**Identification of differentially methylated regions (DMRs) associated with leaf physiological acclimation to experimental long-term drought in holm oak (*Quercus ilex* L.)**

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## Abstract

Shifts in rainfall patterns and increasing temperatures associated with climate change are causing widespread forest decline, especially due to the increase and duration of droughts. Tree species may have to quickly adapt to these changing conditions, and epigenetic modifications are expected to play a key role in regulating rapid acclimation responses. In this study, we measured acclimation physiological responses and methylome responses in mature holm oak trees (*Quercus ilex* L.) subjected to 15 years of experimental accrued drought (-29% of rainfall) and their respective controls with ambient rainfall. We hypothesized that: i) oak trees exposed to long-term drought will exhibit different foliar traits due to adaptative phenotypic plasticity to drought, ii) methylation levels will differ between the drought and control trees allowing the identification of drought-induced differentially methylated regions (DMRs), and iii) these DMRs correlate with the differences in foliar traits. Our results confirmed all hypotheses. The methylome analysis revealed 84 drought-related DMRs among trees from different precipitation treatments, of which 17 DMRs were significantly associated with measured phenotypic responses. This study provides evidence of the role of epigenetic regulation for tree acclimation responses in natural populations of holm oak facing increased droughts and identified candidate genes potentially involved in drought adaptation.

## Introduction

## A significant body of research indicates that increased tree mortality worldwide is linked to human-induced climate changes, which result in higher temperatures and greater water stress (Allen *et al.*, 2010; Choat *et al.*, 2012; Anderegg *et al.*, 2013; Senf *et al.*, 2020). Consequently, there is growing concern that projected future rises in temperature, coupled with heightened variability and uncertainty in water availability—particularly as a result of alterations in the frequency, duration, and intensity of drought events (Intergovernmental Panel On Climate Change, 2023)—pose a significant threat to the composition, structure, and biogeography of forests, as well as the ecosystem services they provide. As sessile organisms with seeds that generally have limited dispersal capabilities, trees must adapt locally to environmental stresses. However, they are especially vulnerable to rapid global changes due to their lengthy lifespans, which hinder their ability to adapt quickly (Petit & Hampe, 2006; Aitken *et al.*, 2008). In this context, phenotypic plasticity is assumed to be a crucial determinant of plant adaptation to stress, both in the short and long term (Nicotra *et al.*, 2015). Epigenetic modifications triggered by environmental stresses are suggested as a rapid alternative mechanism for local phenotypic adaptation in natural populations, independent of genetic variation (Bossdorf *et al.*, 2007; Sork *et al.*, 2022). These epigenetic modifications entail alterations in gene expression and function without changes in the DNA sequence (Richards, 2006). This enable individuals to generate adaptive responses to environmental changes (Angers, et al. 2010; Richards et al. 2017) in the form of phenotypes that are more resistant and resilient to stress (Mirouze & Paszkowski, 2011), effects that may also be inherited across generations (Saze *et al.*, 2003; Hauser *et al.*, 2011; Bose *et al.*, 2020).

Several potential and non-mutually exclusive epigenetic mechanisms have been documented, including (i) changes in the methylation of a cytosine base of the DNA, (ii) changes in the chromatin structure through chemical modifications by histones, and (iii) changes in regulatory processes mediated by small RNA molecules which can regulate gene expression. DNA methylation is recognized as the most extensively researched and stable epigenetic marker (Du *et al.*, 2015). Although the specific functional role of DNA methylation in trees' response to environmental changes is not yet fully understood, evidence suggests that DNA methylation, which affects the regulation of phytohormone-related genes, can be modified by drought (Lafon-Placette *et al.*, 2018). DNA methylation was even proposed as a marker to validate and select the identity, provenance or quality of agro-forestry products in *Populus balsamifera* (Champigny *et al.*, 2020). However, the large majority of the DNA methylation studies stem from experiments conducted on model-species, often using artificial experimental conditions, which questions their generality and external validity (Richards *et al.*, 2017). Studying epigenetic variation in natural populations is challenging because of the existing genetic, phenotypic and ecological differences across individuals growing in the field, due to their different life histories, microhabitats, soil conditions and biotic interactions. Nevertheless, there are now several studies in natural populations that highlight the relevance of DNA methylation in creating adaptative phenotypic variation under stress conditions (Herrera & Bazaga 2013; Herrera & Bazaga, 2011; Nicotra *et al.*, 2015), including in oak species (Rico *et al.*, 2014; Gugger *et al.*, 2016). However, all these studies in natural populations were done with conventional methods like methylation-sensitive markers that allow identifying global methylation patterns, but do not provide information about where differentially methylated regions (DMRs) are placed in the genome and whether they are associated with specific genes or transposable elements that could be involved in local adaptation. Ecological studies mechanistically linking DMRs of the genome with phenotypic plasticity confirming the relationships between epigenetics and phenotypic plasticity are currently missing in the field, despite the importance of assessing the significance of epigenetic variation in microevolution (Richards, 2006). Taken collectively, these findings suggest a potential role for DNA methylation in adaptation to climatic stressors in natural populations, but call for more detailed and functional studies, especially in non-model species.

In this context, this study aims to acquire a deeper understanding of the potential role of DNA methylation in the drought response of *Quercus ilex* L., a non-model tree species of high ecological importance in the Mediterranean region. We used a long-term field experiment in which precipitation was reduced by 29% since 2003, alongside a control treatment with ambient precipitation, in a natural *Q. ilex* forest in the south of France (Limousin *et al.*, 2009). We performed concurrent measurements of genome methylation and foliar traits in the two precipitation treatments 15 years after the onset of the experiment. We focused on foliar traits because leaves are the organs responsible for controlling tree photosynthesis and transpiration, so they play a central role in tree performance and sensitivity to water stress. Furthermore, as new leaf cohorts are produced by the trees every growing season before the seasonal summer drought, their phenotypic differences before stress exposure are indicative of a tree drought-memory and are therefore good candidates to investigate epigenetic responses. We used our ecophysiological and molecular dataset to search for potential links between DMRs and different leaf physiological responses, in order to explore the potential contributions of methylome changes to tree phenotypic acclimation to drought. We hypothesized that i) oak trees exposed to long-term drought treatment will exhibit different foliar traits due to carry-over phenotypic plasticity to drought, ii) that methylation levels within the drought-related DMRs could be identified by comparing methylation levels between trees from the drought and control treatments, and iii) that these DMRs may correlate with the differences in foliar traits. Finally, we aimed at identifying the neighbouring genes and transposable elements (TEs) associated with the DMRs and which may be relevant for the tree’s responses to drought.

