Drought is a stronger driver of soil respiration and microbial communities than nitrogen or phosphorus addition in two Mediterranean tree species

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Highlights

- Effects of drought and nutrients on soils may differ under different trees.
- Soil respiration and microbial community physiological profiles were measured.
- Drought lowered diversity in both species and in *Q. ilex* also reduced respiration.
- Recovery patterns were different for soils of *Q. ilex* and *P. sylvestris*.
- Drought may be larger driver of changes to soil communities than N or P deposition.

Abstract

The drivers of global change, such as increasing drought and nutrient deposition, are affecting soils and their microbial communities in many different habitats, but how these factors interact remains unclear. *Quercus ilex* and *Pinus sylvestris* are two important tree species in Mediterranean montane areas that respond differently to drought, which may be associated with the soils in which they grow. We measured soil respiration and physiologically profiled microbial communities to test the impact of drought and subsequent recovery on soil function and diversity for these two species. We also tested whether the addition of nitrogen and phosphorus modified these effects. Drought was the stronger driver of changes to the soil communities, decreasing diversity (Shannon index), and evenness for both species and decreasing soil respiration for *Q. ilex* when N was added. Soil respiration for *P. sylvestris* during the drought period was positively affected by N addition but was not affected by water stress. P addition during the drought period did not affect soil respiration for either tree species but did interact with soil-water content to affect community evenness. The two species also differed following the recovery from drought. Soil respiration for *Q. ilex* recovered fully after the drought treatment ended but decreased for *P. sylvestris*, whereas the soil community was more resilient for *P. sylvestris* than *Q. ilex*. Nutrient addition did not affect respiration or community composition or diversity during the recovery period. Soil respiration was generally weakly positively correlated with soil diversity. We demonstrate that short-term water stress and nutrient addition can have variable effects on the soil communities associated with different tree species and that the compositions of the communities can become uncoupled from soil respiration. Overall, we show that drought may be a stronger driver of changes to soil communities than nitrogen or phosphorus deposition.

Key words: nitrogen; phosphorus; *Quercus ilex*; *Pinus sylvestris*; BIOLOG EcoPlate; microbial community diversity

1 Introduction

The interactive effects of drivers of global change on soil communities remain mostly unknown. Drought in the Mediterranean region has long been identified as one of the most important threats to soils and their plant communities. Mediterranean ecosystems are adapted to some amount of water stress, especially during summer, but climatic predictions indicate that more frequent and more severe droughts will become increasingly common (Dai, 2011; Field et al., 2014; Touma et al., 2015). Increases in nutrient addition due to greater inputs from industrialisation and farming are an additional disturbance that may interact with drought to affect soils and their biota. Understanding these impacts for predicting the responses of Mediterranean systems and their ecosystem services to the changing future environment is crucial. The combined effects of drought and added nutrients on soil communities, however, remain elusive.

Soil respiration comprises respiration by the microbial community (decomposition of soil organic matter and plant tissues in the bulk soil) and respiration by plant roots, associated mycorrhizal fungi, and rhizosphere microbes (using recently fixed carbon [C] from plants; Kuzyakov, 2006). The effects of drought and nutrients on soil respiration can therefore be complex and are likely to be mediated by the species of plants growing in the soil (de Vries et al., 2019). Soil respiration is a large component of the CO_2 released into the atmosphere, usually representing ~50-75% of total ecosystem respiration in forest systems (Brændholt et al., 2018; Curiel Yuste et al., 2005; Tang et al., 2008), but it also indicates the activity of the soil. Any changes in soil respiration due to drought or increased nutrient deposition could therefore have large implications for the global C balance (Cascio et al., 2017). Increases in soil respiration can indicate that the soil is increasing its capacity as a C source if the additional efflux of CO_2 is derived from organic matter (Kuzyakov, 2006). A lack of soil respiration, however, can suggest that normal soil processes (root and microbial respiration) are not occurring (Allen et al., 2011), which may have future implications for the short-term health of an ecosystem.

Droughts tend to reduce soil respiration, including in Mediterranean areas (Talmon et al., 2011), and precipitation in a six-year study in a Mediterranean Aleppo pine forest was the dominant factor determining soil respiration in water-limited seasons (Grünzweig et al., 2009). Drought reduces respiration in the short term (days to weeks), because it lowers the biological activities of soil microorganisms, animals, and plants. Water stress leads to long-term (months to years) changes in vegetation patterns, such as reducing plant cover, which has knock-on effects for respiration (Talmon et al., 2011). Many studies, however, have reported that microbial biomass can remain relatively constant during periods of drought, so changes in respiration should not be mistaken for changes in biomass (Schimel, 2018).

The availability of soil nutrients can affect soil respiration, but these effects can vary depending on the dose and duration (Cascio et al., 2017). Some ecosystems will be more limited by nitrogen (N), and others will be more limited by phosphorus (P) (Penuelas et al., 2020; Peñuelas et al., 2013), and N and P contents can have interactive effects. These differences depend primarily on the soil, especially its age, and can vary greatly between geographical locations and biome types. While effects of N and P addition have long been shown for aboveground communities, soil microbial communities in various systems tend to be limited more by C (Demoling et al., 2007; Ekblad & Nordgren, 2002; Soong et al., 2019). The impacts of N addition on soil respiration are variable and have been reported either as consistently negative (Ramirez et al., 2010), positive only at low levels of addition (Zhu et al., 2016), or positive overall, except in forests, where negative impacts have been reported (Zhou et al., 2014). The responses of P addition may largely depend on ecosystem type. A recent meta-analysis found increases in soil respiration for tropical forests and cropland but decreases in wetland and no effect in other ecosystems (Feng & Zhu, 2019). P addition did not significantly affect heterotrophic respiration in any of these ecosystems (Feng & Zhu, 2019).

Nutrient addition alters soil respiration by affecting both the plants and the soil microbes. N and P can stimulate root growth, leading to an increase in respiration (Ren et al.,

2016; Zhou et al., 2014). A recent meta-analysis, however, found that N addition consistently led to negative effects on microbial growth and respiration across various global terrestrial ecosystems (T. Zhang et al., 2018). Similarly, N addition reduces microbial biomass (Demoling et al., 2008; Treseder, 2008; Wallenstein et al., 2006), perhaps due to toxic osmotic potential that directly inhibits microbial growth or due to the acidification of the soil from N saturation and subsequent limitation of ions such as calcium or magnesium (Treseder, 2008). The effects of P addition on soil microbes are less straightforward, with different studies reporting positive, negative, or no effect on microbial biomass and activity depending on the type of ecosystem (Guo et al., 2017; Illeris et al., 2003; Keith et al., 1997; Liu et al., 2012).

The composition and diversity of the soil microbial community also change in response to drought and nutrient addition. Drought can directly affect these communities, e.g. by desiccation and reduction of substrate diffusion, leading to the limitation of resources (Naylor & Coleman-Derr, 2017; Schimel et al., 2007; Schimel, 2018). Though note also that on the other hand, drier soils may increase the availability of gaseous and volatile substrates, which may be beneficial (Schimel, 2018). Low levels of soil moisture can therefore decrease microbial activity, such as nutrient mineralisation, enzymatic activity (Sanaullah et al., 2011; J. Sardans & Peñuelas, 2005) and dormancy, allowing microbes to recover after the drought conditions end (Meisner et al., 2018; Schimel, 2018). The impacts of drought will differentially affect different microbes, with some evidence suggesting that fungi are more tolerant than bacteria (Evans & Wallenstein, 2012; Preece et al., 2019).

The impacts of drought on plants can also indirectly affect microbial communities (Bardgett et al., 2008), e.g. by changes in patterns of root exudation, in both quantity and composition (Canarini et al., 2016; Gargallo-Garriga et al., 2018; Preece et al., 2018; Preece & Peñuelas, 2016; Williams & Vries, 2020). Additionally, drought may affect litter inputs from above and below ground, which would likely have knock-on impacts on the microbial community (Bardgett et al., 2008). In terms of microbial diversity, dought has been reported to

have both positive and negative effects (Bouskill *et al.*, 2013; Acosta-Martínez *et al.*, 2014), which may differ for bacteria and fungi (Preece et al., 2019).

Increased N and P in the soil can also affect the composition (Li et al., 2015; Liu et al., 2012; Ramirez et al., 2012) and possibly the diversity (Su et al., 2015) of soil microbial communities, tending to favour copiotrophic, fast-growing bacteria (Leff et al., 2015). Responses to added N and P, however, will likely depend strongly on the concurrent effect on the plants in the system. Interactions between nutrient addition and drought that lead to changes in microbial communities are even less well studied than the individual effects.

Our aim was to assess the interactive effects of drought and nutrient addition on soil respiration and soil community-level physiological profiles (CLPPs). We also assessed the recovery of soil and its community from a drought and whether nutrients would help or hinder the recovery. Finally, we assessed whether the responses differed between two species that coexist in montane Mediterranean regions, *Quercus ilex* L. (holm oak) and *Pinus sylvestris* L. (Scots pine). The two species have very different geographical distributions, with *Q. ilex* common throughout the Mediterranean Basin, whereas *P. sylvestris* is more typical in temperate habitats, with the Mediterranean Basin at the southern limit of its range. In fact, *Q. ilex* is replacing *P. sylvestris* as the dominant canopy species in Mediterranean montane forests (Aguadé et al., 2015; Galiano et al., 2010; Vilà-Cabrera et al., 2013). We therefore expected that *Q. ilex* and its associated soil community would be more resistant to the drought treatment.

We hypothesised that 1) both drought and nutrient addition would decrease soil respiration and microbial diversity, 2) the effects of drought would be lower in the soils with *Q. ilex* than *P. sylvestris*, and 3) the soils with *Q. ilex* would have more signs of recovery from drought, due to differences in the drought tolerance of the two species.

2 Material and methods

2.1 Plant and soil material

A greenhouse experiment was set up in late April 2016 at the Autonomous University of Barcelona (Spain). The experiment contained 64 three-year-old *Q. ilex* saplings and 64 three-year-old *P. sylvestris* saplings (provided by Tres Turons, Barcelona, Spain). The roots of the saplings were gently cleaned using water and small forceps to remove all soil from the previous substrate, and the saplings were then replanted in a new common substrate in 3.5-L pots. This substrate was a natural soil, with stones >1 cm removed. The soil was collected from a natural holm oak forest on a south-facing slope (25% slope) in the Prades Mountains in northeastern Iberian peninsula (41°13′N, 0°55′E; 930 m a.s.l.). All plants were then given adequate water for eight weeks, until late June 2016, to allow them to adjust to the greenhouse environment.

