter later in the season), we expect the difference between minirhizotron RLD and absolute root mass and the different time structure of observations (MR RLD decreased from March 2017 to May 2017 and root biomass increased slightly) to be due to difference in responses of root traits such as RLD when compared to trends in traits such as root diameter between treatments. Stoichiometric changes may provoke responses in terms of root system architecture and traits (Drew, 1975) , potentially due to either a shift in species community or in traits themselves (e.g. root diameter). Thus, plants exploring shallow soil in the P-limited N treatment but producing more primary roots in the NP treatment grasslands alongside general increased production (Figure 3) would explain the methodological difference between treatments. Unlike N ions, P is relatively immobile in soil, so in general, P availability has a major effect on root system architecture, with availability promoting primary root growth at the expense of lateral development (Williamson, 2001) as less surface soil exploration is necessary under high availability of relatively immobile P, reducing the need to access P-rich plant residues (Lynch, 2011) and promoting development to reach deeper soil layers and water-soluble nutrients (such as NO3-). Hence the distinct NP treatment effect from minirhizotrons may be due to more primary roots under the NP treatment, following areas of disturbance, previous root channels or soil objects (Rasse and Smucker, 1998) to allow penetration of deeper soil areas. Indeed, we observed a relative shift towards deeper-located roots in the MR measurements late in the growing season (Mar-May 2017, Figure 2) with most roots lost in shallow soils as they dried out at the onset of summer. This shift could be in terms of traits or overall species composition and associated traits; little information on specific species root

phenology in Dehesas is available although it is clear that both root growth seasonal timings and exploratory properties differ substantially between species In the soil cores, the elevation of both N and NP biomass may be indicative of overall increases in root biomass (including lateral roots) in the N treatment, which are not detected by our minirhizotrons. The methodology allowing us to process large numbers of replicated observations did not allow an assessment of root thickness (potentially

5   leading to exponential increases in mass compared to diameter) and thus potentially, a denser root system under N, supporting foraging for water-mobile N ions in deeper soil. A better pairing between difficult to measure root traits and relatively accessible above-ground plant traits may allow a more diagnostic understanding of root behavior under a highly diverse (Moreno et al., 2016) above-ground system and further differentiation between these treatments.

10   A trait effect of nutrient treatments would also explain the difference observed between OP and TC locations throughout the experiment where directional effects reversed between minirhizotrons and cores. Similar experiments at our site have previously reported changes in canopy functional traits in response to nutrient addition (Migliavacca et al., 2017) but are unable to diagnose changes belowground. A greater abundance in 'high RLD producing' species in OP, where RLD and soil exploration is a critical competitive trait may have led to a greater response for the minirhizotron observatories. On the other hand, an

15   increase in lateral roots under P deficiency (i.e., our N treatment) may have had minimal effects on RLD observed compared to the shift in traits when productivity was increased by extra P (in the NP treatment) restoring stoichiometric ratios. Tree cover has major functional effects on herbaceous layer species (López-Carrasco et al., 2015) with more (relatively high RLD) graminoids compared to legumes and forbs and N abundance tends to shift communities towards grass dominance (Bobbink et al., 2010). As nutrient limitation is lower (due to thick organic layers and relatively high nutrient abundance) in these mi-

20   crohabitats, we may expect an overall weaker response in nutrient-acquiring strategies compared to grasslands when nutrient additions are applied, as generally observed in RLD in this study, even if extra nutrient and re-cycling through litter means root biomass increases, as observed, in shallow soil.

4.2   Changes in Root:Shoot Ratio

25   Conclusions about the NP treatment effect on root architecture rather than absolute amount relative to the N treatment are supported by general increases in RSR in the N treatment (Table 3) These shifts in ratio were particularly extreme in the N treatment but not the NP treatment. In NP treatments, RSR mostly decreased, following standard understanding of resource limitation belowground and decreased whole-plant investment into nutrient acquisition, but ratios did not change so dramatically so this relative decrease in RSR was still a large absolute increase in root biomass. The only large increases in RSR were