## Materials and Methods

*Study site and experimental design*

The study site is located 35 km northwest of Montpellier (southern France) in the Puéchabon State Forest, on a flat plateau (43º44’29’’ N, 3º35’45’’ E, elevation 270m). This forest has been managed as a coppice for centuries and the last clear cut was performed in 1942. Vegetation is largely dominated by the late-successional evergreen oak *Quercus ilex*, with a top canopy height of about 5.5 m, a stand density of *c.* 4900 stems ha-1, and a leaf area index of 2.2. The understorey is a sparse shrubby layer composed of the evergreen species, *Buxus sempervirens, Phyllirea latifolia, Pistacia terebinthus* and *Juniperus oxycedrus,* with a percent cover *c.* 25% and a height of up to *c.* 2m. The soil is extremely rocky, originating from hard Jurassic limestone. The average volumetric fractional content of stones and rocks is about 75% for the top 0–50 cm and 90% below. The stone free fine fraction within the top 0–50 cm layer of the soil is a homogeneous silty clay loam (USDA texture triangle, 38.8% clay, 35.2% silt and 26% sand). The site has a Mediterranean-type climate with about 80% of total annual precipitation occurring between September and April. The mean annual precipitation is 953 mm with a range of 578-1549 mm (1989–2018). Mean annual temperature is 13.5 °C (on-site meteorological station, 1989–2018), the coldest month being January (6.0°C) and the hottest July (22.4°C).

In March 2003, a partial throughfall exclusion experiment was set up on the site. The throughfall exclusion experiment was replicated on three blocks 200 m away one from the other, and situated on a flat area with no lateral runoff. The experiment was composed of one throughfall exclusion treatment (henceforth, Drought treatment) and one control treatment (henceforth, Control), each with a plot area of 140 m2 (14 m x 10 m). Throughfall exclusion is achieved by using 14 m long and 0.19 m wide PVC gutters covering 33% of the ground area underneath the tree canopy. Taking into account interception losses by the canopy and stemflow, the throughfall exclusion treatment effectively reduces the net input of precipitation to the soil by 29% compared with the control treatment (Limousin et al. 2008). On the Control plot, identical gutters are set up upside down so that the albedo and the understorey microclimate are as close as possible in the two treatments. The experimental design reduces significantly the surface soil water content (García de Jalón *et al.*, 2020), and its efficiency in increasing recurrently the tree water stress was evidenced by significantly more negative tree water potentials in most summers since treatment installation in 2003 (Bykova *et al.*, 2018). The trees sampled for this study were exclusively from the two plots equipped with scaffolding, which allowed access to the upper tree canopy.

*Leaf ecophysiological and physicochemical measurements*

Ten foliar traits relevant to drought-tolerance were measured: light-saturated assimilation rate (*Amax*), stomatal conductance (*gs*), dark respiration (*Rd*), mesophyll conductance (*gm*), leaf-level carbon use efficiency (*Rd*/*Amax*), leaf mass per area (LMA), intrinsic water use efficiency (*WUE*i), leaf nitrogen content (LNC), leaf carbon content (LCC) and photosynthetic nitrogen use efficiency (PNUE). Measurements were taken during two field campaigns of three days with similar meteorological conditions in autumn (27th and 28th of November, and 1st of December 2017) on leaves that had experienced the severe 2017 summer drought, and in spring (2nd, 3rd, 6th of July 2018) on newly emerged leaves before the onset of the 2018 seasonal drought. Leaves were sampled from six trees per treatment in 2017, and this number was increased to seven trees per treatment in 2018 to compensate for the fact that some of the trees were identified as clones (see genotyping section). The leaf physiological variables used in correlations with DMRs were from the 2018 spring campaign (and not from autumn 2017), since the goal was to assess the role of DNA methylation induced changes in new emerged leaves without previous drought exposure.

Leaf gas exchange was measured with two intercalibrated LI-6400 Photosynthesis Systems equipped with the LI-6400-40 Leaf Chamber Fluorometer (LiCor Inc., Lincoln, NE, USA). Leaves were first acclimated in the leaf chamber for 20 minutes at ambient temperature, under a regulated ambient CO2 concentration of 400µmol CO2 mol-1air and a saturating photosynthetic photon flux density of 1500 µmol m-2 s-1. Light saturated net assimilation rate (*Amax*) and chlorophyll fluorescence were measured after the 20-min acclimation period. The light source was then switched off for at least 3 min or until stable gas-exchange rates occurred before measuring leaf dark respiration (*Rd*). Leaf mesophyll conductance to CO2 from the sub stomatal cavities to the chloroplasts (*gm*) was calculated from *Amax*, *Rd* and chlorophyll fluorescence measurements following the variable electron transport rate method of Harley et al. (1992):

gm= (Eq.1)

where Ci is the CO2 concentration in the sub-stomatal cavity, is the CO2 compensation point in the absence of mitochondrial respiration taken from Bernacchi et al. (2002), and JETR is the photosynthetic electron transport rate calculated from the chlorophyll fluorescence measurements as in Niinemets *et al.* (2006) and Limousin *et al.* (2010).

Leaf mass per area without petiole (LMA) was measured in the same leaves that were used for the leaf gas exchange measurements. Additional leaves were sampled from 5 adjacent branches, pooled per tree, and used for analyses of leaf nutrient content. LMA was calculated from the projected fresh leaf area measured using a flat-bed high-resolution transmission scanner (EPSON Perfection V800) and the ImageJ software (US NIH, Bethesda, MD, USA, <http://imagej.nih.gov/ij/>), and the leaf dry mass after 3 days at 60ºC in a drying oven. Dry leaves were ground to a fine powder with a mechanical ball mill (MM400; Retsch GmbH, Haan, Germany). Leaf nitrogen content (LNC), leaf carbon content (LCC), and carbon isotope ratio (δ13C) were measured using a continuous flow isotope ratio mass spectrometry system (Euro-EA Eurovector Elemental Analyzer coupled with an IsoPrime Mass Spectrometer, GV Instruments, Manchester, UK) at the Stable Isotope Analytical platform of the Biochemistry and Plant Molecular Physiology Laboratory (INRAE Montpellier, France). Leaf phosphorous content (LPC) was measured colorimetrically with an autoanalyzer (Evolution II, Alliance Instruments, Frepillon-France) using the sodium heptamolybdate coloration method.

Intrinsic water use efficiency (WUEi) was calculated from the δ13C by combining the equations of (Farquhar, *et al.*, 1982; Farquhar & Richards, 1984):

(Eq.2)

where Ca is the atmosphere CO2 concentration taken as 400 µmol mol-1, *a* is the C isotope fractionation during diffusion through the stomata, *b* is the discrimination during carboxylation by the RUBISCO, and  13C atm is the isotopic ratio of C at the atmosphere.