2.2 Experimental design

The experimental design comprised a drought period and a recovery period, with 128 pots in total, divided equally between the two species. During the drought period, a drought treatment (control or drought) and a nutrient treatment (control, N, P and both N and P), were applied to all pots in a factorial design, with equal numbers of replicates for each treatment combination (n = 8). Pots were divided into four blocks (for two replicates of each drought-nutrient combination in each block). The nutrient treatment began on June 10th 2016 with the addition of N, the addition of P, and the addition of both N and P. N was added as ammonium nitrate (NH_4NO_3) at a rate of 50 kg N ha^{-1}, and P was added as calcium phosphate ($Ca_3(PO_4)_2$) at a rate of 25 kg P ha^{-1}. The diameter of the pots required application rates of 0.22 g of $Ca_3(PO_4)_2$ and 0.25 g of NH_4NO_3 as aqueous solutions per pot. A second round of nutrient addition was performed in mid-September. The drought treatment was applied from June 27th 2016 by reducing the addition of water by approximately 50% compared to the controls. Soil-water content (SWC) was measured using an ML3 Theta Probe connected to an HH2 Moisture

Meter (Delta-T Devices, Cambridge, UK) in each pot once a week to maintain the content at approximately 20% for the control plants and 10% for the drought-treated plants. These SWC values were chosen based on previous experiments by the group in similar conditions, that were known to provide sufficient water (20%) and moderate drought (10%) (Preece et al., 2018; Zhang et al., 2017). Soil respiration was measured and soil samples were collected (described in detail below) for all pots after 14-17 weeks (September 30th to October 21st 2016), which signified the end of the drought treatment. Measurements were temporally staggered at one week per block for logistical reasons due to the large number of replicates. The period of drought was considered long enough for the SWC to have stabilised long before the measurements were taken and to avoid differences in soil respiration or CLPPs due to the timing of the measurement. The pots were re-watered at optimal amounts for eight weeks to represent a recovery period, after which respiration was again measured and soil samples were collected (November 25th to December 16th 2016).

Mean air temperature during the experiment, monitored using five EL-USB-2 data loggers (Lascar Electronics, Wiltshire, UK), was 21.3°C. Soil temperature was monitored at a fine scale in four pots across the different soils using a Decagon Em50 data logger with 5TM soil probes (Decagon Devices, Pullman, USA) and averaged 22.3°C throughout the experiment. SWC in each pot was measured at the start of the experiment and at the end of the drought period, and at the end of the recovery period if relevant, using the ML3 Theta Probe connected to the HH2 Moisture Meter. Mean SWC during the drought treatment in the *Q. ilex* pots was 18.6% in the control water-treatment pots and 10.6% in the drought-treated pots. Mean SWC in the *P. sylvestris* pots was 21.3% in the control water-treatment pots and 12.5% in the drought-treated pots. Nutrient addition did not affect SWC.

2.3 Measurements

Soil respiration was measured in each pot using an EGM-4 portable gas analyser connected to an SRC-1 soil-respiration chamber (PP Systems, Amesbury, MA, USA) at the end of the drought and recovery periods. Care was taken when inserting the respiration chamber into the

188 pots to avoid damaging the plants and to ensure a tight seal with the soil. Measurements were
189 taken at least 24 h after watering to limit the effect of water pulses on soil respiration.
190 Respiration was expressed as g CO_2 m^{-2} h^{-1}.

191 CLPPs were assessed using BIOLOG (Hayward, CA, USA) EcoPlates, which measure
192 the metabolism of 31 C sources, to identify changes between microbial communities in the
193 treatments. Soil samples of approximately 10 g were collected from each pot soon after soil
194 respiration was measured, at the ends of the drought and recovery periods. The two replicate
195 samples for each of the eight drought-nutrient combinations per block were pooled and mixed
196 well. A 5 g subsample of soil for each treatment per block was placed in a 100 mL sterile
197 plastic flask, and 50 mL of a sodium pyrophosphate ($Na_4P_2O_7 \cdot 10(H_2O)$) solution (pH 7) were
198 added. This solution was shaken for 20 min, left to settle for 1 min, and then 2 mL were added
199 to 18 mL of Ringer solution and agitated to mix. One hundred and forty microlitres of this new
200 solution were pipetted into each well of an EcoPlate. A microplate reader (Tecan Sunrise,
201 Männedorf, Switzerland) was used to read the absorbance at 590 nm in each well. The plates
202 were incubated in the dark at 25 °C for 4 d, with absorbance readings taken at 0, 24, 48, 60,
203 and 72 h. This process was repeated for each sample.

204 Absorbance readings after 72 h were corrected for initial absorbance for each plate by
205 subtracting the corresponding readings taken at 0 h, with any negative values removed. The
206 mean absorbance for the three wells designated as blanks in each plate was calculated, which
207 was then subtracted from the absorbances for each of the C sources. The mean of these
208 blanked absorbances was calculated to give the average well colour development (AWCD)
209 per plate. All absorbances were then standardised by dividing by the AWCD, and the three
210 replicate samples for each C source were then used to calculate the mean standardised
211 absorbance for each source. Each of these values was divided by the sum of the mean
212 standardised absorbance of all 31 C sources in each sample, to give the relative abundance.
213 This procedure allowed the calculation of the Shannon H index, as a measure of diversity, and
214 evenness per plate (with a plate for each drought-nutrient treatment per block).
215

2.4 Statistical analyses

The data were grouped into two subsets (named as 'experiment' in the statistical models), the drought period and the recovery period. There was no statistical difference between the two species in terms of soil respiration or CLPP diversity (Shannon H and evenness). There was a small difference between species in terms of CLPP composition (PERMANOVA), however the r^2 of this model was very low (<0.04) and the species effect disappeared when other variables (SWC and nutrients) were included in the model. Therefore, all data were analysed separately for each species. The data for soil respiration were log-transformed before analysis, and a constant was first added for the data for *Q. ilex* to improve the normality of the data. All statistical analyses were performed using R (R Core Team, 2018). Linear mixed-effect models were used to test the effect of drought and nutrient (N and P) addition on soil respiration using the *lmer* function in the *lme4* package. Soil respiration per pot was thus the response variable, and the explanatory variables were SWC (as a continuous variable) and N and P addition as two categorical variables. Block was added as a random effect. Interactions between variables were included if they were significant in the model ($P < 0.05$). We also used mixed-effect models for analysing the CLPP data, with the Shannon H index or evenness as the response variable and with SWC, N addition, and P addition as the explanatory variables, with any interactions where significant.

The effect of the recovery period on respiration, Shannon H, and evenness for each species was also tested using linear models, combining the drought and recovery data, with 'experiment' as a fixed factor interacting with the drought treatment. The C sources were grouped into five guilds (Supplementary Material, Table S1), carbohydrates, amino acids, carboxylic and acetic acids, amines/amides, and polymers (following Weber & Legge, 2009), to identify the effect of the drought or nutrient addition on the potential activity (as a percentage of all C sources) of each C guild. The simplest model was always selected using the Akaike information criterion. A permutational multivariate analysis of variance (PERMANOVA) was also performed, using *adonis2* in the *vegan* package, to test the effects of SWC and N and P addition on the composition of the carbohydrate sources that could be metabolised. The

relative abundance of the mean standardised absorbance was the response variable, and as before, block was included as a random effect. A principal component analysis (PCA) was performed to visualise the relationships between the potential metabolisms of the 31 C sources and SWC during the drought treatment and recovery period. The nutrient treatments were not included, because the PERMANOVA indicated that they were not relevant to the changes in potential community composition. Significant differences between the control and drought groups were identified using an analysis of variance (ANOVA). The relationship between soil CLPP diversity (Shannon H) and respiration for all data was tested using a linear mixed-effect model, with species as a random effect. Finally, to analyse the correlations between SWC, nitrogen and phosphorus and the individual carbon sources, a partial least squares regression was performed (*pls* function in *MixOmics* R package), with results shown in clustered image maps (*cim* function in *MixOmics* R package, Rohart et al., 2017).

3 Results

3.1 Effects of drought and nutrient addition on soil respiration and CLPPs

Soil respiration ranged from 0.01 to 0.54 µmol C m^{-2} s^{-1}. Respiration in the drought period was lower in the soils with *Q. ilex*, but only when N was added (Table 1, Fig. 1a; interaction between SWC and N, $P < 0.05$). Although respiration in *P. sylvestris* soil was unaffected by drought, it was positively affected by N addition (Table 1, Fig. 1b; effect of N, $P < 0.05$). P addition during the drought treatment did not affect soil respiration for either *Q. ilex* or *P. sylvestris*.

PERMANOVA indicated that the CLPPs did not differ significantly between the soils with *Q. ilex* and *P. sylvestris* under control conditions. SWC strongly affected the CLPPs for both species (Table 1; effect of SWC, $P < 0.001$ for both). Similarly, the PCA clearly separated the control and water-stressed groups for both species during the drought period, with an ANOVA indicating that PC1 differed significantly between the two groups, and PC2 also for *P. sylvestris* (Fig. 2a, PC1, $P < 0.001$; PC2, $P = 0.07$ for *Q. ilex*; Fig. 2b, PC1, $P < 0.01$; PC2, $P < 0.05$ for *P. sylvestris*).

The drought treatment negatively affected CLPP diversity for both species (Table 1, Fig. 3a, b; effect of SWC, $P < 0.05$ for both). CLPP evenness for *Q. ilex* during the drought period was positively correlated with SWC (Table 1, Fig. 3c; effect of soil-moisture content, $P < 0.01$). CLPP evenness in pots with *P. sylvestris* during the drought period was affected by an interaction between SWC and P addition (Table 1, Fig. 3d; $P < 0.05$), such that SWC was strongly positively correlated with evenness when no P was added but was not correlated when P was added.

In soils with *Q. ilex*, drought led to increases in the potential metabolism of carbohydrates (Supplementary Material, Table S2, negative effect of SWC, $P < 0.001$), in particular D-cellobiose, pyruvic acid methyl ester and D-mannitol (Supplementary Material, Fig S1). Drought led to decreases in the amine/amide group (positive effect of SWC, $P < 0.01$) and polymers ($P < 0.05$), with no significant nutrient effects. In pots with *P. sylvestris*, drought increased the potential metabolism of amino acids ($P < 0.001$), in particular L-asparagine (Supplementary Material, Fig S2), and there was an interaction between soil moisture and phosphorus addition; when there was no additional P, there was a positive effect of the drought (negative effect of increasing SWC) and when there was additional P, there was a negative drought effect (interaction between SWC and P, $P < 0.05$).

3.2 Recovery of soil respiration and CLPPs

Soil respiration did not differ significantly between the drought period and the recovery period. Respiration in the soils with *Q. ilex* fully recovered when watering recommenced at normal levels (Table 2, Fig. 4a). Respiration in the soils with *P. sylvestris* had a delayed effect of drought, because it was affected by SWC during the period of drought (Table 2, Fig. 4b; effect of SWC, $P < 0.001$). The addition of the nutrients had no effect on soil respiration for either species during the recovery period.

The CLPP patterns during the recovery from drought were more complex. The PERMANOVA indicated that *Q. ilex* CLPP was affected by SWC during recovery and the

298 previous drought period (Table 2; $P < 0.01$). *P. sylvestris* CLPP was also affected by SWC,
299 but only from the drought period ($P < 0.05$). Nutrient addition did not affect the CLPPs
300 (diversity, evenness or composition) for either species in the recovery period. The PCA
301 indicated that the communities overlapped more than after the drought treatment, and the
302 ANOVA for *Q. ilex* found no difference between the control and drought groups, although the
303 first principal component (PC1) for *P. sylvestris* differed significantly between the control and
304 drought groups (Fig. 5b, $P < 0.05$).