30   in the N treatment, potentially indicating that plants were investing more belowground to alleviate the induced P limitation. Nutrient stress may increase RSR if these deficits are the major growth constraint (Erikson, 1993; Ågren and Franklin, 2003) both on the ecosystem and individual plant level (Fichtner and Schulze, 1992), suggesting that while the overall system may have shifted in terms of traits or community in the NP treatment, as observed in RLD, the system was more 'nutrient stressed' under the N only treatment, resulting in greater investment of C belowground. The difference between control and N treatment

RSR was notably not found in May in OP, potentially due to the faster late-season dry down in more exposed pasture locations. Generally, the ratios (15-30:1) found in this experiment were very large and driven by a high root biomass at our site (despite being calculated based on only the top 13 cm of soil), peaking at around 8000 kg ha-1. Other studies in similar landscapes in southwestern Europe found maximum root masses of around 2000 kg ha-1 (dependent on cover type; Rolo and Moreno, 2012),
5   2500 kg ha-1 or under in nearby walnut forestry, 300-400 ka ha^{-1} (López-Carrasco et al., 2015), despite pasture production being around 2 x higher than our site (G. Moreno, pers. comm). Root:shoot ratios at these sites were considerably lower than ours (e.g, 4, (Jongen et al., 2013) and more consistent with global means ('temperate grassland' mean 4.2, and 'savanna' mean 0.6 (Mokany et al., 2006)). We are confident in the magnitude of root masses reported in this study due to their consistency both over time and between treatments (Figure 4) and RSR in systems such as Dehesas can be shifted in favour of roots by
10   multiple factors such as the (seasonally) dry environment, (Chapin et al., 1993) where root systems tend to be shallow and wide (Schenk and Jackson, 2002), and grazing of above-ground vegetation (McNaughton et al., 1998) which reduces LAI and transpiration, hence favouring nutrient rather than water acquisition, but also requiring as root foraging for nutrient for this removed biomass. As the year progressed towards the summer drought and grazing pressures increased, RSR shifted towards roots, and root profiles became more evenly vertically distributed (Figure 2), agreeing with this explanation of the ratios at the
15   site, while a combination of weather and grazing pressure during the year studied may have shifted the balance in towards root production. However, similar root: shoot ratios to ours have been reported earlier in the season (Puerto, 1992) at dehesa sites. More frequent sampling is necessary to disentangle whether these ratios are representative of the rest of the growing season.

4.3   Seasonality of Root Biomass and Linkage to Above-Ground Phenology

20   Our site has a highly seasonal climate with severe deficits of water in summer and an excess in winter (Perez-Priego et al., 2017). As well as a long-term seasonal cycle of root biomass observed from all treatments, in the control treatment we observed that that root growth responded to short-term rain events in the late growing season (Figure 5) as RLD increased in all soil depths following a rain pulse in May. This event was paired by a clear response in both NDVI and GCC interrupting the general decline in the late growing season dry down as both shoots and roots responded simultaneously. However, measurements in
25   autumn implied a considerable desynchronization between above- and below- ground during this time in both 2016 and 2017. Most of the root production appeared to occur after the measurement in December and before the measurement in March, indicating that the key periods of root production were overwinter rather than early or late in the growing season. The initiation of major periods of root growth had not begun by December in either year, while both GCC and NDVI of grassland areas had reached growing season levels by this point in all treatments (Figure 5). This difference was presumably due to high water
30   availability but decreasing light availability in autumn leading to prioritizing of above-ground LAI development compared to decreasing water availability but abundant light in spring. As both GCC and NDVI are commonly used to track plant phenology (e.g. in a semi-arid grassland Browning et al., 2017), this was particularly interesting as grassland systems are expected to be relatively highly synchronized above- and below- ground (Steinaker and Wilson, 2008). This difference was substantially larger than the 2-4 weeks observed in other grasslands (Steinaker et al., 2010) , and, coupled with the implicit high root: shoot

ratio during the later growing season, indicates that of overall productivity and C status (i.e. the coupling between GPP and RECO) of this system may depend largely on the below-ground system. Additionally, unlike the four Mediterranean studies in Abramoff and Finzi (2015)'s meta-analysis, this desynchronisation also indicated that leaf growth was before root growth in our system. This may be linked to the severe summer drought that our site experiences, with extensive root systems being more