Photosynthetic nitrogen use efficiency (PNUE) was calculated as *Amax* divided by the amount of nitrogen per unit leaf area (PNUE = *Amax* \* 100 / LNC \* LMA). Finally, the leaf-level carbon use efficiency was approached by the ratio of dark respiration to maximum photosynthetic assimilation *Rd*/*Amax* (Limousin *et al.*, 2015).

The effect of the rainfall exclusion experiment on tree water stress was verified regularly since 2003 by measuring the predawn leaf water potential (Ψpd). Measurements were carried out seven times in 2017 and six times in 2018 between the months of June and October. Three leafy shoots were sampled before dawn on five trees per treatment. Shoots were stored in a sealed plastic bags in a dark cooler, and Ψpd was measured within two hours after sampling with a pressure chamber (PMS1000; PMS Instruments, Corvallis, OR, USA). The soil water balance model described in (Cabon *et al.*, 2018) was used to simulate daily Ψpd between measurements and in periods when field measurements were not available. The relationship between measured and simulated Ψpd had an R² = 0.85 (RMSE = 0.58 MPa) for the control treatment, and R² = 0.89 (RMSE = 0.57 MPa) for the dry treatment.

*Genotyping*

Tree genotyping at 70 single nucleotide polymorphism markers (SNPs) was used to identify potential clones among the sampled trees motivated by their proximity in the field. Genotyping was performed by the Genome Transcriptome Facility in Bordeaux, France (PGTB) using MassARRAY (System Agena Bioscience technology). This analysis used 70 validated SNP markers (Bonal *et al.*, 2019) developed from ddRAD-Seq data following the methods described in (García *et al.*, 2018). The clonal membership was assigned with the cervus software (Marshall, 1998) based on an identity analysis that compares each genotyped individual against all the others. Individuals were considered as clones if they had more than 50 loci with exact matching of their SNP markers (Gavinet *et al.*, 2020). The analysis identified three tree clones, and consequently two were discarded from the identification of DMRs, which reduced the number of trees used for calculating the methylation level in the Drought treatment to 4 trees, against 7 trees in the Control treatment.

*Methylome and bioinformatic analysis*

Leaf samples for DNA methylation analyses were sampled in spring 2018 by randomly selecting from 5 different branches of the upper canopy of the trees. Collected leaves were immediately kept on dry ice for transportation to the laboratory. DNA extraction of leaves was done using DNeasy 96 Plant Kit following the manufacturer’s protocol (Qiagen N.V., Hilden, Germany) and quantified using the ND-1000 Spectrophotometer (NanoDrop Technologies Llc, Wilmington, USA) and Qubit Fluorometric Quantification (Thermo Fisher Scientific Inc., Waltham, USA). Extracted DNAs samples were sent to the Novogene company (Beijing, China) for whole genome bisulfite assessment. Library preparation, sequencing and data quality control was done by Novogene. Bisulfite conversion rate was above 99.85% for all samples. Quality control of FASTQ files provided by the company was evaluated using FastQC tool (Babraham Bioinformatics, Cambridge, UK). Sequences were trimmed using Trimmomatic 0.36 version (Bolger *et al.*, 2014). Bisulfite treated reads were aligned to *Quercus robur* PM1N reference genome already indexed (Plomion *et al.*, 2018) and bisulfite converted using “Bismark Bisulfite Mapper v0.22.1” (Krueger & Andrews, 2011). Bismark also performed PCR duplicates removal and methylation call for every single cytosine analysed. The Bismark methylation extractor generated the output detailing the following for each cytosine: its position, the context (CpG, CHG, or CHH), and the methylation status. Methylated cytosines are marked as forward reads (+), whereas non-methylated cytosines are indicated as reverse reads (-). Output genome-wide cytosine methylation reports were extracted with Bismark and imported to “ViewBS” for methylation data visualization (Huang et al. 2018) and DMRs identification was done using the R package “DMRcaller” (Catoni *et al.*, 2018). The most conservative method/ noise filter for DMR detection provided by DMRcaller was used with the strictest parameters (window size = 100 bp, P-value threshold = 0.01, kernelFunction = "triangular", test = "score", minimum Cytosines Count = 4, Proportion Difference minimum = 0.2, minGap = 0, minSize = 50, minimum Reads Per Cytosine = 4, cores = 1). Each DMR found was checked with a graph amplifying the DMR region in order to discard the DMRs that were wrongly detected due to intermittent mapping. Methylation level (number of methylated reads / total number of reads) for each DMR of each tree was calculated using “computeMethylationProfile” option from DMRcaller, and this variable was used for correlations with the leaf physiological traits. Identification of DMRs was done with genetically different individuals (7 from Control and 4 from the Drought treatment) after identification of clones among the measured trees (see Genotyping section). However, methylation level at each DMR was calculated in all trees including non-genetically different individuals (7 from Control and 7 from Drought) in order to have a high sample size for the correlations with leaf physiological traits. Gene functions in promoter regions of the DMRs were identified using the database of Oak Genome Sequencing site (<http://www.oakgenome.fr/>) from the *Quercus robur* genome PM1N (Plomion *et al.*, 2018).

*Statistical analyses*

All statistical analyses were performed with the R software version R 3.4.1 (R Core Team, 2013). The effect of the rainfall exclusion treatment on physiological variables was analysed using a mixed linear model with tree as random effect using the *lmer* function from the ‘lme4’ package version 1.1–21 (Bates *et al.*, 2014). An example of the syntax for a model looking at photosynthesis in the spring season is as follows: “*model<-* *lmer (Amax ~ PR\_treat + (1|Tree), data=Spring\_2018)”.* Minimal adequate models with the lowest Akaike information criterion (AIC) were obtained following the guidelines of Zuur *et al.*, (2009) with the help of the “buildmer” package version 1.4 (Voeten, 2019). Correlations between methylation proportion within DMRs and the physiological variables were tested using a Pearson’s correlation coefficient with the false discovery rate correction of Benjamin & Hochberg (1995) to reduce the likelihood of identifying false positives.

