



Exploring the effects of microhabitat, ectomycorrhiza and epigenetic regulation on the establishment and performance of holm oak (*Quercus ilex* L.) in a drier world

Laura Garcia de Jalon

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THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

Filière EFSA – Ecologie Fonctionnelle et Sciences Agronomiques

École doctorale GAIA

Unité de recherche Centre d'Ecologie Fonctionnelle et Evolutive

Exploring the effects of microhabitat, ectomycorrhiza and epigenetic regulation on the establishment and performance of holm oak (*Quercus ilex* L.) in a drier world

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Le 11 décembre 2020

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A mis abuelos

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Avant-propos

Cette thèse est rédigée sous la forme d'une thèse sur articles composée d'une introduction générale, suivi de trois chapitres en forme d'articles et une discussion générale toutes parties rédigés en anglais. A la fin du document, il y a un résumé en français de la thèse de 10 pages. Les travaux du chapitre 1 sont déjà publiés dans la revue PLOSone, les travaux du chapitre 2 sont prévus d'être soumis au New Phytologist, et finalement les travaux du chapitre 3 sont prévues d'être soumis au eLive. Cette thèse a été financée par une bourse ANR (Agence Nationale de la Recherche, France) TRANSfER projet (ANR-16-CE32-0002). Les travaux de recherche présentés dans cette thèse ont été effectués au Centre d'Écologie Fonctionnelle et Évolutive (CEFE) à Montpellier dans l'équipe BIOFLUX (Biodiversité, flux et changements globaux). L'ensemble du travail de recherche de la thèse a été dirigé par Alexandru Milcu de la même équipe que moi Bioflux, par Jean-Marc Limousin (de l'équipe du CEFE FORECAST FOREts, Changements globaux, et Adaptation : Simulations et expérimentations,) et Franck Richard (de l'équipe du CEFE Equipe Taxonomie et Biogéographie des Interactions) qui a fait que j'ai pu me bénéficier du contact et partage avec des gens d'autres équipes au sein du CEFE. Une partie des analyses biomoléculaires épigénétiques ont été réalisées en collaboration avec Marie Mirouze et Nathalie Picault dans le Laboratoire de Génome et Développement de plantes à Perpignan. J'ai encadré deux stagiaires ; un étudiante de Master 1 et un étudiante de BTS. J'ai été chargé d'enseignement vacataire dans le TP d'Ecologie Fonctionnelle (27 h) à la Faculté de Sciences de Montpellier.

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Résumé

Les forêts méditerranéennes sont confrontées à une augmentation de l'intensité et de la durée des sécheresses en raison du changement climatique, avec de potentielles conséquences néfastes sur la mortalité des arbres et sur leur régénération. Cette thèse étudie les effets de la sécheresse pouvant affecter l'établissement, la performance et la survie des semis de chêne vert (*Quercus ilex* L.), ainsi que les modifications physiologiques et épigénétiques des arbres adultes. Plus précisément, elle étudie (i) les effets du microhabitat et de la colonisation ectomycorhizienne sur l'établissement et la survie des semis, (ii) les effets de la sécheresse sur la diversité et la composition des communautés fongiques et leurs conséquences indirectes sur la survie des semis, et (iii) les effets de la sécheresse sur les modifications épigénétiques et les réponses physiologiques chez les arbres mères ayant la capacité d'être adaptatives et d'influencer la survie des semis de la génération suivante. Cette thèse représente une approche interdisciplinaire combinant l'épigénétique, les interactions biotiques et les effets environnementaux dans une expérience de sécheresse à long terme (15 ans) avec une réduction de 29% des précipitations annuelles dans une forêt de *Q.ilex* dans le sud de la France. Nos résultats montrent que la sécheresse estivale est la principale cause de mortalité des semis. Cependant, le traitement de réduction des précipitations a augmenté la survie des semis par rapport aux conditions contrôles. Ce résultat surprenant a été mis en relation avec une augmentation de la richesse spécifique ectomycorhizienne et une augmentation des espèces d'ectomycorhizes plus adaptés à la sécheresse dans le traitement sec. Par ailleurs, les arbres mère ont présenté des réponses d'acclimatation à la sécheresse corrélée aux modifications épigénétiques, pouvant induire une transmission adaptative à la génération suivante. Cette thèse met en évidence la résilience remarquable des espèces forestières méditerranéennes face à la sécheresse, grâce à l'émergence de stratégies d'adaptation à long terme en réponse aux récurrentes fluctuations environnementales vécues.