5   important for water uptake in the late growing season than nutrient uptake for biomass production in the early growing season. On the other hand, both roots and above-ground vegetation responded to a rain pulse in May (during the dry down late in the growing season). This indicated that at some points in the year, likely when water stress is high, short-term events are highly coupled. While our MR data was not high resolution enough to study this at other points, seasonal effects did not appear to be so closely tied to rain pulses above-ground at other points in the year (Figure 6), and ecological effects of seasonal and fertility-

10   related changes are also likely heavily modified by annual variation (Vaughn and Young, 2010). Hence, the expectation of root production in direct synchronization with shoot production is clearly incorrect at least in some times of the year. High resolution measurements are necessary to capture such events and advancements in minirhizotron technology including autonomously operational, frequent image capture (matching above-ground proximal remote sensing) and methods to analyze these data, will allow much greater understanding of seasonal cycles in root production and their link to above-ground productivity.

15   **5   Conclusions**

Much of our ecosystem-level understanding of plant seasonality and responses to global change is drawn from above-ground measurements but it is not clear how well this understanding holds belowground. In this study, from both minirhizotron and direct soil measurements, we found a seasonal cycle of root dynamics broadly matching the above ground 'growing' season and seasonal patterns of above-ground biomass as inferred from near-surface remote sensed measurements. However there

20   was a notable delay in root production in the early growing season with most root growth not commencing until well after the regreening of the system. Nutrient treatments (N and NP addition) increased root biomass in productive periods for understory vegetation, indicating that there was not an effect of altering ecosystem-level stoichiometry on root production, and changes were a result of increased N availability. However, NP treatments in grassland areas did show increased RLD over other treatments indicating a stoichiometry-driven shift in root traits or plant community following fertilization. Further work should

25   focus on i) understanding community and plant trait responses to ecosystem stoichiometry, especially in highly diverse and seasonal communities with a large pool of species able to exploit changes and ii) increasing temporal density of belowground observations. Phenology responses to global change factors may be on the scale of days and hence high frequency sampling is essential to understand the belowground response to such forcing and the mechanistic effects of global change on such communities.

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5   interpretation. Gerardo Moreno provided both above-ground data, and fieldwork assistance. Marion Schrumpf and Mirco Migliavacca helped design the experiment, all authors helped in manuscript preparation.