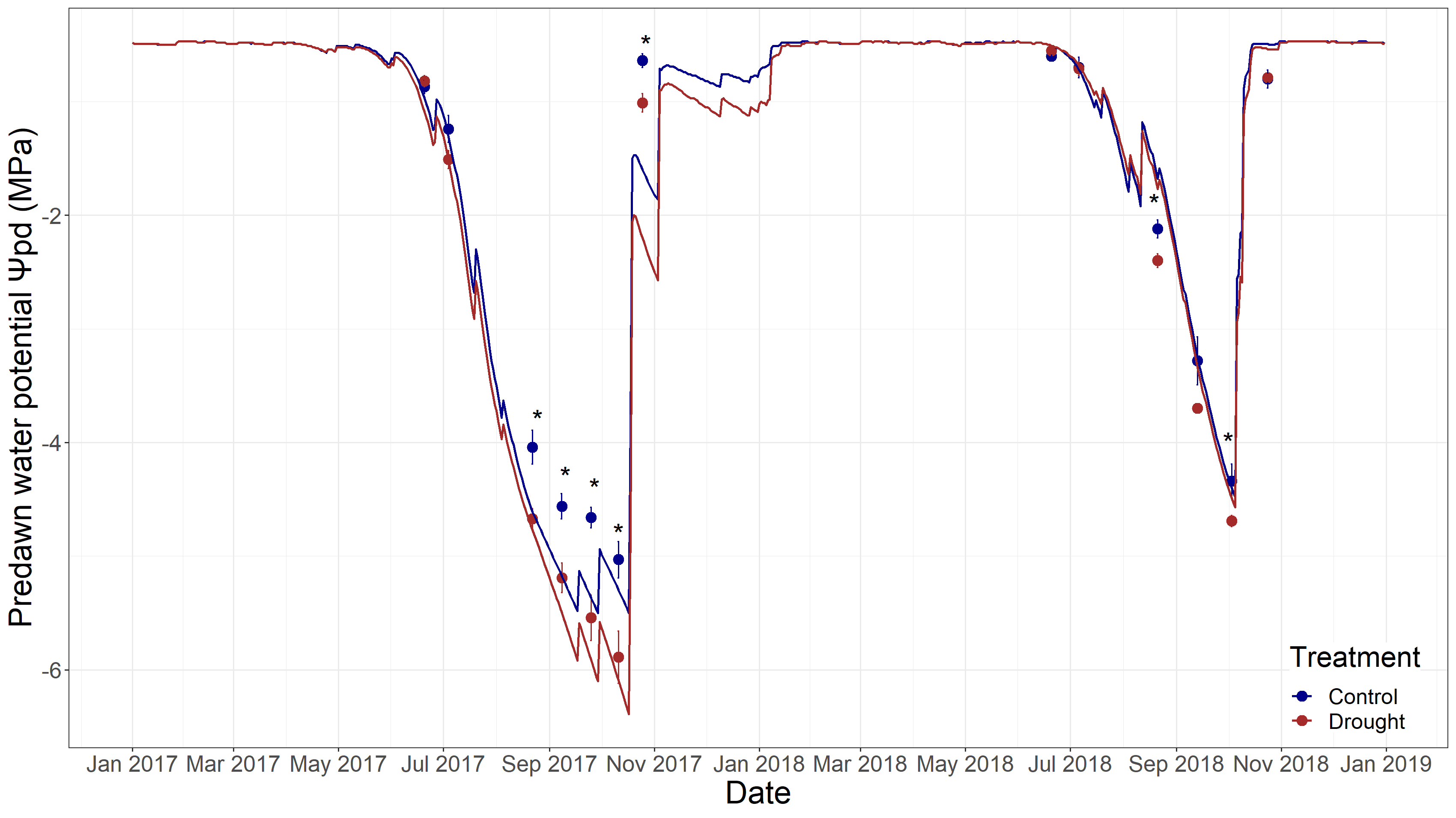
## Results

*Precipitation reduction effect on tree water stress*

The experimental drought treatment applied at the Puéchabon site has consistently induced significantly more severe tree water stress during the summer droughts, across most years since its initiation in 2003; see Bykova *et al.*, (2018) for a summary of results from 2003 to 2017. The 2017 summer drought, just before the start of this study, was the most severe since 2003 and predawn water potential reached -5.03 ± 0.16 MPa in the Control treatment and -5.89 ± 0.23 MPa in the Drought treatment on October 11, 2017. Over the 7 Ψpd measurements performed in 2017, the five campaigns between August and October exhibited significantly more negative Ψpd in the Drought treatment (*P* < 0.05; Fig. 1). In 2018, the drought was milder and minimum Ψpd recorded at the drought peak were characteristic of the average water stress for the site. The treatment effect on Ψpd was nevertheless significant in two of the six campaigns (in August and October, *P* < 0.05; Fig. 1).

[ **Insert Fig. 1**]

**Figure 1:** Predawn leaf water potential (Ψpd in MPa) measured in 6 trees from the Control (blue circles) and Drought treatment (red circles) in 2017 and 2018. The continuous lines show the daily Ψpd simulated by the water balance model in the two treatments for an indication of the seasonal dynamic of the water stress (blue line for Control, and red line for the Drought treatment). Leaf physiological measurements were carried out in autumn 2017 and spring 2018. Asterisks show the statistical significance at P < 0.05.