305 The diversity indices did not differ significantly between the drought period and the
306 recovery period for either species. The drought, however, had a continued effect for *Q. ilex*
307 during the recovery period; the drought treatment had a negative impact on Shannon H when
308 measured at the end of the recovery period (Table 2, Fig. 6a; $P < 0.01$). Diversity, however,
309 tended to recover for *P. sylvestris*, as SWC did not affect H (Table 2, Fig. 6b). Similarly, with
310 evenness, soil under *Q. ilex* showed a continued negative effect of SWC during the drought
311 period (Table 2, Fig. 6c; $P < 0.05$), but not for *P. sylvestris* (Table 2, Fig. 6d). Nutrient addition
312 was not important for determining the Shannon diversity or evenness during the recovery
313 period.

314 The five guilds of C sources responded differently to SWC in the pots with *Q. ilex* at
315 the end of the recovery period, with interactive effects between SWC during drought and
316 recovery for the carboxylic and acetic acids group and for polymers (Supplementary Material,
317 Table S2, $P < 0.01$ and $P < 0.05$, respectively). SWC in the two periods was positively
318 correlated with the potential metabolism of carboxylic and acetic acids and negatively
319 correlated with the potential metabolism of polymers. Regarding individual C sources, L-serine
320 (amino acid) was strongly positively correlated with SWC in drought, and D,L-alpha-glycerol
321 phosphate (a carbohydrate) was strongly positively correlated with SWC in recovery, while
322 there was a strong positive effect of drought on the polymer alpha-cyclodextrin
323 (Supplementary Material, Fig S3). Nutrient addition did not affect the metabolism of the five C
324 guilds in the pots with *Q. ilex*. The results for the pots with *P. sylvestris* were slightly more

complicated. Carbohydrate metabolism was affected by an interaction between the SWC in the drought treatment and the recovery period ($P < 0.05$). Amino acid metabolism was positively affected by N addition, particularly L-arginine (Supplementary Material, Fig S4), and carboxylic and acetic acid metabolism was affected by an interaction between P addition and SWC during drought ($P < 0.05$) and by an interaction between the SWC in the drought period and the recovery period ($P < 0.01$).

3.3 Are diversity and respiration linked?

The combined data for both periods of the experiment (drought period and recovery period) indicated a positive correlation between CLPP diversity (Shannon H) and soil respiration (Fig. 7, $P < 0.05$).

4 Discussion

4.1 Effects of drought on soil respiration and CLPPs

Soil respiration tended to be resistant to drought, at least in the short term, with no change for *P. sylvestris* and a negative effect for *Q. ilex* only when extra N was added. Previous studies have consistently found that drought tended to decrease soil respiration, including in Mediterranean regions (Curiel Yuste et al., 2007; Misson et al., 2010). The drought treatment in our study may not have been severe enough (in duration or in water reduction) to induce significant changes in soil respiration, particularly autotrophic respiration by the sapling roots. In comparison with our drought treatment, studies which found reductions in soil respiration tended to have lower soil moisture than was achieved in our study (e.g. less than 5% in Curiel Yuste, 2009 and less than 10% in Mission et al., 2010 compared with 10.6 and 12.5% mean moisture in *Q. ilex* and *P. sylvestris* pots respectively in our study). This result was supported by indications that foliar greenness (chlorophyll fluorescence measurements, data not shown)

was not greatly affected by the drought, and this resistance may have extended to the other organs of the plant, such as the roots. The decrease in respiration in the droughted soils with *Q. ilex* in the presence of high levels of N could indicate a decrease in the activity of the soil microbial community when the N was added, which is a common response to N addition (Ramirez et al., 2010). The respiration of the system then decreased at low levels of soil moisture with the additional stress of the drought.

The use of EcoPlates allowed us to monitor the potential microbial communities by measuring the metabolic responses to different C sources. Reducing SWC had strong negative effects on the CLPPs for both species, reducing diversity and evenness and affecting the composition. Some evidence suggests that Mediterranean soils may be resistant to mild long-term water stress (Curiel Yuste et al., 2014), but microbial communities may still be responsive to shorter-term changes in water availability (Preece et al., 2019) before a new steady state is reached, especially because bacteria and fungi tend to have fast turnover times of days to weeks (Blazewicz et al., 2014; Rousk & Bååth, 2011). The drought treatment was a 50% reduction in water for approximately four months, similar to other studies using EcoPlates to study the effects of drought and which have detected changes in CLPPs (e.g. Kassem *et al.*, 2008; Hueso *et al.*, 2012), supporting our use of this method. Drought had a negative, positive, or no impact on the C guilds, and these effects were not consistent between the two species. The drought treatment thus affected the potential composition of microbial communities, but not consistently. The role of plant species in shaping the rhizosphere has been confirmed in previous studies that have showed that distinct microbial communities occur in the root-zones of different species (Burns et al., 2015), mediated, at least in part, by root exudates (Bais et al., 2006; Sasse et al., 2018). Thus, differences in the effects on CLPPs between the soils of *Q. ilex* and *P. sylvestris* in our study may well be due to differences in their root exudation patterns.

4.2 Recovery after drought

Soil respiration is known to show a large pulse of CO_2 efflux when drought conditions end, known as the Birch effect, which is thought to be due to the microbially mediated decomposition of soil substrates or dead microbes that accumulate during dry periods, or to the mobilisation of stable soil C that can be respired by the surviving microorganisms (Birch, 1958; Göransson et al., 2013; Schimel, 2018). Respiration fully recovered in the soils with *Q. ilex* but not *P. sylvestris*, where the effect of drought was delayed. We do not know how long a period of recovery, within the constraints of our study, would be needed for soil respiration with *P. sylvestris* to return to pre-drought levels, but soil respiration was less resilient for *P. sylvestris* than *Q. ilex*.

The CLPPs had opposite patterns between the species, with diversity and evenness for *Q. ilex* negatively affected by drought with no signs of recovery at the end of the recovery period. SWC did not affect diversity or evenness during the recovery period for *P. sylvestris*, indicating that the microbial community had either recovered or had adjusted to the new conditions. Our experiment could not determine which of these options was correct, but the fact that there was also a change in recovery CLPP composition due to the prior drought treatment suggests a change to a new stable condition. The uncoupling of the responses of the CLPPs and soil respiration to drought suggests that autotrophic respiration may have been more important in our experiment, because the respiration data was often not explained by the CLPP data.

Previous studies have demonstrated the high capability of respiration to recover in soil. For example, soil respiration in a field study with beech (*Fagus sylvatica*) trees recovered within three days of the end of a drought treatment, even exceeding control values as the recovery period progressed, compensating for the decrease in respiration during drought (Hagedorn et al., 2016). In that study it was reported that the majority of soil respiration was plant derived as opposed to microbe derived, and the quick recovery was thought to be due

to the accumulation of carbon compounds in the roots, such as sugars, starch, and amino acids, which enabled the fast re-activation of trees when re-watering began (Hagedorn et al., 2016). This finding was supported by another mesocosm experiment in a montane grassland, which also found that C from root exudates accumulated in the rhizosphere during drought, accompanied by a low activity of soil microbes, and in re-wetting this C disappeared and microbial activity returned (Karlowsky et al., 2018). Root exudates from droughted plants can also stimulate soil respiration compared to controls, presumably due to changes in exudate composition (Gargallo-Garriga et al., 2018). Experimental work has shown that the effect of these exudates on soil respiration can be much greater than the effect of a soil having a legacy of drought, illustrating that plant mediated signals during recovery might be critical for sustained and successful soil-ecosystem function after drought (de Vries et al., 2019). Additionally, it has been shown, in *Q. ilex*, that exudate amount and composition varies between drought and recovery periods, changing from higher exudation rates, dominated by secondary metabolites in drought to lower exudation rates of mainly primary metabolites in recovery (Gargallo-Garriga et al., 2018; C. Preece et al., 2018). This has important implications for the composition of the rhizosphere microbiome, which can be directly affected by exudation patterns (Williams & Vries, 2020), suggesting that differences in CLPP profiles in the current study may be largely driven by changes in root exudation.

4.3 Does nutrient addition affect soil respiration and CLPPs during drought and recovery?

Although the Mediterranean region is thought to be primarily limited by water, in terms of plant growth and microbial activity, it is also often limited by N and P (Ochoa-Hueso et al., 2011; Rutigliano et al., 2009; Jordi Sardans et al., 2004). This may explain the main effect of nutrient addition on soil respiration in our study, which was the positive impact that N addition had in the soil with *P. sylvestris* during the drought period. This finding was inconsistent with previous studies where N and P addition have had widespread negative effects on soil respiration

(Bowden et al., 2004; Guo et al., 2017; Ramirez et al., 2010; T. Zhang et al., 2018), although responses varied with site and treatment. These effects included both heterotrophic and root (autotrophic) respiration and were larger at less productive sites, where N was more likely to limit plant growth (Janssens et al., 2010). This finding is associated with long-term (chronic) N fertilisation, so results in short-term experiments such as ours may be less likely to have any impacts. Moreover, a previous study in a Mediterranean system showed that an increase in nitrogen supply can lead to an increase in water use efficiency and reduced water loss of *Pinus pinaster* (Fernández et al., 2006). Thus, in our study, positive effects of nitrogen addition on *P. sylvestris* could lead to feedbacks in the soil, such as maintenance of root exudation, which could have a follow-on benefit on soil respiration.

In addition to soil respiration, the effects of N and P addition on the soil community were also identified by analysing CLPP composition, diversity and evenness. The effects of nutrient addition on CLPP were generally small and varied depending on the species and the SWC, and overall, the potential soil community composition was mostly unaffected by the addition of both N and P. A previous large-scale experiment found that adding N to a variety of bare soils directly and consistently affected bacterial communities and decreased the activities of extracellular enzymes, indicating a decrease in soil microbial activity (Ramirez et al., 2012). Our experiment, however, found no evidence of an effect of nutrient addition on the structure of the soil communities, perhaps due to the larger impact of the drought.

Drought in our study, as in many similar experiments, was a treatment whose effects increased over time, concurrent with the length of time that the plants had less than adequate water, whereas the effects of N and P addition decreased in intensity over time, due to the uptake by plants and soil microbes and to leaching during watering. This may help to explain why drought effects seemed to be more important in their impacts on soil respiration and the CLPPs compared with nutrient addition. We found no evidence that nutrient addition improved respiration or the diversity indices during the recovery from drought for either species.