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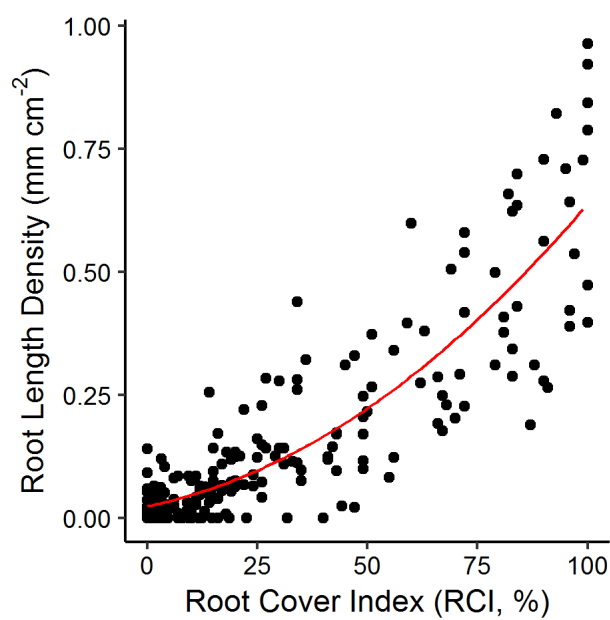
Figure 1. Root Cover Index (RCI) against root length density (RLD) for a random sample of images (n = 300) from three imaging sessions at our site. A simple model fit to these data with an r2 of 0.77 was used to predict RLD for all other figures.
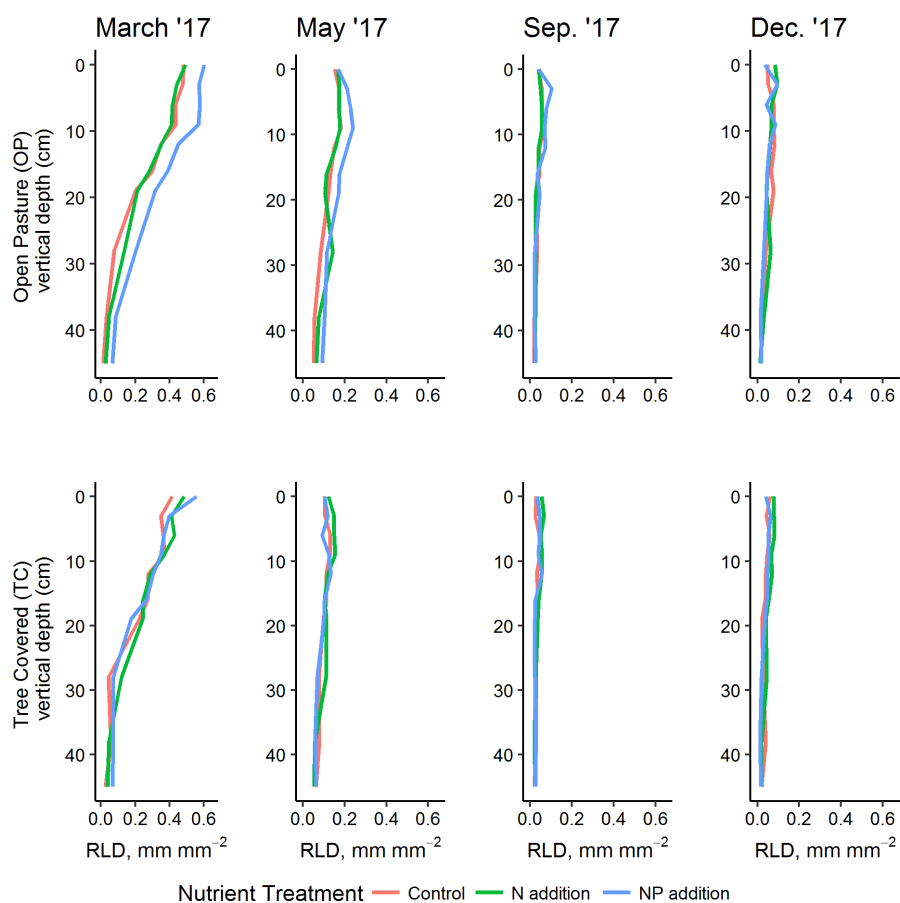
Figure 2. Vertical root profiles (mean of minirhizotron measurements) for four dates in 2017. NP treatments tended to diverge in the grassland, especially in shallow soil where RLD was highest. Treatment effects in shallow soil were reflected throughout the depth profile.
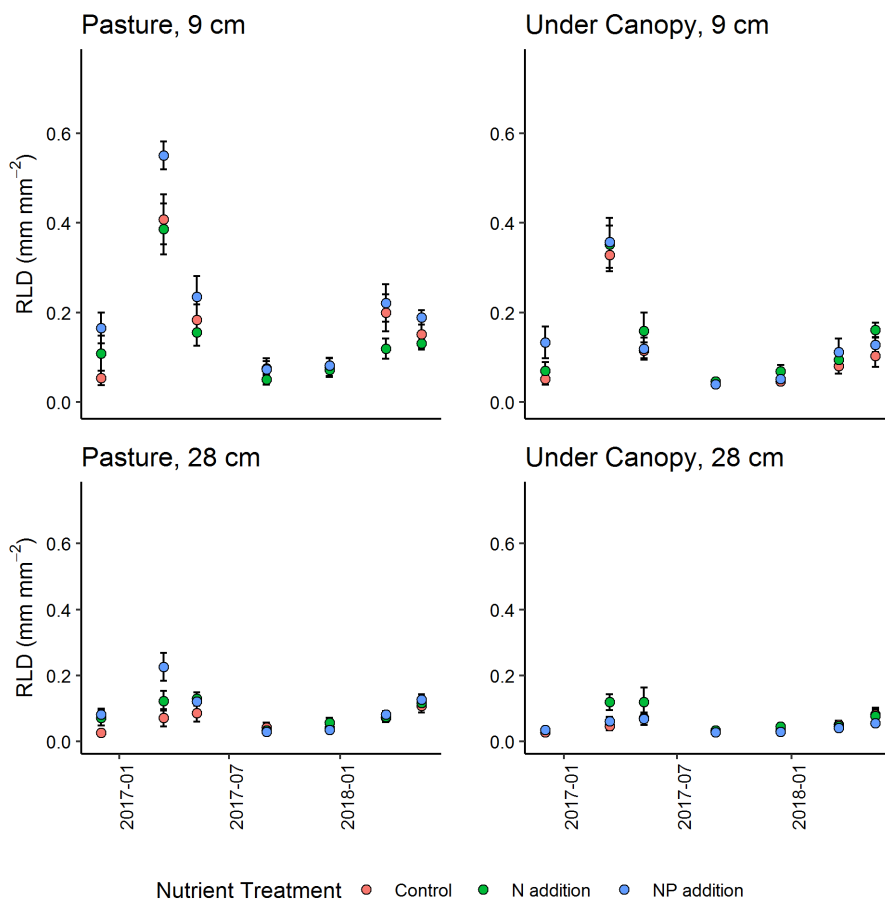
Figure 3. Seasonal cycle of minirhizotron-calculated RLD between treatment at two selected depths. NP treatments tended to diverge, especially in spring 2017. Error bars show ± SE. A mixed effect model revealed to these data revealed a significant effect of the NP treatment (P < 0.005) but N did not differ from control on this metric.
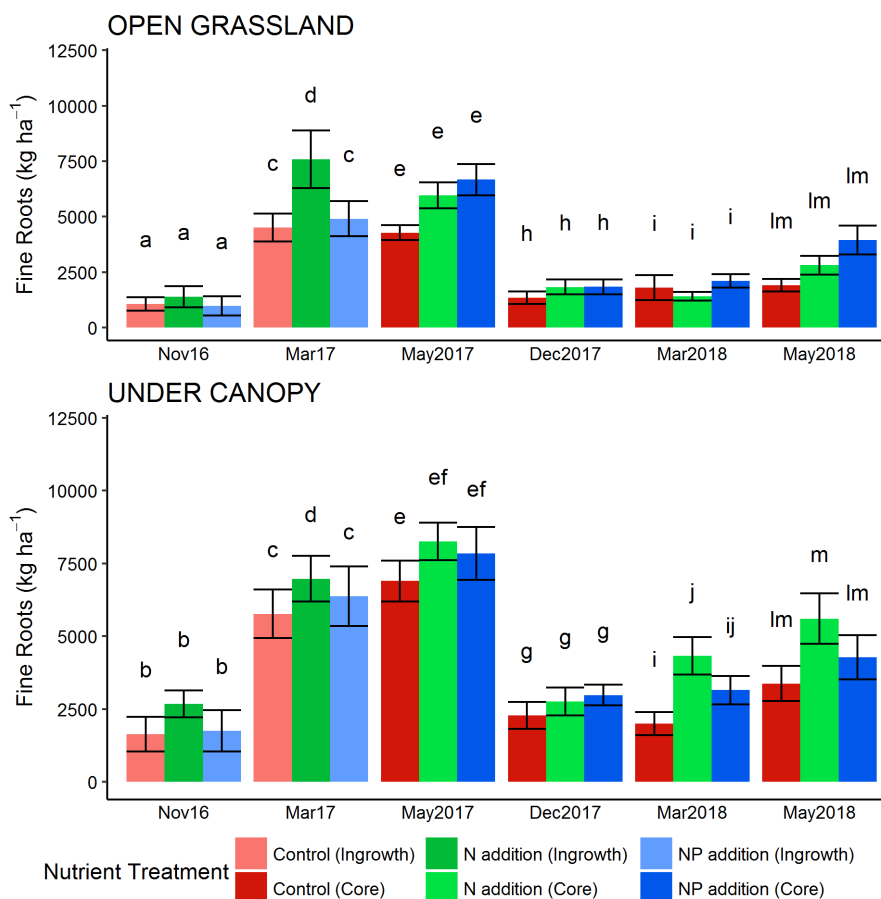
Figure 4. Root biomass in top 13 cm from ingrowth cores (all installed Dec. 2016 and removed then in Nov 16 and Mar 17) and direct soil cores to obtain standing living root biomass during the sampling period. Direct measured