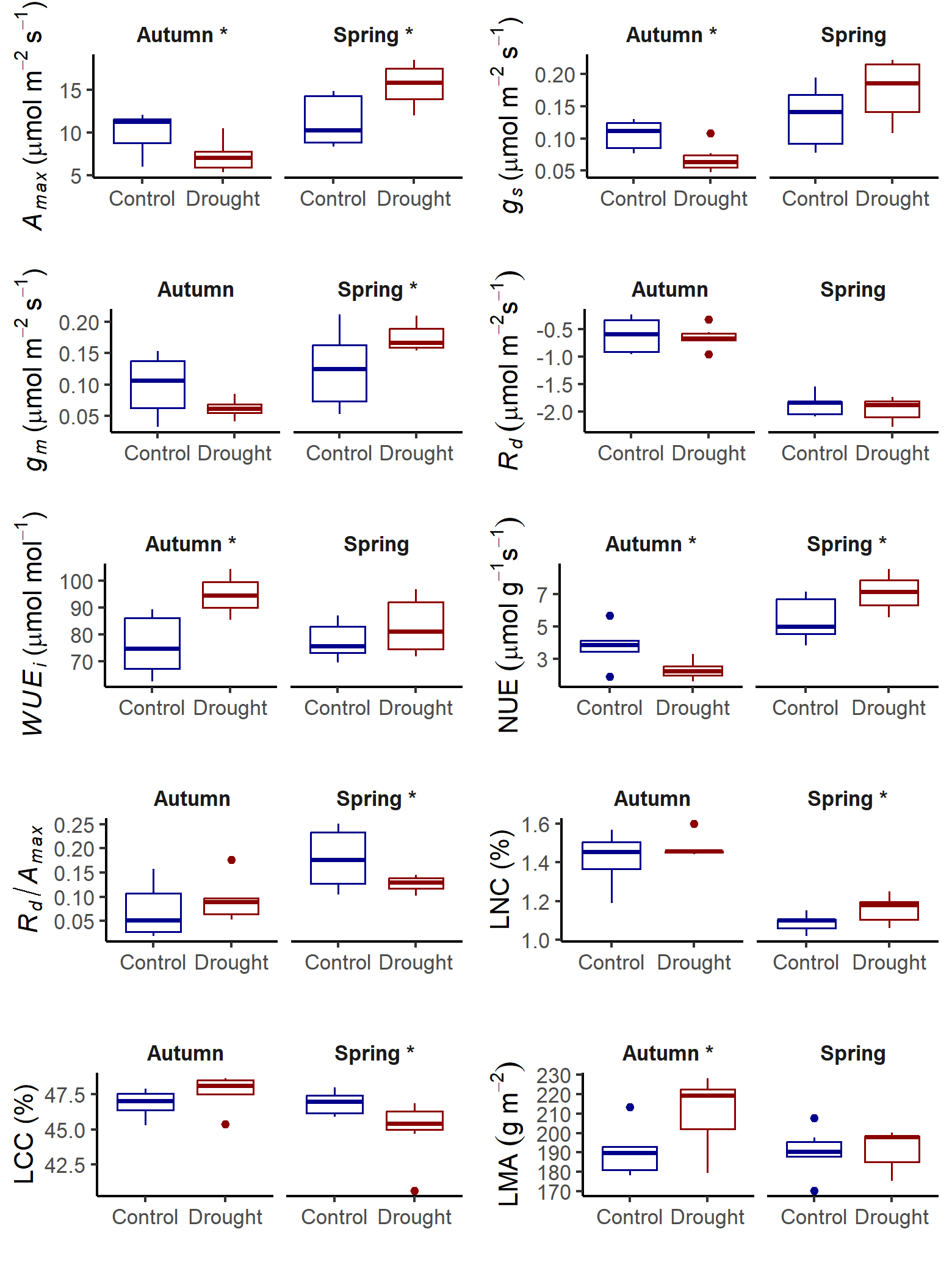


*Phenotypic and physiological responses to drought*

In autumn 2017, following the summer water stress, trees in the Drought plot exhibited a significant (P < 0.05) reduction in maximum photosynthetic rate (*Amax*) and stomatal conductance (*gs*) by 28% and 35%, respectively, compared to trees in the Control plot (Fig. 2). Mesophyll conductance (*gm*) was also lower in the Drought treatment, although not significantly.

[**Insert Fig. 2**]

**Figure 2:** Boxplots of leaf phenotypic traits according to the precipitation treatment (Control or Drought): maximal CO2 assimilation rate (*Amax*), stomatal conductance to water vapour (*gs*), mesophyll conductance to CO2 (*gm*), dark respiration (*Rd*), intrinsic water use efficiency (*WUEi*), nitrogen use efficiency (NUE), respiration to assimilation ratio (*Rd/Amax*), leaf nitrogen content (LNC), leaf phosphorous content (LPC), leaf carbon content (LCC) and leaf mass per area (LMA). Statistically significant differences at *P* < 0.05 are indicated with “\*” for each campaign (n = 12 trees in autumn 2017, n = 14 trees in spring 2018).



In contrast, in spring 2018 leaf gas exchange rates were significantly increased in trees from the Drought plot compared to Control, with increases of +27% in *Amax* (*P*<0.05), +24% in *gs* (P > 0.05) and +30% in *gm* (P < 0.05). The intrinsic water use efficiency (*WUEi*), which is indicative of the amount of carbon assimilated per amount of water transpired, was significantly increased by +20% (P < 0.05) in autumn 2017 in trees from the Drought plot compared to those in the Control. In contrast, *WUEi* of newly emerged leaves was not significantly different between treatments in spring 2018, although there was a slight tendency for an increased *WUEi* in trees from the Drought plot.

Regarding the leaf chemical composition, we found no changes between treatments in leaf nitrogen, phosphorous and carbon in autumn. Conversely, we observed that trees exposed to enhanced Drought presented significantly higher leaf nitrogen content (+6%) and significantly lower leaf carbon content (-4%) compared to the Control in the leaves produced in spring 2018. Leaf mass per area (LMA) was increased by +10% in the Drought plot in autumn 2017, but this trait was not different between treatments in newly produced leaves in spring 2018.

# DNA methylation responses to drought

The mapping efficiency obtained after mapping bisulfite reads of *Quercus ilex* samples against the *Quercus robur* reference genomewas about 25%, which means that our data will only concern the most conserved part of the genome between the two species. On average, the detected levels of cytosine methylation were 40% for CG context, 33% for CHG context and 5% for CHH context, respectively. Whole genome methylation levels were higher in trees coming from the Drought plot compared to trees coming from the Control at all cytosine contexts (+9.1% at CG, +9.4% at CHG, +9.2% at CHH).

Within the remaining 25% of the mapped genome, we found 84 differentially methylated genome regions (DMRs) between trees from the two precipitation regimes (Table 1; Figure 3).

[**Insert Fig. 3**]

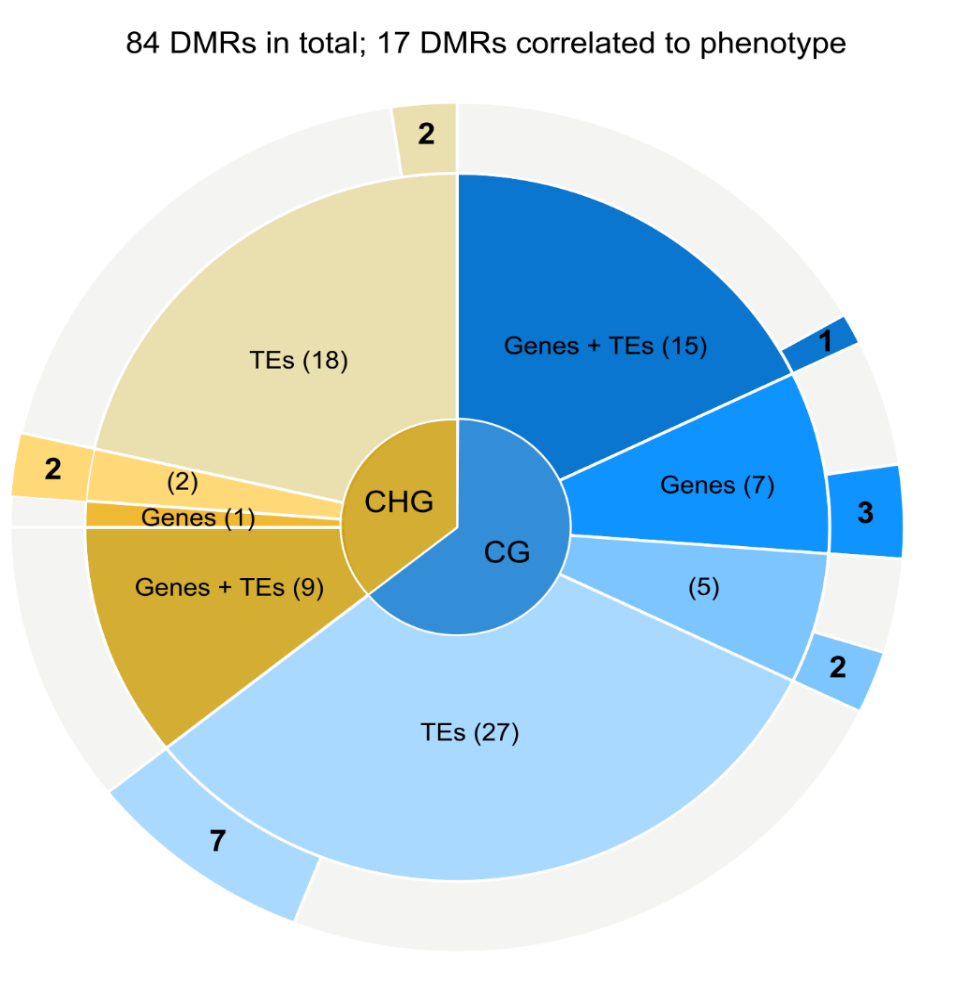
**Table 1** Summary description of the 84 DMRs found between trees from Drought (n = 4) and Control (n = 7) treatments according to the cytosine. Number of DMRs with percent in brackets, hypermethylation, and number of DMRs in the proximity of ± 2000 bp of genes and TEs (transposable elements).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **All C contexts** | **CG** | **CHG** | **CHH** |
| **No. of DMRs** | 84 | 54 (64%) | 30 (36%) | 0 |
| **Hypermethylation** | 60 (71%) | 35 (42%) | 25 (30%) | 0 |
| **Genes ± 2000bp** | 32 (38%) | 22 (26%) | 10 (12%) | 0 |
| **TEs ± 2000bp** | 69 (82%) | 43 (51%) | 27 (32%) | 0 |