4.4 *Are responses the same for* Q. ilex *and* P. sylvestris*?*

The Mediterranean region is characterised by dry summers to which the vegetation is adapted, but this adaptation does not prevent the plants and soils from responding to environmental changes. Although the two study species can coexist in montane Mediterranean regions, *Q. ilex* has been shown to be replacing *P. sylvestris* as the dominant species (Aguadé et al., 2015; Galiano et al., 2010; Vilà-Cabrera et al., 2013). It was therefore expected that *Q. ilex*, and its associated soil community would be the more resistant to the drought treatment. In contrast to this hypothesis, the drought treatment negatively affected soil respiration faster for *Q. ilex* than *P. sylvestris*, but only when N was added. However, the drought treatment did not affect respiration at the end of the drought period for *P. sylvestris.* The CLPP analysis indicated that the potential compositions of the soil communities did not differ significantly between the species under control conditions, excluding the presence of measurably distinct soil communities (at the CLPP level) prior to the drought treatment, which led to the divergent responses to drought. In terms of CLPP, the species responded similarly to drought, which negatively affected diversity and strongly affected community functional composition. Therefore, overall, we do not have support for our second hypothesis, that the effects of drought would be lower in the soils with *Q. ilex*.

It was also hypothesised that soils with *Q. ilex* would recover more quickly from the drought compared with soils with *P. sylvestris*, which is supported by the soil respiration data. Respiration in soils with *Q. ilex* returned to control levels during the recovery period, whereas in soils with *P. sylvestris*, respiration was negatively affected during the recovery period, presumably a delayed effect of the previous water stress. Both species continued to show the effects of the drought (SWC during the drought period) on community composition into the recovery period, although these effects for *Q. ilex* were also linked to SWC at the time of measurement (recovery). However, the Shannon index and evenness recovered completely

for *P. sylvestris* but not for *Q. ilex*, contradicting our prediction that soils with *Q. ilex* would be able to recover better than the soils with *P. sylvestris*.

4.5 Are soil diversity and respiration linked?

The relationship between the composition and function of soil microbial communities is still not completely understood. Some evidence suggests a positive relationship between microbial diversity and soil respiration (Ren et al., 2018) and that information about the composition of microbial communities can improve predictions of soil respiration (Cleveland et al., 2007; Liu et al., 2018), but other studies report a lack of effect of diversity on respiration (Griffiths et al., 2001; Nannipieri et al., 2003). CLPP diversity in our study was positively correlated with soil respiration, but the correlation was weak, likely due to both the method for assessing the communities (CLPPs with the EcoPlates) and the influence of other experimental factors, particularly SWC, that had a large impact on both respiration and diversity. Our results generally reinforced the potential for using CLPP techniques to identify links between soil diversity and soil functioning, but our relatively coarse assessment of the communities indicates that data must be carefully interpreted.

5 Conclusions

This study has demonstrated that responses to drought in Mediterranean forest soils may vary strongly depending on the dominant tree species and the timescale of the drought. Respiration in the soils with *Q. ilex* decreased during water stress but recovered quickly, whereas respiration in the soils with *P. sylvestris* had a delayed effect and a subsequent slower recovery. Interestingly, the opposite pattern was found, for the two species, regarding the diversity of the microbial communities, indicating that changes in soil respiration may be more closely linked to autotrophic (root) than heterotrophic (microbial) respiration. The effects of nutrient addition on the soils were generally weaker than the effects of drought, reaffirming the

505 importance of water availability for soil function and community diversity in Mediterranean
506 ecosystems.

507

Figure Captions

Fig. 1. Effect of SWC and N and P addition on soil respiration at the end of the drought period for pots with (a) *Q. ilex* and (b) *P. sylvestris*. Each datapoint represents a different pot.

Fig. 2. PCA of the CLPP data for (a) *Q. ilex* and (b) *P. sylvestris* at the end of the drought period. Control pots are blue points, and drought-treated pots are yellow points. Carbon sources are colour-coded as follows: red = carboxylic & acetic acids, orange = carbohydrates, green= amines/amides, blue = amino acids, purple = polymers.

Fig. 3. Effect of SWC and N and P addition on diversity (Shannon H index) for pots with (a) *Q. ilex* and (b) *P. sylvestris* and on evenness for pots with (c) *Q. ilex* and (d) *P. sylvestris* at the end of the drought period. Each datapoint represents a different pot.

Fig. 4. Effects of SWC and N and P addition on soil respiration at the end of the recovery period for pots with (a) *Q. ilex* and (b) *P. sylvestris*. Each datapoint represents a different pot. NS, not significant.

Fig. 5. PCA of the CLPP data for (a) *Q. ilex* and (b) *P. sylvestris* at the end of the recovery period. Control pots are blue points, and drought-treated pots are yellow points. Carbon sources are colour-coded as follows: red = carboxylic & acetic acids, orange = carbohydrates, green= amines/amides, blue = amino acids, purple = polymers.

Fig. 6. Effects of SWC and N and P addition on diversity (Shannon H index) for pots with (a) *Q. ilex* and (b) *P. sylvestris* and evenness for pots with (c) *Q. ilex* and (d) *P. sylvestris* at the end of the recovery period. Each datapoint represents a different pot. NS, not significant.

Fig. 7. Relationship between soil respiration and CLPP diversity (Shannon H) ($P < 0.05$) for all pots in the experiment (drought period and recovery period).

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802

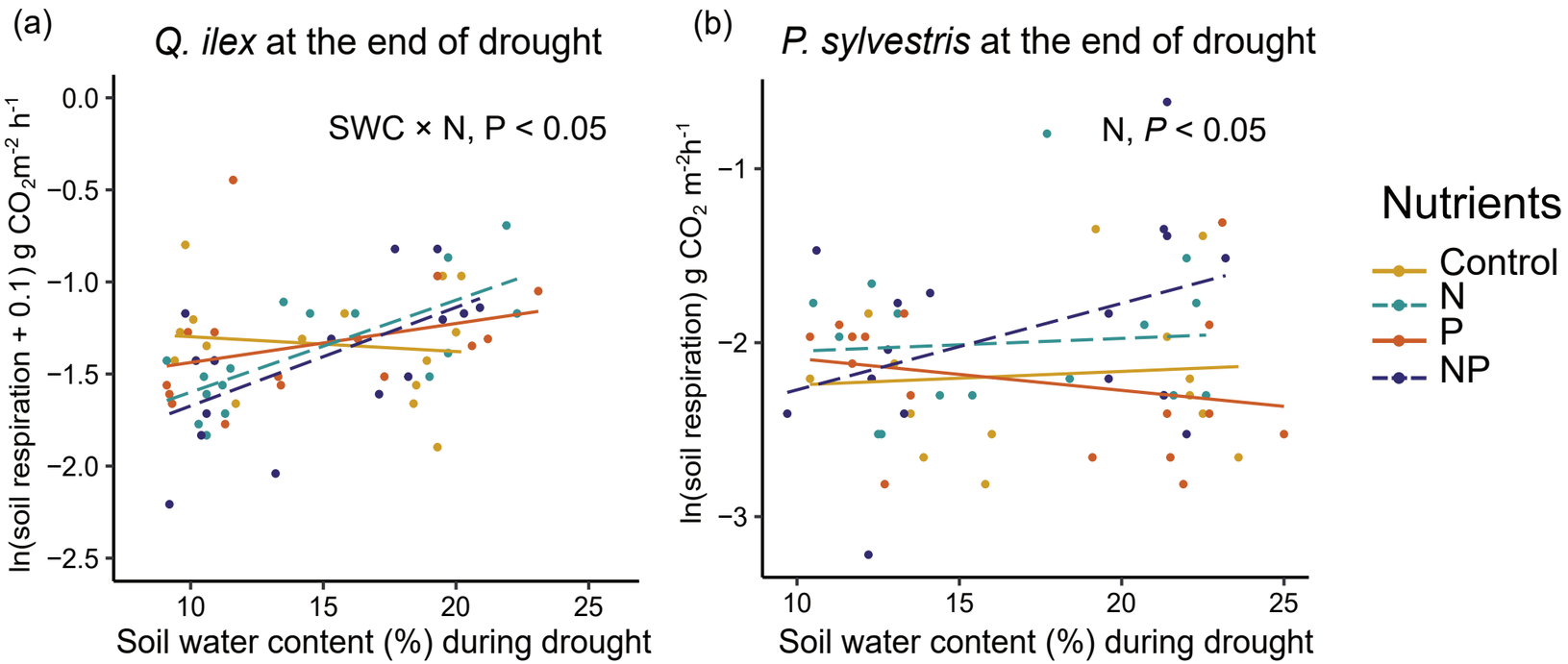
803

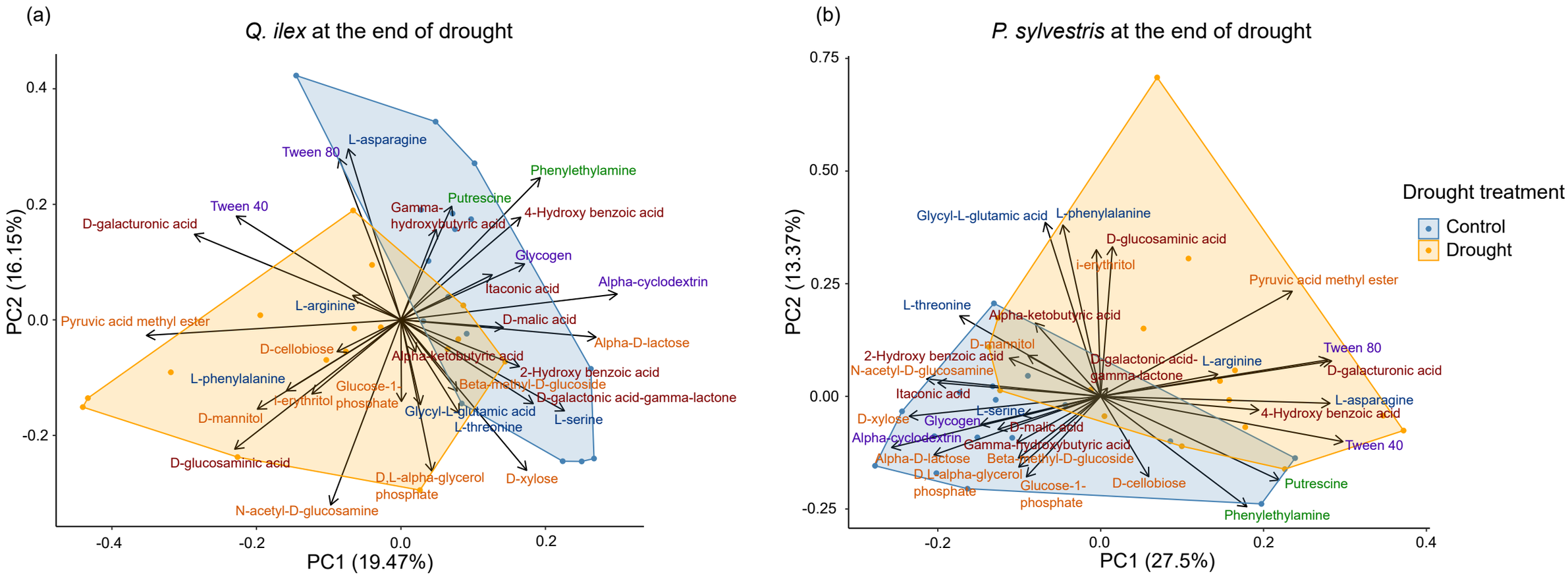
Table 1. Effects of SWC and N and P addition on soil respiration and CLPP diversity (Shannon H) and evenness during the drought period.