root biomass showed a seasonal cycle and also differences between treatments, with more roots in general under canopies and in fertilized plots. Error bars show ± SE and letters indicate Tukey-HSD groupings for most parsimonious models within treatment for both Open Pasture and Under Canopy locations combined in individual sessions. Both N and NP treatments tended to have more root biomass than control treatments. Across the whole dataset, N had significantly ($P < 0.05$) more fine roots than the other two treatments.
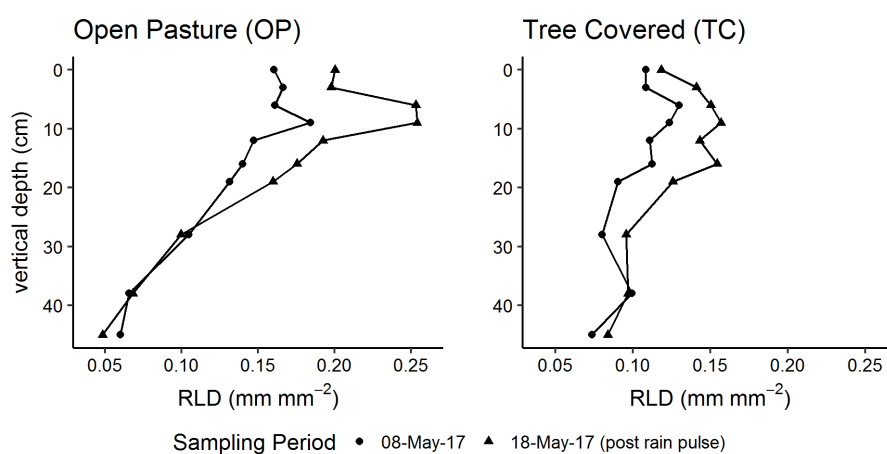
Figure 5. Control Treatment response to rain pulse in May 2017 (Section 3.5). Repeat MR sampling showed increases in RLD throughout the soil profile (P < 0.001 for both locations).
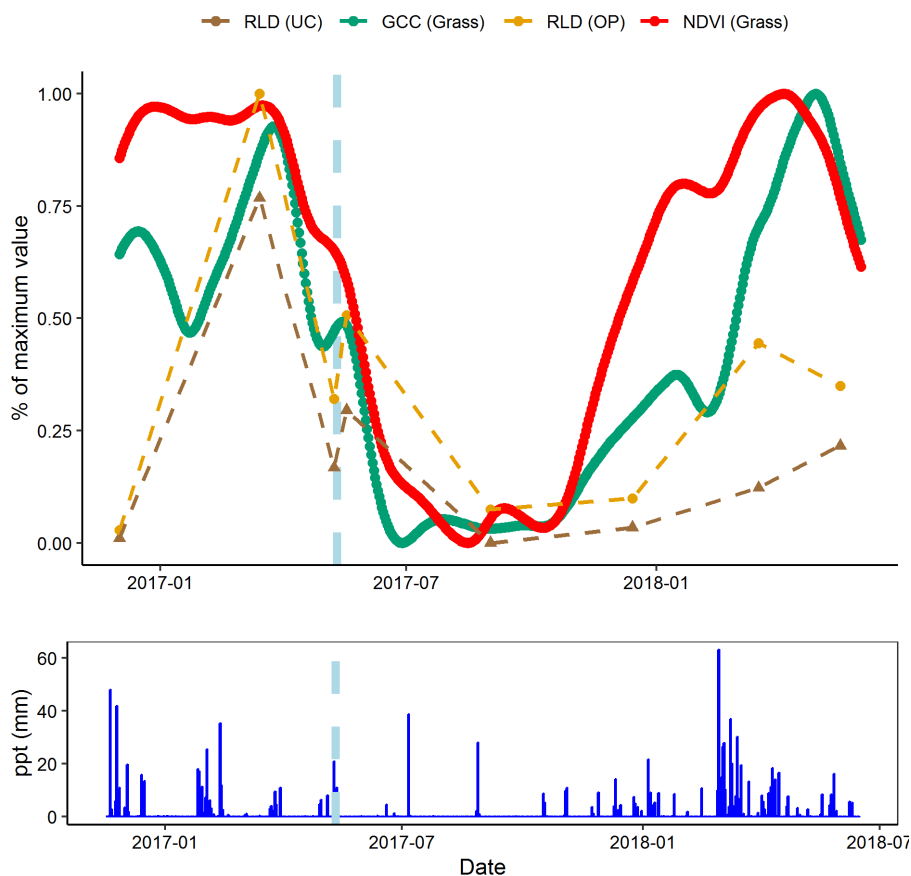
Figure 6. Comparison of minirhizotron RLD dynamics for open pasture with site-level grassland Normalized Difference Vegetation Index (NDVI) and grassland Greenness Colour Coordinate (GCC). After the rain pulse in May 2017 (indicated on graph, light blue), minirhizotron measurements could detect the similar response to above-ground (shown in more detail figure 5). Desynchronization was evident in both autumn periods where proximal-remote sensed metrics reached near-peak levels while RLD remained low.