Most of the DMRs found where in the CG context (64%), whereas 36% were in the CHG context. No DMR was detected in the CHH context. Around two thirds of the DMRs (71%) were hypermethylated (higher methylation in the Drought compared to the Control) whereas one third (29%) of the DMRs were hypomethylated (lower methylation in the Drought compared to the Control).

In order to analyse the functionality of the DMRs we searched for gene-promoter regions and transposable elements (TEs) at their proximities defined as ± 2000 base pairs (bp). Of the 84 identified DMRs, 38% were found near genes, 82% near TEs, and 29% near both genes and TEs (Table 1, Fig. 3); note that the percentages exceed 100% because DMRs nearby both genes and TEs are double counted.

**Figure 3:** Summary of the 84 differentially methylated regions (DMRs) observed in response to Drought treatment. The pie chart is divided first by cytosine context, then by the DMR's position relative to genes, transposable elements (TEs), or both (within ±2000 bp), and finally by whether the DMR methylation proportion is significantly correlated with phenotypic traits. The numbers indicate the number of DMRs.



Genes with DMRs in their promoter regions or within their gene bodies were identified and their functions were summarized in Table S1. Among the 32 DMRs associated to genes, 56% were in the gene body, 19% downstream the genes, and 25% upstream the genes. Among the functions of these genes, 33% of the genes were related to the regulation of enzyme or protein activities involved in biochemical reactions related to DNA transcription, DNA replication and chromatin remodelling. Around 15% of the genes functions were related to the biosynthesis and transport of carbohydrates of any group (Cx(H20)y). The remaining gene functions were linked to general biochemical reactions (18%), cell-cycle regulators like ion transport, signal transducers, or homeostatic pathways (19% of the genes), and unknown functions (15% of the genes).

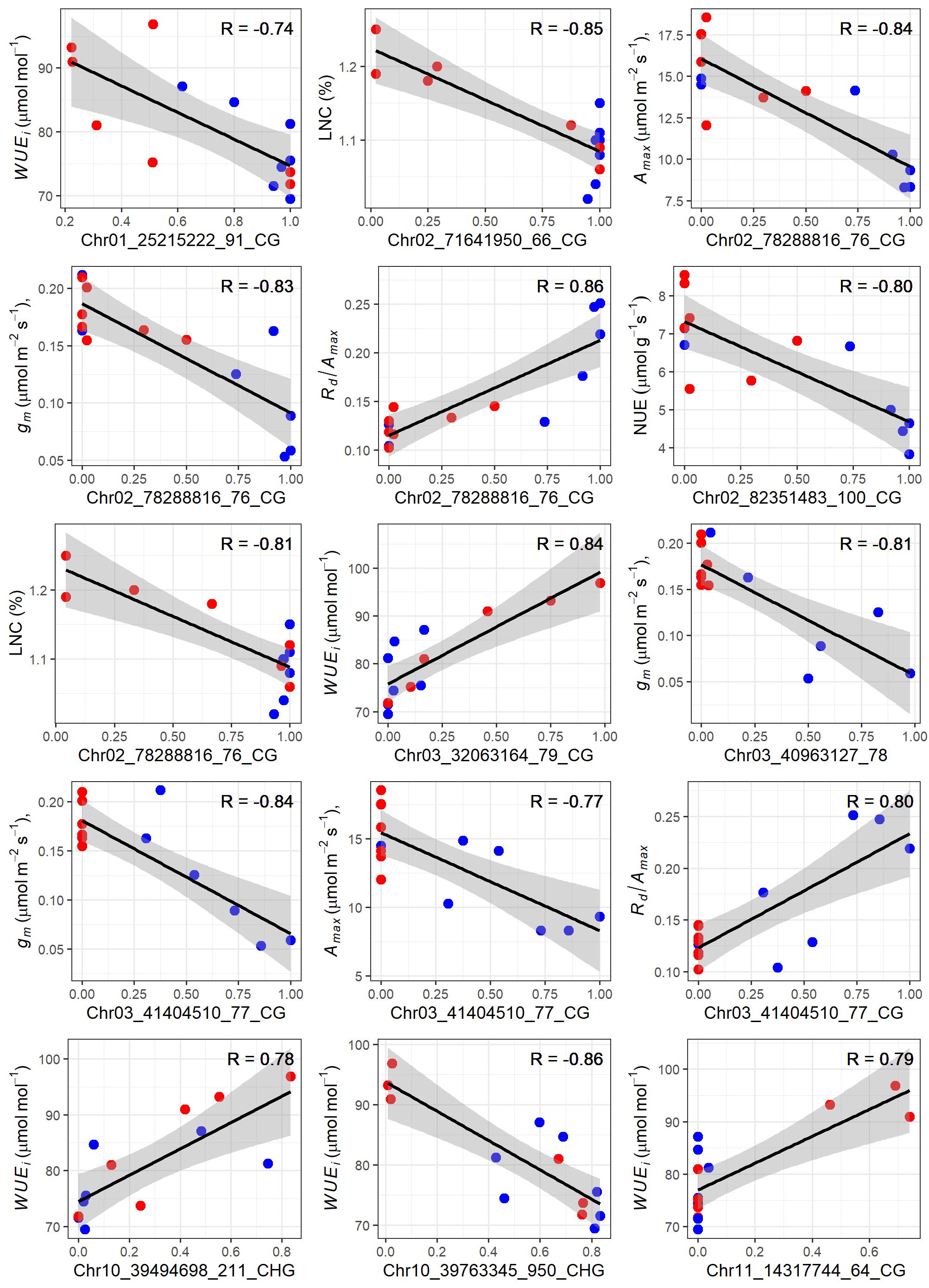
# Correlations between DMRs and physiology

Our conservative analysis (using a false discovery rate correction of Benjamin & Hochberg (1995)) of the correlations between the methylation level in the 82 identified DMRs and the leaf phenotypic variables (measured in spring 2018) identified 22 significant correlation involving 17 DMRs (13 in CG context and 4 in CHG context). Notably, all correlations retained as significant had correlation coefficients either above 0.75 or below -0.75 (Fig. 4).