Mots clés : sécheresse, ectomycorhizes, méthylation ADN, microhabitat, survie, *Quercus ilex*

Abstract

Mediterranean forests are facing an increase in the intensity and duration of droughts due to climate change, with potentially severe consequences for tree mortality and regeneration dynamics. This thesis investigates the potential carry-over drought effects that may affect the establishment, performance and survival of holm oak (*Quercus ilex* L.) seedlings alongside physiological and epigenetic modifications in mature trees. Specifically, it explores (i) the effects of microhabitat and ectomycorrhizal colonization on the establishment and survival of seedlings, (ii) the effects of drought on the diversity and composition of fungal communities and the subsequent consequences on seedling survival, and (iii) the effects of drought on the epigenetic modifications and physiological responses manifested in mother trees, which have the potential to be adaptive and enhance seedling survival. This thesis represents an interdisciplinary approach combining epigenetics, biotic interactions and environmental effects in a long-term drought experiment (15 years) with a reduction of 29% in annual precipitation in a *Q.ilex* forest in the south of France. Our results showed that even though summer drought was the main cause of seedling mortality, the precipitation reduction treatment increased the survival of seedlings compared to ambient conditions. This unexpected result was related to an increase in the ectomycorrhizal richness and an increase of drought adapted ectomycorrhizal species under drier conditions. Mother trees presented acclimation responses to drought that were correlated to changes in the methylome which suggest a potential role of epigenetics regulating these responses and a potential intergenerational transmission. This thesis provides evidence of the remarkable resilience to drought stress of the Mediterranean forests species, where recurrent environmental fluctuations may have favoured the emergence of long-term adaptation legacies.

Keywords: drought, ectomycorrhiza, DNA methylation, microhabitat, survival, *Quercus ilex*

GENERAL INTRODUCTION

Suelen pasar desapercibidos, pero el mundo natural emite continuos mensajes informativos sobre sucesos acaecidos durante las últimas horas, semanas o años. Esa mariposa con un par de muescas triangulares, perfectamente simétricas, en el borde de sus alas anteriores nos cuenta que logró escaparse, en el último momento, del pájaro que la había atrapado. Las hojas de rosal con nítidas muescas circulares nos narran que una abeja cortadora del género “Megachile” las mutiló para obtener material con el que construir su nido. Si aprendemos a leerlos, estos y muchos otros vestigios nos informarán sobre el pasado reciente del lugar que visitamos, los organismos que lo habitan y las interacciones ecológicas que los entrelazan...

Carlos Herrera “A vueltas con los vestigios: recuerdos que se hederan”

1.1 The conquest of dry terrestrial ecosystems

The colonization of land by plants, around 450 million years ago, is one of the most significant evolutionary episodes in Earth history, leading to the emergence of actual terrestrial ecosystems. The photosynthetic rates of the newly emerged terrestrial plants contributed to the regulation of atmospheric CO₂ concentration and the global water cycle (Beerling and Berner, 2005). This exceptional innovation of plant life forms came with many new challenges to solve, of which the independence from a constant source of water is perhaps the most notable one. The ancestors of terrestrial plants lived bathed in water with their cells in direct contact with water. As some early land plants still do, they depended on water for reproduction, since the propagation of their gametes occurred in water. It is argued that the closest ancestors of land plants (*Charophyta* algae) lived under ephemeral aquatic habitats with water fluctuations that imposed a strong evolutionary pressure towards tolerance to desiccation and terrestrialization (Lewis and McCourt, 2004). Beneficial symbiotic interactions between plant/algae and fungi certainly helped plants to colonize new substrates, by increasing their ability to capture nutrients and water and to resist to seasonally dry environments (Delaux et al., 2015) (Box 1).