	Q. ilex	***P. sylvestris***
Soil respiration	SWC × N addition interaction, $P < 0.05$. Positive effect of SWC with added N	Positive effect of N addition, $P < 0.05$
Composition (PERMANOVA)	Effect of SWC, $P < 0.001$	Effect of SWC, $P < 0.001$
Shannon H	Positive effect of SWC, $P < 0.05$	Positive effect of SWC, $P < 0.05$
Evenness	Positive effect of SWC, $P < 0.01$	SWC content × P addition interaction. Positive effect of SWC with no added P, $P < 0.05$

Table 2. Effects of SWC on soil respiration and CLPP diversity (Shannon H) and evenness at the end of the recovery period. NS, not significant.

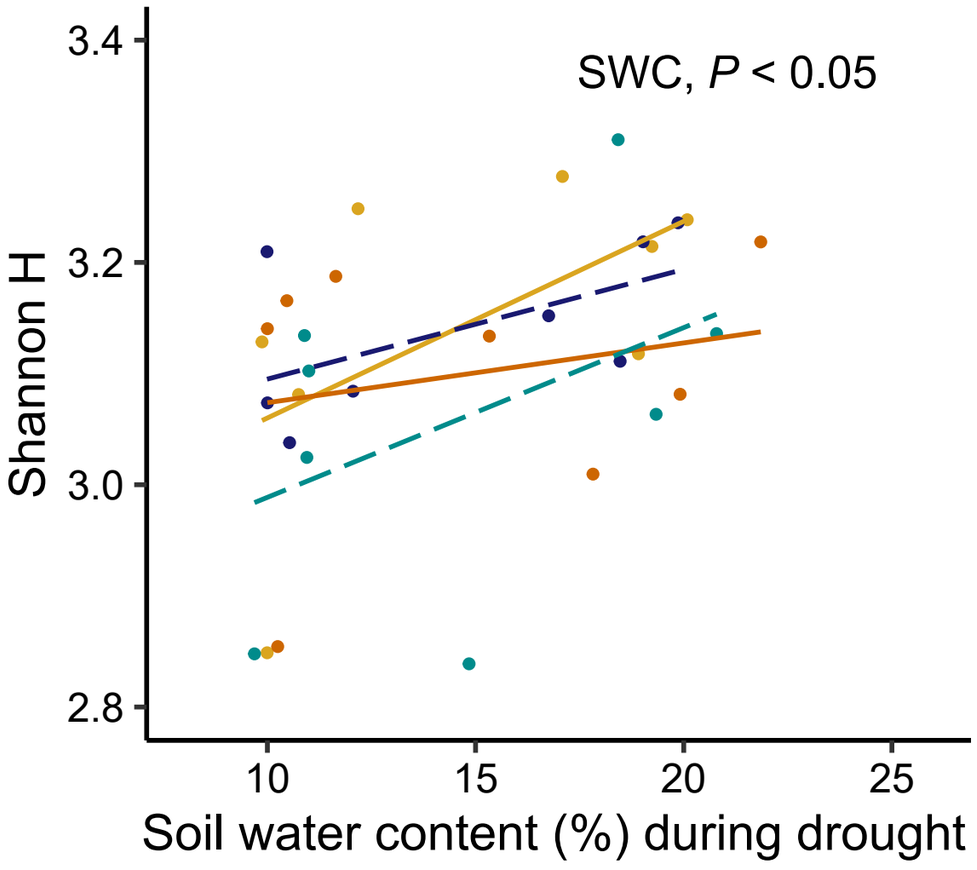
	Q. ilex	*P. sylvestris*
Soil respiration	NS	Positive effect of SWC during drought, $P < 0.001$
Composition (PERMANOVA)	SWC during drought × SWC during recovery, $P < 0.01$	Effect of SWC during drought, $P < 0.05$
Shannon H	Positive effect of SWC during drought, $P < 0.01$	NS
Evenness	Positive effect of SWC during drought, $P < 0.05$	NS


(a)
Q. ilex at the end of drought
SWC × N, P < 0.05
ln(soil respiration + 0.1) g CO2m-2 h-1
0.0
−0.5
−1.0
−1.5
−2.0
−2.5
10
15
20
25
Soil water content (%) during drought
(b)
P. sylvestris at the end of drought
N, P < 0.05
ln(soil respiration) g CO2 m-2h-1
−1
−2
−3
10
15
20
25
Soil water content (%) during drought
Nutrients
Control
N
P
NP


(a)
Q. ilex at the end of drought
PC2 (16.15%)
PC1 (19.47%)
0.4
0.2
0.0
-0.2
-0.4
-0.2
0.0
0.2
L-asparagine
Tween 80
Phenylethylamine
Tween 40
Gamma-hydroxybutyric acid
Putrescine
4-Hydroxy benzoic acid
D-galacturonic acid
Glycogen
Itaconic acid
Alpha-cyclodextrin
L-arginine
Pyruvic acid methyl ester
D-malic acid
Alpha-D-lactose
D-cellobiose
Alpha-ketobutyric acid
2-Hydroxy benzoic acid
L-phenylalanine
Beta-methyl-D-glucoside
Glucose-1-phosphate
D-galactonic acid-gamma-lactone
i-erythritol
Glycyl-L-glutamic acid
L-serine
D-mannitol
L-threonine
D-glucosaminic acid
D,L-alpha-glycerol phosphate
D-xylose
N-acetyl-D-glucosamine
(b)
P. sylvestris at the end of drought
PC2 (13.37%)
PC1 (27.5%)
0.75
0.50
0.25
0.00
-0.25
-0.2
0.0
0.2
0.4
Glycyl-L-glutamic acid
L-phenylalanine
D-glucosaminic acid
i-erythritol
Pyruvic acid methyl ester
L-threonine
Alpha-ketobutyric acid
D-mannitol
2-Hydroxy benzoic acid
N-acetyl-D-glucosamine
D-galactonic acid-gamma-lactone
L-arginine
Tween 80
D-galacturonic acid
Itaconic acid
L-asparagine
L-serine
4-Hydroxy benzoic acid
D-xylose
Glycogen
D-malic acid
Alpha-cyclodextrin
Gamma-hydroxybutyric acid
Tween 40
Alpha-D-lactose
Beta-methyl-D-glucoside
D,L-alpha-glycerol phosphate
Glucose-1-phosphate
D-cellobiose
Putrescine
Phenylethylamine
Drought treatment
Control
Drought

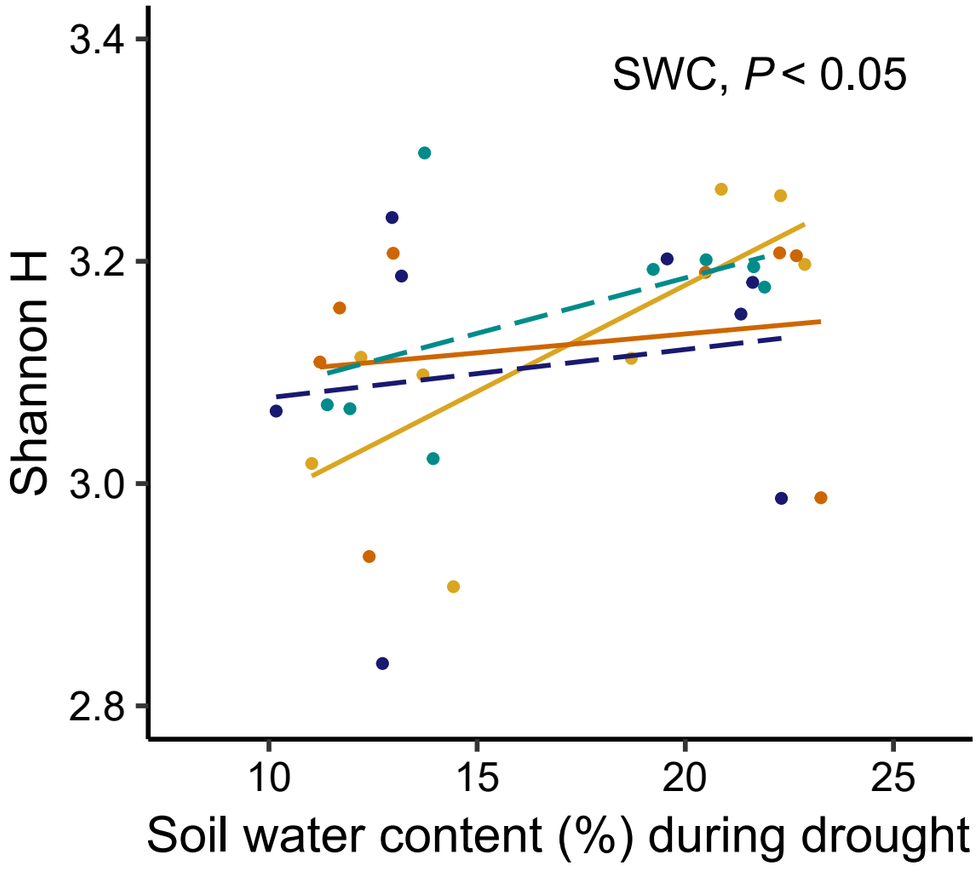
(a)

Q. ilex at the end of drought



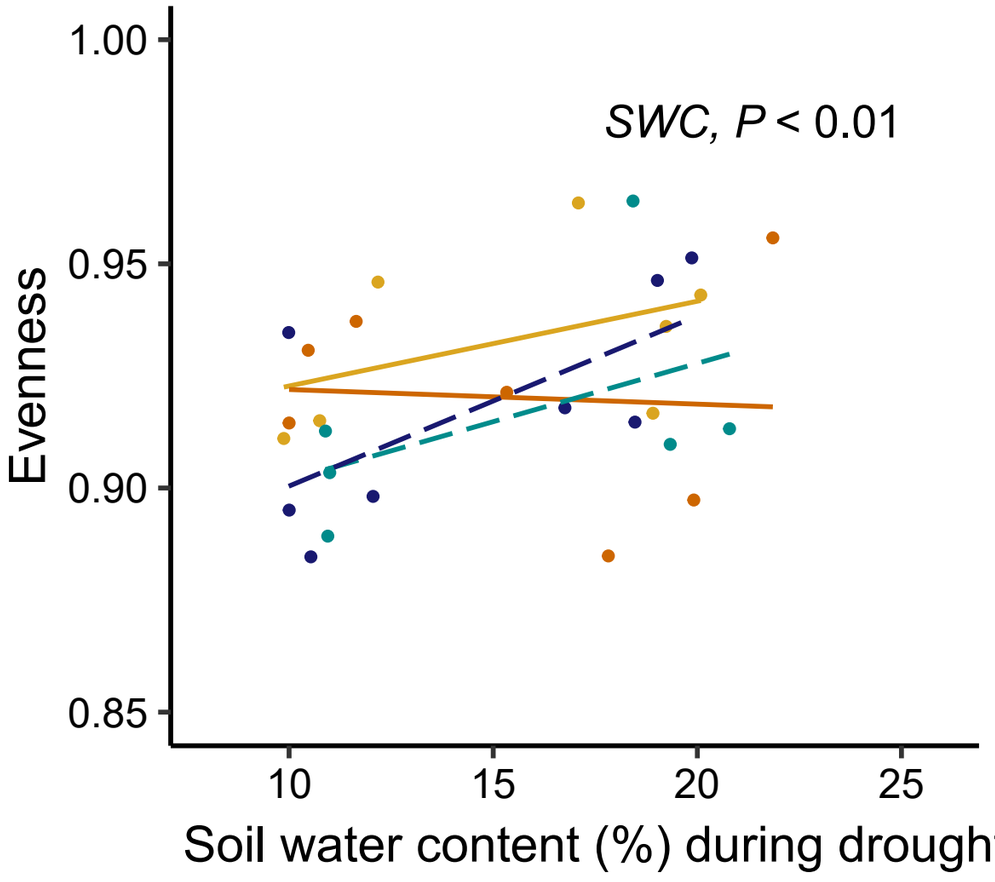
(b)