[**Insert Fig. 4**]

Among these, 1 DMR was located in the promoter region of both genes and TEs, 4 were located in promoter regions of genes, 8 were located in promoter regions of TEs, and 3 were located in other regions of the genome. The phenotypic variables that showed correlations with DMRs were WUEi, LNC, *Amax*, *gm*, *Rd*/*Amax*, NUE, LCC, correlating with 5, 2, 2, 3, 2, 1 and 7 DMRs, respectively. For details on 15 of the 22 correlations, see Fig. 4, and for the remaining correlations, refer to Supplementary Fig. S1.

**Figure 4.** Correlations among methylation levels (%) in differentially methylated regions (DMRs) and foliar traits: light-saturated assimilation rate (Amax), stomatal conductance (gs), dark respiration (Rd), mesophyll conductance (gm), leaf-level carbon use efficiency (Rd/Amax), leaf mass per area (LMA), intrinsic water use efficiency (WUEi), leaf nitrogen content (LNC), and photosynthetic nitrogen use efficiency (NUE). Correlations with leaf carbon content (LCC) are available in Fig. S1. Points represent individual trees (n = 14), with Control and Drought treatments shown in blue and red, respectively. Chromosome positions (Ch1 to Ch12) and contexts of DMRs are marked on the x-axis.



## Discussion

Trees exposed to precipitation reduction in our long-term Drought treatment presented phenotypical responses common to plastic responses to water stress that could induce a better acclimation to drought compared to the trees from the Control. In addition, we observed larger plasticity between seasons in the Drought treatment where some physiological and morpho-chemical traits, such as *Amax*, *gs*, *gm*, NUE, LNC differed significantly between autumn 2017 and spring 2018, whereas they exhibited less variable values in the Control treatment (Fig. 2). In autumn 2017, when trees were still recovering from the severe summer drought, we found a significant reduction in *Amax* (-27%) and *gs* (-35%) in Drought compared to the Control treatment (Fig. 2). This reduction of leaf gas-exchange rates in the Drought treatment might be due to the fact that the soil water content, and hence the tree water stress, had not recovered to the same extent as in the Control treatment in autumn 2017 (Fig. 1). In addition, it might also be due to carry-over drought effects from the past summer mediated either by damages to the leaf cells, chloroplasts or enzymes (Flexas *et al.*, 2006; Chaves *et al.*, 2009), by hydraulic limitation due to the loss of hydraulic conductance in the leaf or in the branches (Resco *et al.*, 2009; Peguero-Pina *et al.*, 2018), or by the selective shedding of more drought resistant but less physiologically performant leaves (Niinemets, 2015; Li *et al.*, 2018). Consistently with this last conjecture, leaves sampled in autumn in the Drought treatment were significantly higher in LMA (+10%), a trait commonly associated with a better tolerance to leaf desiccation (Poorter *et al.*, 2009; Niinemets, 2015), and presented higher intrinsic water use efficiency (+20% *WUE*i). Higher *WUEi*means that these leaves assimilated their C at a lower water cost than in the control treatment, and also that they exhibited a stronger stomatal regulation of transpiration (Fig. 2; Wright *et al.*, 2003; Limousin *et al.*, 2010). In contrast, the newly emerged leaves sampled in the following spring had not experienced any water stress yet at the time of sampling. They exhibited nonetheless a significant increase in leaf gas exchange compared to the control trees with a +28%, +24% and +30% increase for *Amax*, *gs* and *gm*, respectively. This pattern is consistent with the higher leaf nitrogen content (+6%) in the new leaves of the Drought treatment, although the significantly higher PNUE indicates that leaf nutrient concentration is not solely responsible for the higher gas exchange rates (Fig. 2; (Evans, 1989; Limousin *et al.*, 2010). Moreover, besides being more efficient in water use and nitrogen use, we also observed that the new leaves in the Drought treatment had a lower *Rd/Amax* ratio which indicates a higher carbon use efficiency (Limousin *et al.*, 2015). These results suggest that even without being exposed to water stress, the young leaves produced by trees in the Drought treatment were more efficient in resource use than in the control treatment. This points out to a conservative resource use strategy to face water limitation as trees from the Drought treatment also support a lower leaf area (Limousin *et al.*, 2012; Gavinet *et al.*, 2019). Moreover, our results show an increase in plasticity to respond to and recover from drought stress in the trees from the Drought plot. The mechanisms involved in this increased plasticity were not analysed, but one might hypothesize an epigenetic role in the signalling and pathways underlying these changes. For example, an epigenetic memory of summer drought through modifications in DNA methylation patterns that increased the transduction of growth hormone signalling was observed in apical meristems of winter-dormant shoots in *Populus trichocarpa* growing in field (Lafon-Placette *et al.*, 2018; Sow *et al.*, 2020).

Our genome-wide DNA methylation results revealed a tendency for genome-wide hypermethylation in oak trees exposed to the Drought treatment compared to Control trees in all three cytosine contexts (+9.1% at CG, +9.4% at CHG, +9.2% at CHH). Hypermethylation was also observed at the locus level, with 71% of the identified drought-related DMRs being hypermethylated (Table 1). These results are comparable to those of Rico *et al.*, (2014) who found an increased methylation of the hypermethylated loci (CmCmGG/GGCmCm ) and a decreased methylation of the fully methylated loci (CCmGG/GGCmC) in *Q. ilex* trees exposed to a precipitation reduction treatment for 12 years (being Cm a methylated cytosine, C a non-methylated cytosine and G a Guanine). Differences between the results of the two studies can, at least in part, be attributed to the different methodologies, as the inherent additional limitations of MS-AFLP fingerprinting technique used by (Rico *et al.*, 2014) is known to underestimate the total level of genome methylation (Schrey *et al.*, 2013). In contrast, the WGBS method used in our study permits the analysis of methylation patterns at different cytosine contexts along the whole genome, which allows for different interpretations of methylation according to the cytosine context (Dubin *et al.*, 2015). In our study, most of the DMRs (64%) were found in CG context, followed by 36% in CHG context, and none was found in CHH context. The role of methylation in CG context has been identified as participating in local adaptation to temperature in a study where DNA methylation patterns of *Quercus lobata* were analysed across different climates, while methylation at CHG or CHH context was not found to play a comparable role (Gugger *et al.*, 2016). Similarly, gene body methylation at CG was correlated to the latitude and climate of origin in different accessions of *Arabidopsis thaliana* from different regions of Sweden, suggesting also a role in local adaptation to climate, in contrast to the other C contexts that were not sensitive to environmental variables (Dubin *et al.*, 2015).