Table 1. Mean RLD at 9 cm midpoint (8-12 cm depth) ± standard error for each treatment during the growing period of 2017 and 2018. Letters indicate TukeyHSD groupings for the most parsimonious linear model for each individual sampling date. Nitrogen and Phosphorus addition together tended to diverge from N and control and open pasture RLD tended to be higher than RLD below tree canopies. Nutrient effects were stronger in 2017 than 2018

	Open Pasture (OP) RLD (mm mm^{-2})					
	Control	N	NP	Control	N	NP
March '17	0.33 ± 0.0 [ab]	0.41 ± 0.1 [ab]	0.35 ± 0.1 [a]	0.39 ± 0.1 [ab]	0.36 ± 0.0 [ab]	0.55 ± 0.0 [b]
May '17	0.12 ± 0.0 [c]	0.18 ± 0.1 [c]	0.16 ± 0.1 [c]	0.16 ± 0.0 [d]	0.12 ± 0.1 [d]	0.23 ± 0.1 [d]
March '18	0.08 ± 0.0 [e]	0.20 ± 0.0 [e]	0.09 ± 0.0 [e]	0.12 ± 0.0 [f]	0.11 ± 0.0 [f]	0.22 ± 0.0 [f]
May '18	0.10 ± 0.0 [g]	0.15 ± 0.0 [g]	0.16 ± 0.0 [g]	0.13 ± 0.1 [g]	0.13 ± 0.0 [g]	0.19 ± 0.0 [g]