P. sylvestris at the end of drought



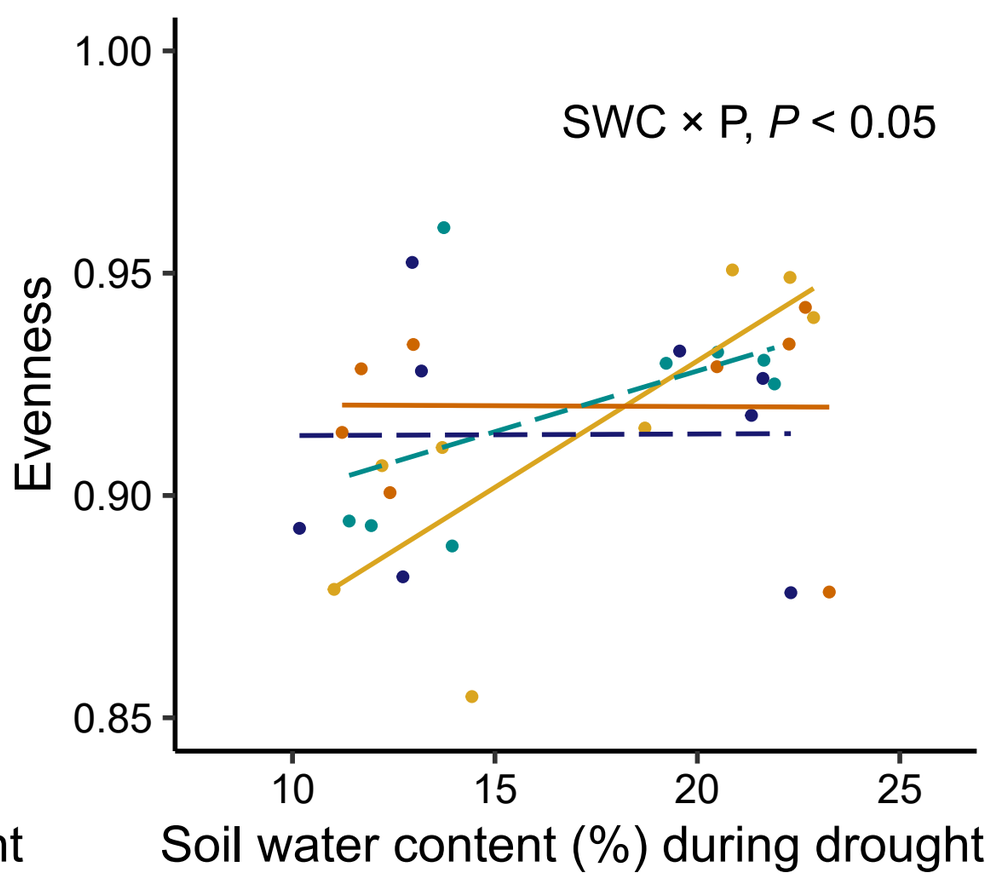
(c)

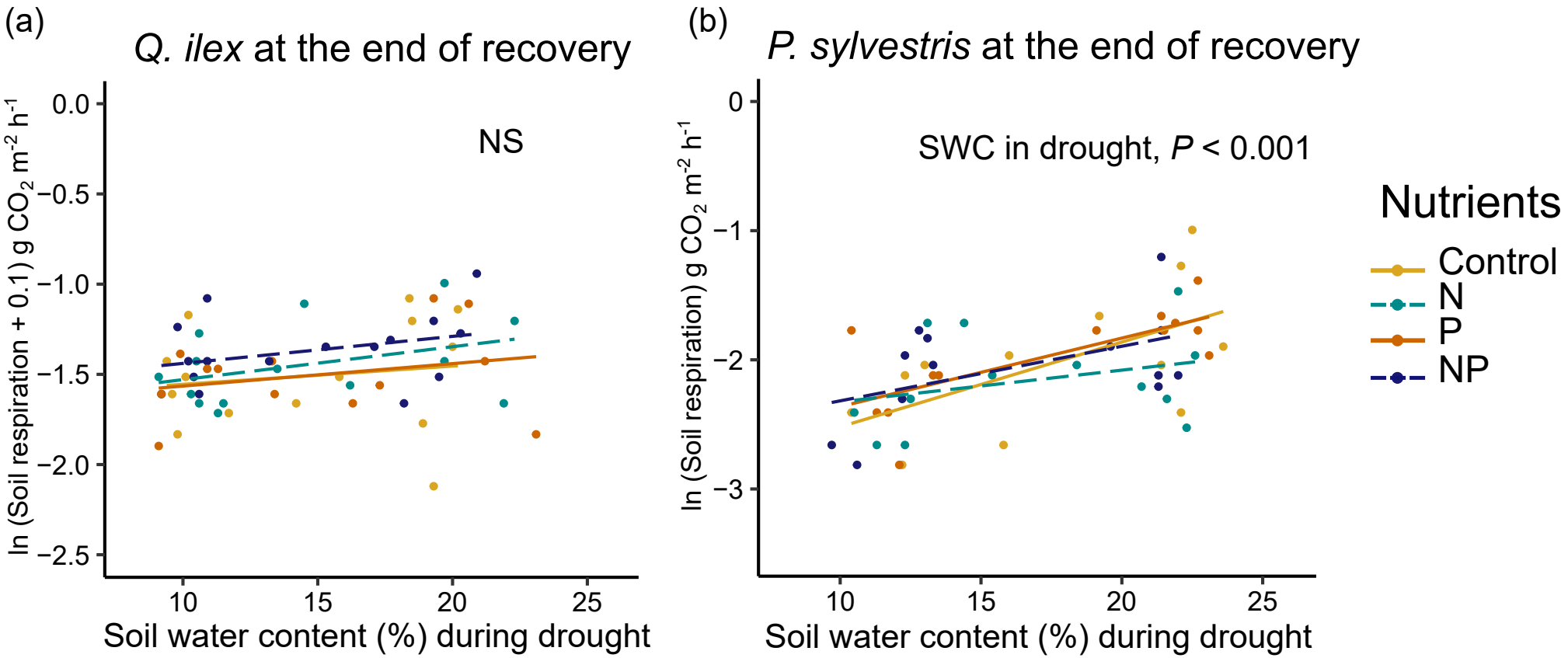
Q. ilex at the end of drought

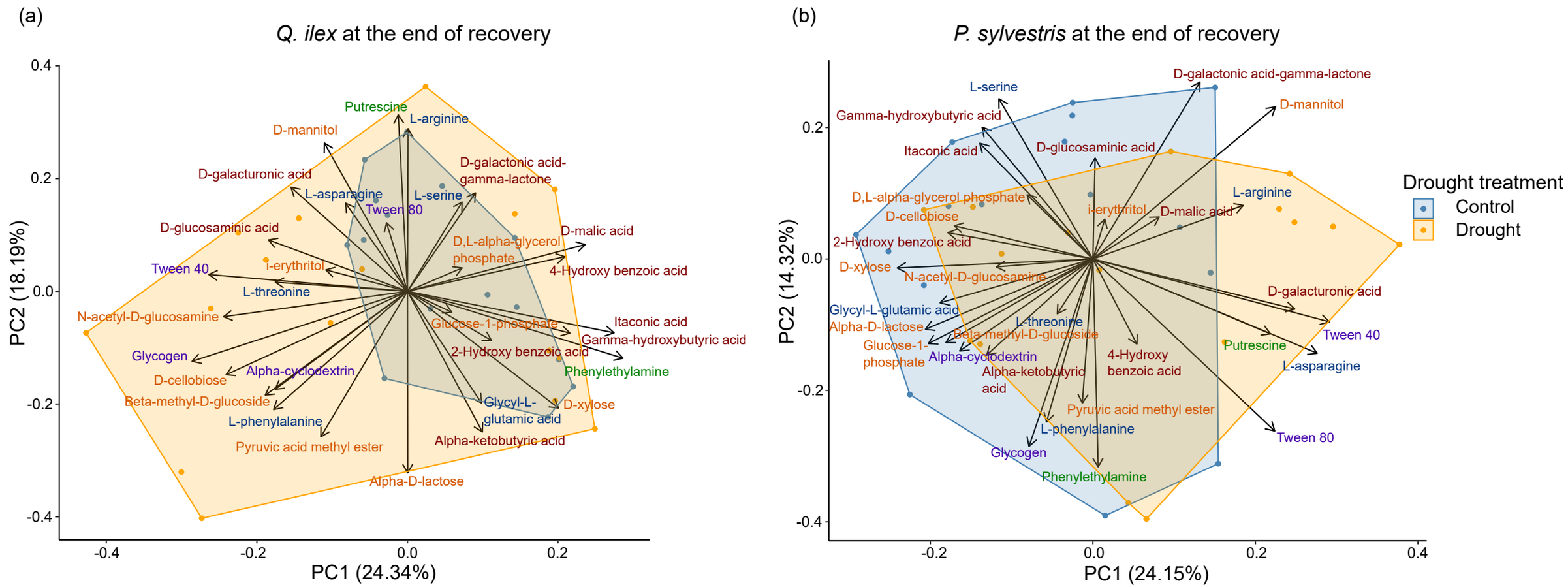


(d)

P. sylvestris at the end of drought




(a)
Q. ilex at the end of recovery
NS
ln (Soil respiration + 0.1) g CO2 m-2 h-1
0.0
−0.5
−1.0
−1.5
−2.0
−2.5
10
15
20
25
Soil water content (%) during drought
(b)
P. sylvestris at the end of recovery
SWC in drought, P < 0.001
ln (Soil respiration) g CO2 m-2 h-1
0
−1
−2
−3
10
15
20
25
Soil water content (%) during drought
Nutrients
Control
N
P
NP