In order to colonize land, it was necessary to be protected against desiccation, so superficial epidermal cells evolved into a waxy hydrophobic layer of cells protecting plant organs from water loss and UV radiation, which corresponds to the emergence of the cuticle. Furthermore, it was necessary to regulate the exchange of gases and water with the atmosphere. Emergence of pores over the cuticle evolved in order to regulate the plant water losses, the stomata. Once the cuticle and stomata were established, the evolution of water-conducting cells completed an important part of the adaptation to the terrestrial environment. In early land plants, such as bryophytes (mosses), the transport of water was performed by diffusion through gradients of concentration from the substrate (soil or rock) to the extremities of the plant. This transportation system was not efficient and was only possible in small plants as it depended highly on the moisture of the substrate. More recent lineages of plants thus evolved vascular transport systems and specific organs to assimilate water (Pittermann, 2010). The appearance of plants with vascular systems (Tracheophytes) represented a significant evolutionary step in the efficiency of water transport, whilst simultaneously providing mechanical support for heavy aerial parts such as woody trunks and crowns. Tracheids were

the first vascular elements in earlier-evolved vascular plants (ferns, horsetails, lycopods), that latter evolved into vessels with bigger diameters in angiosperms allowing water to move rapidly and over long distances from roots to shoots.

Box 1: The role of fungal mutualism in the terrestrialization

Land colonization by plants would probably not have been possible without fungi. In fact, plant-fungus mutualism is suggested to have played a key role in dealing with the limited water supply and the scarcity of soluble minerals of the new colonized territories (Selosse & Le Tacon, 1998). The fossil records confirm the existence of mutualistic fungi in the Devonian (417 millions of years ago), at the time when plants colonized terrestrial ecosystems. In the figure 1.1, we can see a primitive vesicular-arbuscular mycorrhiza* (Glomales) colonizing the parenchymal cells of a 400 million year old fossil plant (*Aglaophyton major*) (Taylor et al., 1995). Arbuscular mycorrhizas have their origins possibly over 1000 million years ago. Another study revealed that the algal ancestor of terrestrial plants was pre-adapted to symbiosis (Delaux et al., 2015) suggesting a potential use of this symbiosis for the land colonization by the algae.

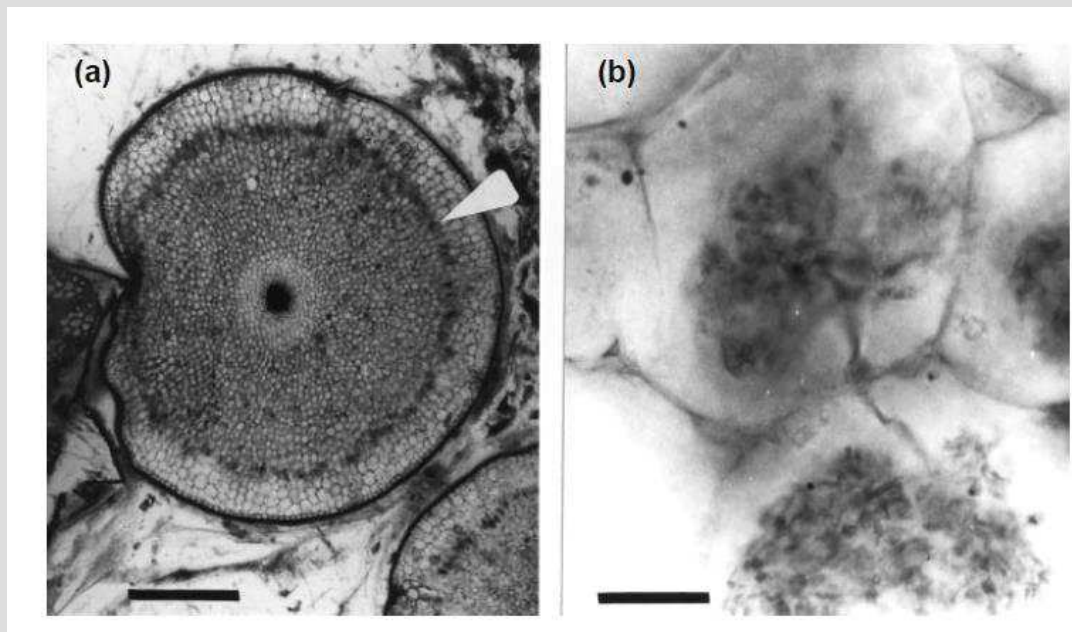


Figure 1.1. Colonization of hyphae and vesicles of an arbuscular mycorrhizae (Glomales) in the cells of *Aglaophyton major* plant fossil (a) Section of an *Aglaophyton* plant fossil rhizome (b) two parenchymal cells colonized by the fungus. Figure from Taylor et al. 1995.