DNA methylation can repress transcription when methylation is found in gene promoter regions, and conversely, genes can be upregulated by the loss of methylation that reactivates transcription (Zilberman *et al.*, 2007). The loss of methylation in the repeated sequences or TEs can lead to the reactivation and transposition influencing gene expression in other regions of the genome (Cokus *et al.*, 2008). In our study, we explored the potential role of the identified DMRs in the expression of genes and TEs (Table 1), and found that 38% of the identified DMRs were located in the vicinity of genes and 82% of them in the vicinity of TEs (± 2000 bp.; Table 1). We can hypothesize that the genes involved in the biosynthesis and transportation of carbohydrates or other organic compounds Cx(H20)y, and located near 4 in the vicinities of the identified 4 DMRs may be potentially involved in the increased plasticity of trees under Drought. An efficient regulation of the use, storage and transport of carbohydrates can be crucial under drought conditions because it enhances both drought resistance, through osmoregulation, maintenance of metabolism and defence against pests, and drought recovery, through the supply of energy and carbon for regrowth, repair of damaged tissues and belowground biotic interactions with rhizosphere mycorrhiza and bacteria (McDowell, 2011; Hartmann & Trumbore, 2016; Ouyang *et al.*, 2021). In addition, there were 9 DMRs associated to genes involved in transcription and chromatin remodelling which could suggest different general activations of epigenetic pathways between trees from different treatments. An important number of identified DMRs (83%) were located nearby TEs, suggesting a potential effect of the treatment on TE control. Future studies will be needed to evaluate the impact of these DMRs on gene expression and on TE transposition. Long-lived trees are known to present a high proportion of TEs in their genomes (52% of the *Quercus robur* genome consist of diverse TEs, Plomion *et al.*, 2018). This might indicate the cumulated stresses suffered across their long lifespan, lead to accumulation of many epimutations across time, as could be the case for the *Q. ilex* trees investigated here after 15 years of drought treatment.

The present study provides evidence for an impact of long-term drought treatment on DNA methylation in natural populations of *Q. ilex.* Moreover, we have observed correlations between variations in DNA methylation levels at specific loci and variations in phenotypic traits, opening new perspectives for future studies. Indeed, the methylation level for 17 drought-related DMRs was significantly correlated to physiological or morpho-chemical traits (Fig. 3). However, whether these DMRs are transmitted to the next generation remains an open question in our study and should be explored by future research. Notably, evidence for Scots pine trees suggests that memory of environmental conditions is transferred across generations and affects the phenotypic acclimation potential of seedlings (Bose *et al.*, 2020). Studies have demonstrated the role of DNA methylation in creating plastic phenotype responses, within and inter generations, however the causal role of DNA methylation in the transgenerational effect remains to be demonstrated (Rendina González *et al.*, 2018). In a study of six generations with repeated drought stress in the model herb *Arabidopsis thaliana,* although transgenerational memory was found, the identified DMRs were not conserved in the next generation, impeding the causal link between DNA methylation and transgenerational memory (Ganguly *et al.*, 2017). The same was found by Van Dooren *et al.*, (2020) where changes in methylomes of parents exposed or not to drought were not inherited in the next generation. However, these studies were done under controlled conditions with artificial stress, which could reduce the transferability of the results to natural populations.

While the present study represents a step forward in understanding the role of DNA methylation in the acclimation to drought in natural populations, several caveats common to studies on natural systems with low genomic resources need to be acknowledged (Bossdorf *et al.*, 2007; Richards *et al.*, 2017). First, the unavailability of *Q. ilex* reference genome at the time of the analysis forced us to map *Q. ilex* reads to the *Quercus robur* genome. Despite evidence for high synteny between the *Quercus* *sp.* (Rey *et al.*, 2023) indicating conserved evolution, this certainly reduces the power of the study because the part of the *Q. ilex* genome that we were unable to analyse might have contained important loci involved in drought acclimation. Indeed, *Quercus robur* is a deciduous species from temperate forests, whereas *Q. ilex* is an evergreen species from drought-prone Mediterranean forests with a long history of adaptation to drought (Niinemets & Valladares, 2006; Lobo *et al.*, 2018). However, in spite of the large fraction of the *Q. ilex* genome that could not be mapped, the average values of DNA methylation at the genome-wide level in the control (40% at CG, 32% at CHG, and 7% at CHH) were close to those found in other woody species like *Populus trichocarpa*  (44% at CG, 30% at CHG, and 12% at CHH context, (Liang *et al.*, 2019). Second, we did not take in account the genetic relatedness of the measured trees (despite the fact that *Quercus sp.* is known to have a high genetic diversity; Plomion *et al.*, 2018), because of the difficulties and costs of disentangling the genetic and epigenetic variation. Nevertheless, despite these limitations, we were able to find, in eleven genetically distinct trees, 17 DMRs significantly correlated with a set of relatively low number of phenotypic traits, which arguably strengthens the validity of our results.

In conclusion, this study presents the first genome-wide analysis of the impact of drought on the methylome of holm oak trees from a long-term precipitation reduction experiment. By combining the identification of DMRs with a suite of phenotypic variables showing a plastic response to drought we successfully identified 17 DMRs whose methylation levels were significantly correlated to foliar traits. This suggests that epigenetic regulation could be involved in tree acclimation to drought in natural forest ecosystems facing climate change. More experiments are required to confirm the potential transgenerational inheritance of these DMRs, as well as validation in other tree species and RNA-seq tests to confirm the role of the identified DMRs in altering the expression of the adjacent genes. Upon positive validation, these DMRs could serve as foundational elements for the establishment of epigenetic markers, facilitating the assessment of drought stress within natural tree populations.

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**Data availability statement**

Before the final version of the article is published, the raw data associated with the article will be made available in a public repository.

**Author contributions**

A.M. acquired the funding; L.G.J., J-M.L., A.G. and A.M. designed the experiment; L.G.J., J-M.L., A.G., M.M., N.P and AM provided methods; L.G.J., M.M. and N.P performed the bisulfite analyses; L.G.J. and A.M. analysed the data and led the writing of the manuscript with the important contributions from all authors.

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