(a)
Q. ilex at the end of recovery
PC2 (18.19%)
PC1 (24.34%)
0.4
0.2
0.0
-0.2
-0.4
Putrescine
L-arginine
D-mannitol
D-galactonic acid-gamma-lactone
D-galacturonic acid
L-asparagine
L-serine
Tween 80
D-glucosaminic acid
D,L-alpha-glycerol phosphate
D-malic acid
Tween 40
i-erythritol
4-Hydroxy benzoic acid
L-threonine
N-acetyl-D-glucosamine
Glucose-1-phosphate
Itaconic acid
Gamma-hydroxybutyric acid
Glycogen
2-Hydroxy benzoic acid
Alpha-cyclodextrin
Phenylethylamine
D-cellobiose
Beta-methyl-D-glucoside
Glycyl-L-glutamic acid
D-xylose
L-phenylalanine
Pyruvic acid methyl ester
Alpha-ketobutyric acid
Alpha-D-lactose
(b)
P. sylvestris at the end of recovery
PC2 (14.32%)
PC1 (24.15%)
0.2
0.0
-0.2
-0.4
D-galactonic acid-gamma-lactone
L-serine
D-mannitol
Gamma-hydroxybutyric acid
Itaconic acid
D-glucosaminic acid
L-arginine
D,L-alpha-glycerol phosphate
D-cellobiose
i-erythritol
D-malic acid
2-Hydroxy benzoic acid
D-xylose
N-acetyl-D-glucosamine
D-galacturonic acid
Glycyl-L-glutamic acid
Alpha-D-lactose
L-threonine
Tween 40
Beta-methyl-D-glucoside
Glucose-1-phosphate
Alpha-cyclodextrin
Putrescine
L-asparagine
4-Hydroxy benzoic acid
Alpha-ketobutyric acid
Pyruvic acid methyl ester
Tween 80
L-phenylalanine
Glycogen
Phenylethylamine
Drought treatment
Control
Drought

(a)

Q. ilex at the end of recovery

SWC in drought, $P < 0.01$

Shannon H

3.4
3.2
3.0
2.8

10
15
20
25

Soil water content (%) during drought

(b)

P. sylvestris at the end of recovery

NS

Shannon H

3.4
3.2
3.0
2.8

10
15
20
25

Soil water content (%) during drought

Nutrients
Control
N
P
NP

(c)

Q. ilex at the end of recovery

SWC in drought, $P < 0.05$

Evenness

1.00
0.95
0.90
0.85

10
15
20
25

Soil water content (%) during drought

(d)

P. sylvestris at the end of recovery

NS

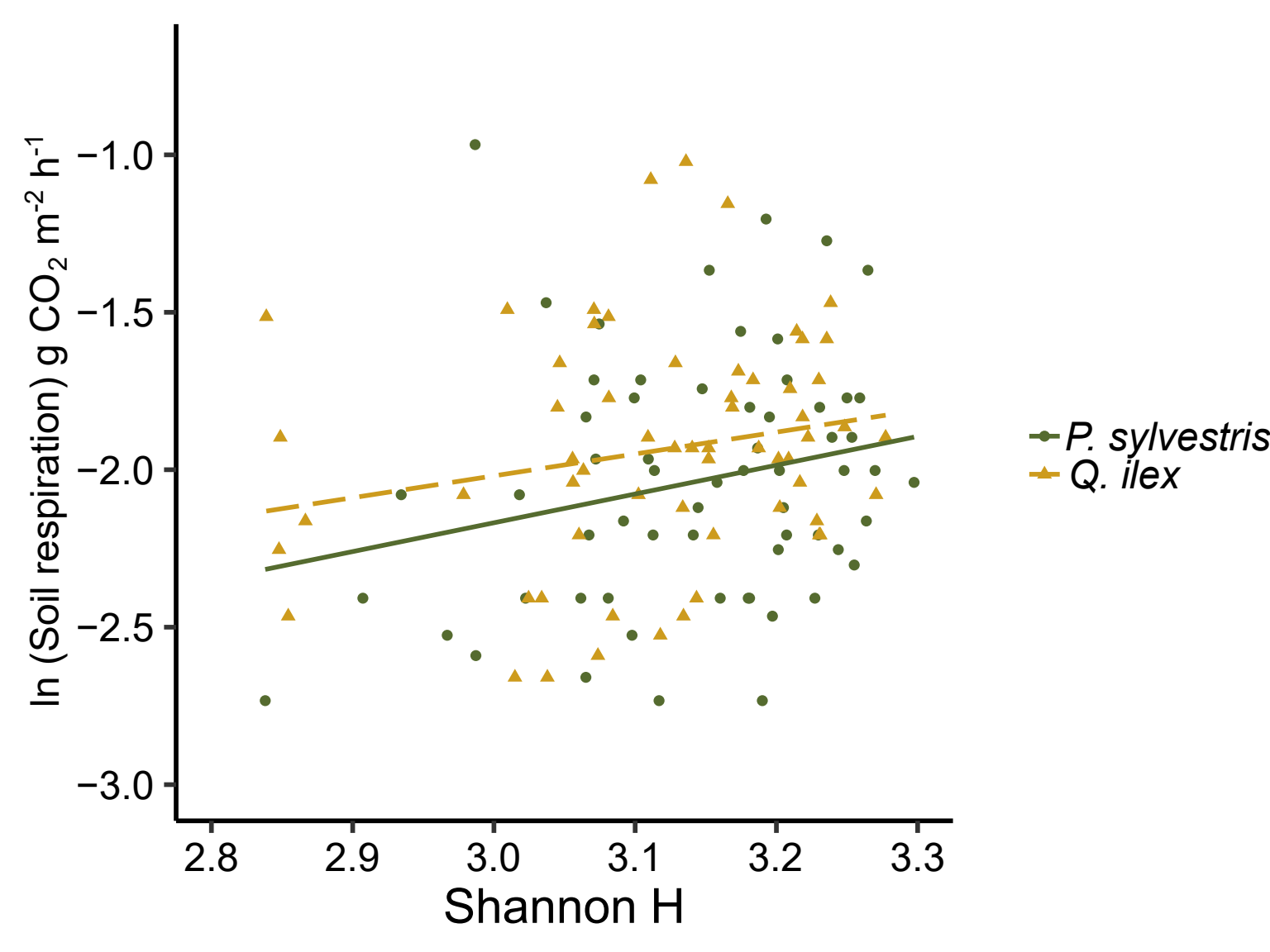
Evenness

1.00
0.95
0.90
0.85

10
15
20
25

Soil water content (%) during drought

Nutrients
Control
N
P
NP


ln (Soil respiration) g CO_2 m^{-2} h^{-1}
−1.0
−1.5
−2.0
−2.5
−3.0
2.8
2.9
3.0
3.1
3.2
3.3
Shannon H
P. sylvestris
Q. ilex

Drought is a stronger driver of soil respiration and microbial communities than nitrogen or phosphorus addition in two Mediterranean tree species – Supplementary Material

Table S1 Guild groupings of the BIOLOG EcoPlate carbon sources (following Weber and Legge, 2009).

Carbon source	Guild
Alpha-D-lactose	Carbohydrates
Beta-methyl-D-glucoside	Carbohydrates
D-cellobiose	Carbohydrates
D-mannitol	Carbohydrates
D-xylose	Carbohydrates
D,L-alpha-glycerol phosphate	Carbohydrates
Glucose-1-phosphate	Carbohydrates
i-erythritol	Carbohydrates
N-acetyl-D-glucosamine	Carbohydrates
Pyruvic acid methyl ester	Carbohydrates
Alpha-ketobutyric acid	Carboxylic & acetic acids
2-Hydroxy benzoic acid	Carboxylic & acetic acids
4-Hydroxy benzoic acid	Carboxylic & acetic acids
D-galactonic acid-gamma-lactone	Carboxylic & acetic acids
D-glucosaminic acid	Carboxylic & acetic acids
D-galacturonic acid	Carboxylic & acetic acids
Gamma-hydroxybutyric acid	Carboxylic & acetic acids
Itaconic acid	Carboxylic & acetic acids

D-malic acid	Carboxylic & acetic acids
L-arginine	Amino acids
L-asparagine	Amino acids
L-phenylalanine	Amino acids
L-serine	Amino acids
L-threonine	Amino acids
Glycyl-L-glutamic acid	Amino acids
Phenylethylamine	Amines/amides
Putrescine	Amines/amides
Alpha-cyclodextrin	Polymers
Glycogen	Polymers
Tween 40	Polymers
Tween 80	Polymers

7

Table S2. Effects of drought and nutrient addition on the potential metabolisms of the five C guilds identified by linear models, with species (*Q. ilex* and *P. sylvestris*) and experiment (drought and recovery) separated. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant. Close to significant P-values are also shown. Positive and negative refers to the type of correlation between the relative abundance of the mean standardised absorbance for each guild with the explanatory variable. Note that when there is a significant interaction, the direction (positive or negative) of the individual treatment effect is not described.

Drought period					
	Carbohydrates	**Amino acids**	**Carboxylic and acetic acids**	**Amines/amides**	**Polymers**
Quercus ilex					
SWC	**Negative *** **	NS	NS	**Positive ** **	**Positive * **
N addition	NS	NS	NS	NS	NS
P addition	NS	NS	NS	NS	NS

Pinus sylvestris					
SWC	NS	**Negative *** **	NS	**	NS
N addition	NS	NS	NS	NS	NS
P addition	NS	NS	NS	*	NS
Moisture content × P addition	NS	NS	NS	**Moisture content has positive effect with P addition but negative effect without P addition ***	NS

Recovery period					
	Carbohydrates	**Amino acids**	**Carboxylic and acetic acids**	**Amines/amides**	**Polymers**
Quercus ilex					
SWC during drought	NS	NS	**Positive ****	NS	**Negative ***
SWC during recovery	NS	NS	**Positive *****	NS	**Negative ****
N addition	NS	NS		NS	
P addition	NS	NS		NS	
SWC (drought) × SWC (recovery)	NS	NS	******	NS	*****
SWC (recovery) × P addition	NS	NS		NS	
SWC (drought) × N addition	NS	NS		NS	
SWC (drought) × P addition	NS	NS		NS	

Pinus sylvestris					
SWC during drought	*****	*P* = 0.07	**Positive ****	NS	NS
SWC during recovery	*****	*P* = 0.05	**Positive ****	NS	NS
N addition	NS	**Positive ***		NS	NS
P addition	*P* = 0.08		**Positive ***	NS	NS
SWC (drought) × SWC (recovery)	*****	*P* = 0.06	**	NS	NS
SWC (drought) × N addition		*P* = 0.09		NS	NS
SWC (drought) × P addition			*	NS	NS
SWC (recovery) × P addition	*P* = 0.08			NS	NS

Figure S1. Clustered image map showing the results of the PLS analysis for *Quercus ilex* in the drought period.