Further in time, plants developed different adaptations to dry conditions through morphological, biochemical, physiological and molecular responses. The adaptations to drought can be divided into three plant strategies: drought tolerance, drought avoidance and drought escape (Abobatta, 2019; Volaire, 2018). The **tolerance** strategy allows plants to tolerate dehydration through morphological, physiological and anatomical traits that confer the ability to continue growing under sub-optimal water supply. The **avoidance** strategy or dehydration avoidance, allows plants to increase their water uptake or minimize their water loss during the transient periods of drought stress (ex. dormancy strategies). Finally, the drought **escape** strategy allows plants to complete their life cycle before the onset of drought, for example by reducing the vegetative growth period and producing seeds early before environmental stresses. These different strategies and adaptations to dry conditions allowed plants to colonize lands with different climates, creating different vegetation types and founding the Earth's biomes.

1.2 The role of climate determining the Earth's biomes

Biomes are the largest scale at which ecologists classify vegetation. Biomes differ in the composition of dominant species of their vegetation, which are determined by the climatic conditions, particularly mean and variability of temperature and precipitation, (Gurevitch et al., 2002) (Figure 1.2). Plants have evolved a wide variety of adaptations to climatic conditions across the Earth biomes. These adaptations range from the evergreen needle leaves in coniferous trees and shrubs dominating the coldest tundra and taiga biomes, passing by the deciduous broad-leaved of angiosperms that shed their leaves during the colder season dominating the temperate forest, by the sclerophyllous leaves of evergreen woodlands adapted to drier climates, or the broad-leaves of evergreen tropical rainforests. And finally the adaptations of the driest and hottest biome, the desert, with leaves evolved to resist a very high degree of desiccation, such as the spines of cactus.

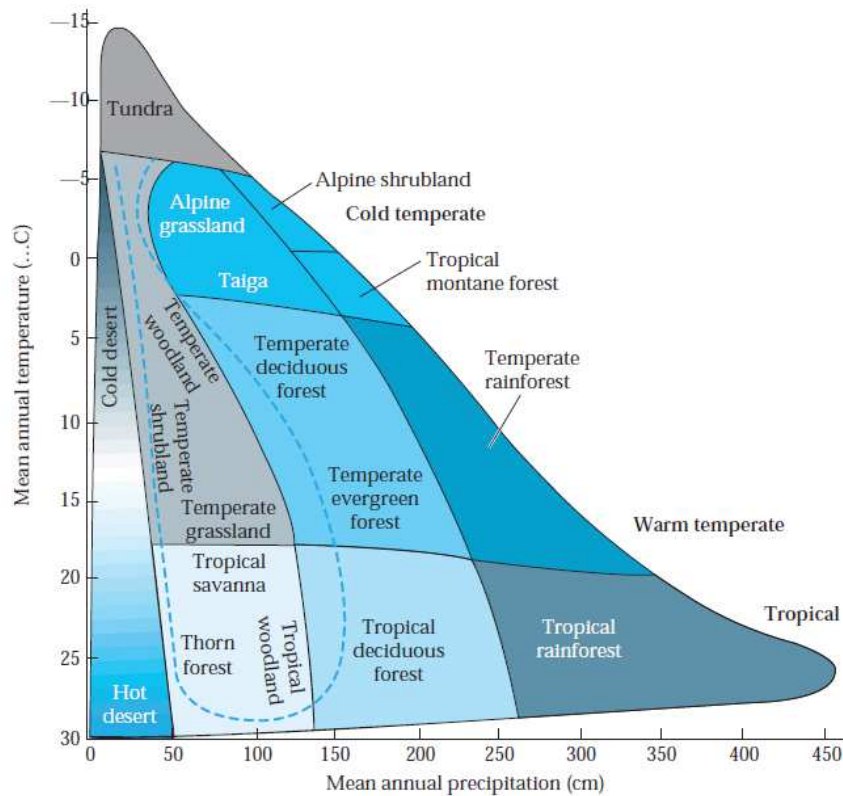


Figure 1.2. The distributions of biomes is determined by climate, particularly by the mean annual precipitation and temperature. The dashed lines represent biomes impacted by other factors than climate (fire, grazing, and seasonality of the precipitation). Figure extracted from Gurevitch et al. 2002.