Table 2. Absolute (Herbaceous layer) Biomass Measurements and Root:Shoot Ratios (RSR) at two points in 2017. Root shoot ratio increased later in the growing season but tended to decrease in nutrient added treatments, with the exception of N:OP in March and N:TC in May which exhibited unusually high RSR.

March 2017

Nutrient	Vegetation	Aboveground Biomass	Belowground Biomass	Root:Shoot Ratio
		kg ha^{-1}		
kg ha^{-1}				
Control	TC	290 ± 20	5770 ± 320	20 ± 2 : 1
Control	OP	290 ± 20	4510 ± 250	15 ± 1 : 1
N	TC	340 ± 20	6890 ± 390	21 ± 2 : 1
N	OP	290 ± 20	7590 ± 420	26 ± 3 : 1
NP	TC	380 ± 30	6380 ± 350	17 ± 2 : 1
NP	OP	300 ± 30	4900 ± 270	16 ± 2 : 1

May 2017

Nutrient	Vegetation	Aboveground Biomass	Belowground Biomass	Root:Shoot Ratio
Control	TC	260 ± 40	6900 ± 700	26 ± 5 : 1
Control	OP	370 ± 60	4280 ± 330	12 ± 2 : 1
N	TC	220 ± 30	8250 ± 640	38 ± 6 : 1
N	OP	400 ± 50	5960 ± 580	15 ± 2 : 1
NP	TC	360 ± 40	7850 ± 900	22 ± 3 : 1
NP	OP	440 ± 30	6670 ± 700	15 ± 2 : 1