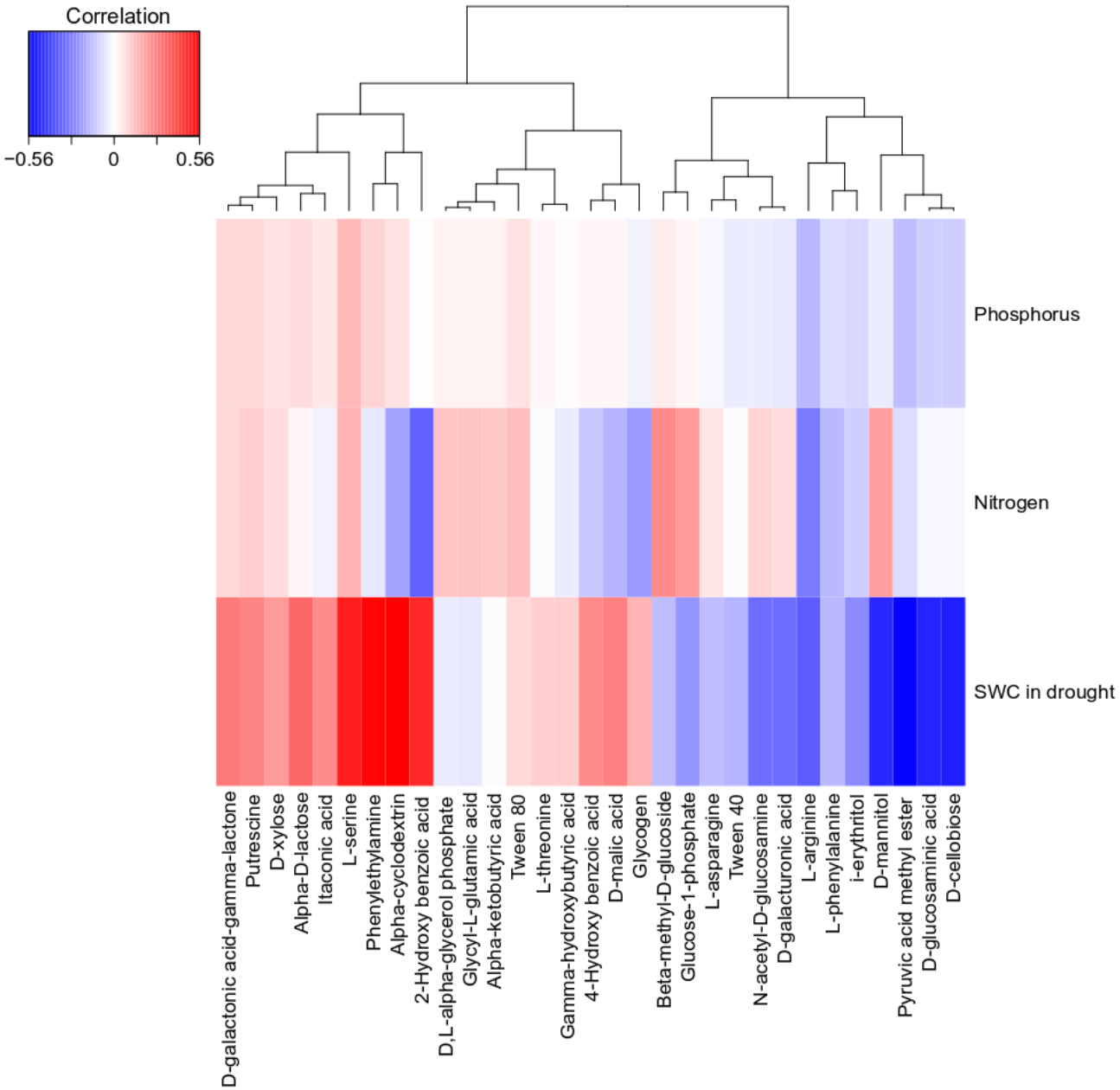


Figure S2. Clustered image map showing the results of the PLS analysis for *Pinus sylvestris* in the drought period.

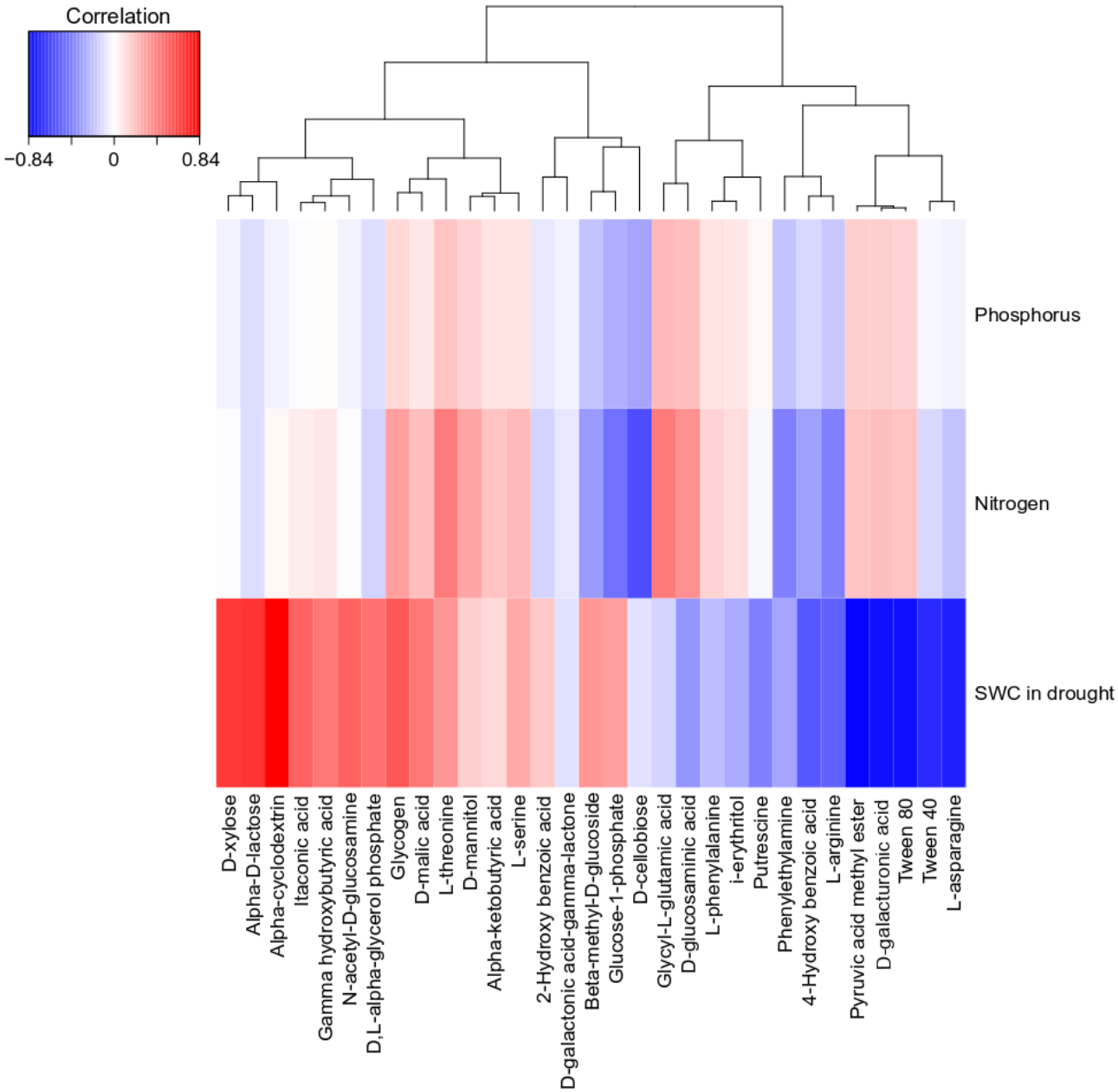


Figure S3. Clustered image map showing the results of the PLS analysis for *Quercus ilex* in the recovery period.

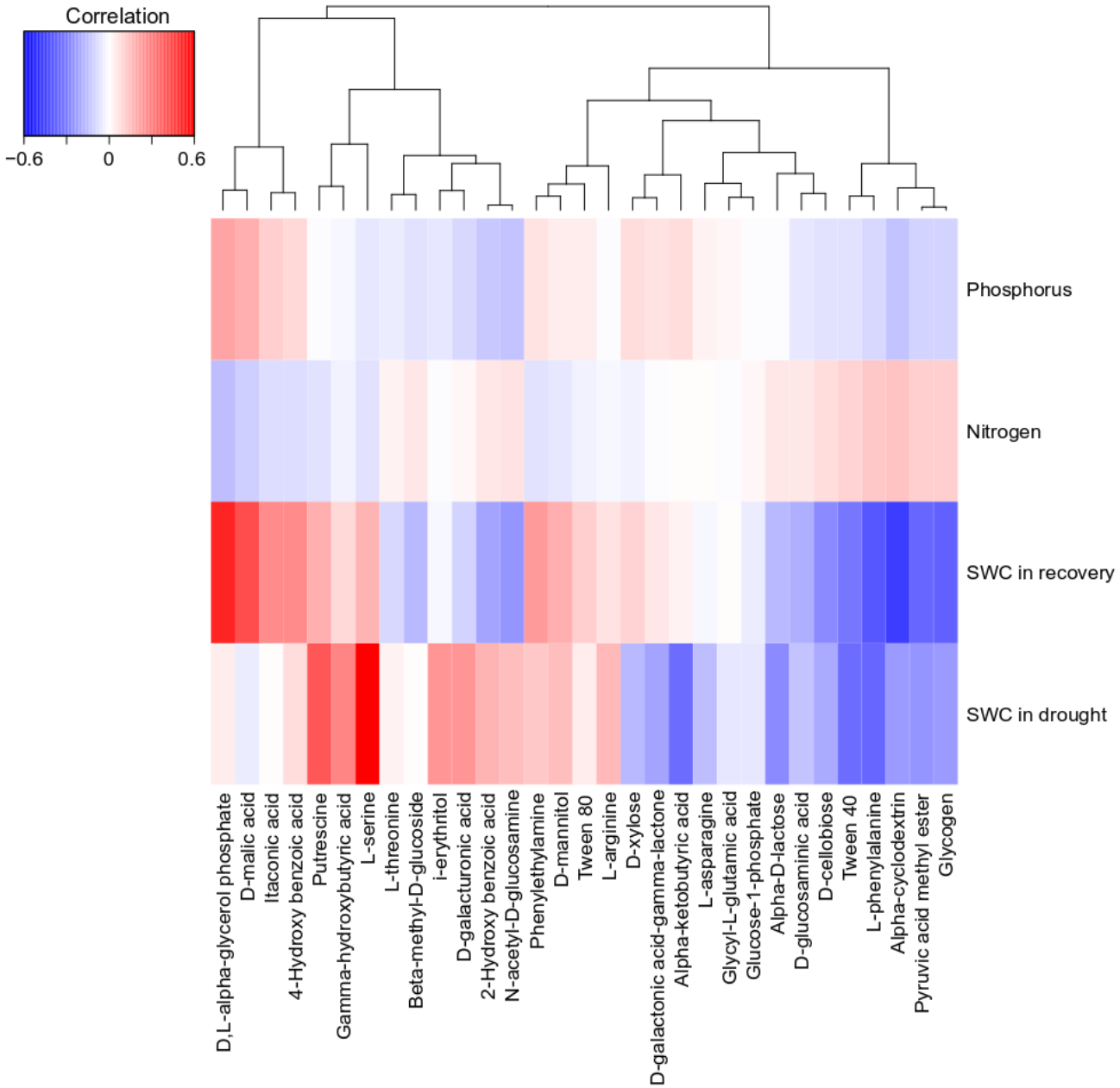


Figure S4. Clustered image map showing the results of the PLS analysis for *Pinus sylvestris* in the recovery period.