Given the dependency of vegetation to climate, changes in climate could lead to modifications in the type of vegetation, and if they persist, modifications in the distributions of some biomes. For example, the Mediterranean region is at the intersection between two biomes (temperate evergreen forest and temperate grasslands). These biomes are known to be affected by climate and other factors like fire, grazing and the seasonality of precipitation (dashed line of the Figure 1.2). If one of these factors changes drastically, such as the severity of seasonal droughts, the distribution of Mediterranean forest could be reduced in favour of savannas and shrublands (Acácio et al., 2007).

1.3 Climate change effects on forests

The adaptations of plants during land colonization towards an increased ability to live in dry environments have evolved over approximately 300 millions of years (from the emergence of cuticle until the angiosperms vessels). Nowadays, the accelerating climate

change induced by anthropogenic activities is forcing plants to respond to a rapidly changing environment, jeopardizing the potential ability of plants to adapt to the new conditions.

Human activities since the mid-20th century (industrialization, deforestation, large-scale agriculture) are releasing greenhouse gases into the atmosphere at an unprecedented rate, and thereby driving global climate changes (IPCC, 2014). Climate change projections for Europe suggest an increase in temperature and changes in precipitation regimes with an increased occurrence of extreme events such as droughts and flooding, especially in the Mediterranean region (IPCC, 2014). Climate change is thus expected to produce changes in the distributions, composition and diversity of forest species, with consequences on ecosystem functioning and forest goods and services (Lindner et al., 2010).

The increases in temperature combined with drought stress, have already been reported to decrease tree growth and increase tree mortality around the globe (Allen et al., 2010; Choat et al., 2012; McDowell et al., 2011; Steinkamp and Hickler, 2015) (Figure 1.3; Box 2). This increased tree mortality is decreasing the carbon (C) storage capacity of forests and releases C into the atmosphere, which further accelerates the global warming. The ongoing climate change will not only increase tree mortality but it is also expected to impact seedling establishment and performance and thus to threaten natural regeneration and future compositions of forests (Lloret et al., 2004). The investment of trees in reproduction is also expected to decline under increasing droughts thereby endangering the regeneration of forests (Pérez-Ramos et al., 2010). In response to these rapid environmental changes, epigenetic regulation seems to have a crucial role to increase the adaptation capacity of plant species especially for long-lived organisms such as trees (Bräutigam et al., 2013). Because these epigenetic changes can occur much more rapidly than genetic mutations, they may play a key role in the adaptive responses to rapid changes in climate (Bossdorf et al., 2007).

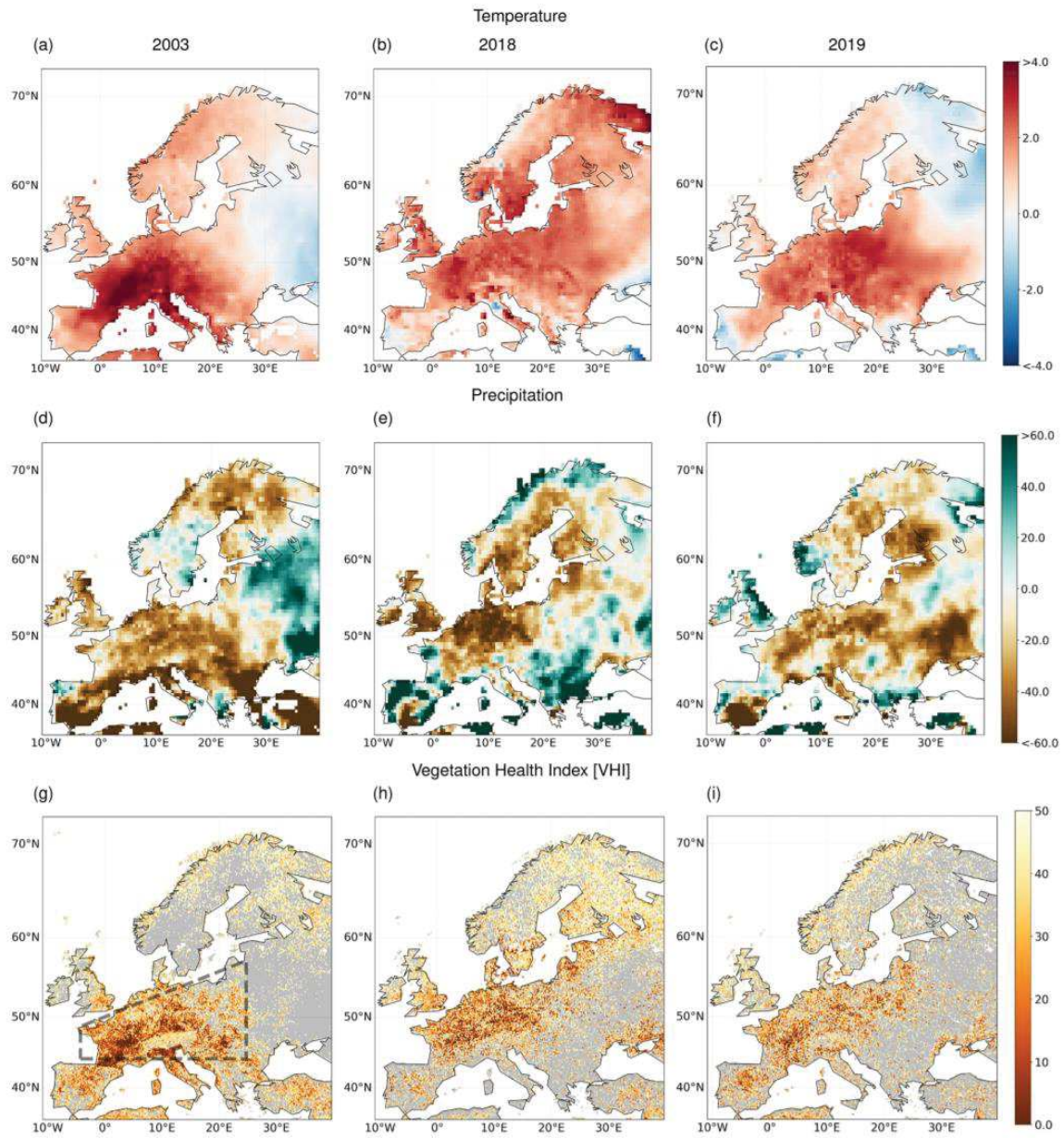


Figure 1.3. Temperature anomalies ($^{\circ}\text{C}$), Precipitation anomalies (%), and Vegetation Health Index in Europe during summer droughts (July-August) of 2003, 2018, and 2019 based on the 1980-2010 climatology. Vegetation Health evaluates the impact of drought on vegetation health. A high value of VHI indicates healthy vegetation, a VHI between 50-30% indicates vegetation suffered moderate drought, and a VHI below 30% means vegetation suffered severe drought. (Figure extracted from (Hari et al., 2020)).

Furthermore, new climatic conditions are also altering the soil biochemical cycles through modifications of microbial activity. Microbial activity is essential for plant nutrition, because it is responsible for the decomposition of organic matter and the transformation of nutrients into chemical compounds that can be easily assimilated by plants (Baldrian, 2017). Since microbial activity is very dependent on soil moisture and on resource availabilities

(particularly in C and N) (Bu et al., 2018), microbial communities are expected to be strongly affected by drought.

Box 2: Tree mortality mechanisms

Hydraulic failure has been recognized as the primary mechanisms for tree mortality (Anderegg et al., 2013; McDowell et al., 2008, 2011), although water deficit also leads to lower carbon assimilation which may ultimately prevent cell metabolism and cause tissue mortality (Sevanto et al., 2014). Water deficit in plants occurs when water losses from transpiration are higher than the uptake by the roots, thereby leading to plant dehydration. Plants are then forced to limit their physiological activity, especially by closing their stomata and thus ceasing photosynthesis. The supply of water from the soil (rhizosphere) to the sites of photosynthesis (leaves) may be stopped by hydraulic failure if dehydration is severe enough to create gas embolism inside the xylem. Under long and intense droughts, the available storages of carbohydrates may also become depleted because photosynthesis cannot resupply the carbohydrates used for cell metabolism (respiration), eventually leading to carbon starvation in tissues. Furthermore, the reduction of water availability could also limit phloem transportation, impeding cells to receive and use carbohydrates from distant plant reserves for their metabolism (McDowell et al., 2011). Under these conditions trees become increasingly vulnerable to biotic attacks since they cannot maintain cellular and defensive metabolism, which could also ultimately cause their death (McDowell et al., 2008; Zweifel et al., 2009).

Understanding how forest will respond to the expected future climate conditions is essential to better anticipate the future of forests, and develop appropriate forest management strategies. In this thesis, I aim to contribute to this field by studying how droughts of increased severity in a Mediterranean forests will affect *Quercus ilex* regeneration and survival as well as its associated fungal community due to its role in counteracting drought effects.

1.4 Factors affecting tree recruitment

The success of tree recruitment is firstly dependent on the characteristics of the produced seeds (seed provisioning) such as the quantity of reserves (sugars, proteins, hormones...) that mother trees allocate to offspring, and (Khan and Shankar, 2001; Quero et al., 2007). In addition, there are also other external factors that influence tree recruitment, such as (i) the microhabitat where acorn germinate and seedling establish, and (ii) the biotic interactions with species of their environment.

1.4.1 Microhabitat conditions

The abiotic and biotic factors affecting seedling establishment are highly heterogeneous in space and vary among geographical areas, regions, habitats and microhabitats within habitats (Gómez-Aparicio et al., 2005). Even the finest spatial scale of microhabitat, which is normally not larger than the largest individual plant in the population, the scale of a few square meters, is suggested to influence the patterns of seedling establishment (Kollmann, 2000). Within a forest, microhabitats differ with the heterogeneity of the canopy cover that creates a range of conditions from gaps to deep shade. These microhabitats can vary widely in light availability and soil moisture. Seedlings emerging under tree canopy could benefit from a reduced temperature and evaporative demand compared to seedlings under canopy gaps, which could reduce the local water deficit and seedling drought stress. In addition, seedlings living under canopy may also benefit from the transfer of nutrients and symbiotic species through the belowground mycelium network established by nearby trees that facilitates seedling establishment (Selosse et al., 2006) (see below in Ectomycorrhizae section). Forest microhabitats can be modified by tree mortality and tree falls that creates gaps in the canopy (Suarez and Kitzberger, 2008) or by changes in the land-use modifying the microclimate conditions and the suitable conditions for seedling recruitment.

1.4.2 Biotic interactions (mycorrhizae)

Biotic interactions can (i) positively influence seedling performance, like in the case of mycorrhizal symbiosis and plant-plant facilitation, or (ii) negatively influence seedling performance like herbivory or pathogen infection. Hereafter, I will focus on the former because of its key role in seedling establishment.

Mycorrhizal symbiosis is arguably the most important symbiosis on Earth (Brachmann and Parniske, 2006). Mycorrhizas are symbiotic associations between fungi and roots of plants where carbohydrates such as glucose and sucrose fixed by the plant photosynthesis are provided to the fungus, and increased nutrients and water uptake is provided by the fungus to the host plant, thereby potentially increasing host resistance to stresses (drought, salinity, pathogens). About ~20% of the C produced by the plant is transferred to mycorrhizal fungus (van der Heijden and Sanders, 2003). Undeniably, mycorrhizas have a key role in coping with drought (Lehto and Zwiazek, 2011). The astonishing dynamics of the mycelium and the ability of fungi to dissolve rocks (mineral weathering), allow roots to uptake nutrients and water in places where plant could not. In addition, the enhanced water uptake by mycorrhizas helps maintaining root hydraulic conductance, stomata conductance and photosynthesis activity during drought (Lehto, 2006). Depending on whether or not their hypha penetrate inside the plant cells, mycorrhizas are divided into endomycorrhizas (hyphae penetrate plant cells) or ectomycorrhizas (hyphae do not penetrate plant cells). In this thesis, I focus on ectomycorrhizas because they are the type associated with most temperate woody perennials, such as the holm oak, the model species of my PhD.

1.4.2.1 Ectomycorrhizae

Ectomycorrhizas (ECM) account for a great diversity with about 20,000-25,000 species of fungi forming ectomycorrhizas (Tedersoo et al., 2010) (Figure 1.4). A single plant species may associate with hundreds of fungal species over its natural geographic range. Plants are known to have a lack of specificity for fungal partners that allows roots to increase their chances to find compatible fungal partners wherever they go and potential substrates to colonize (van der Heijden and Sanders, 2003). In turn, most ECM fungi are generalist and few are host-specific. ECM diversity usually concentrates in the most superficial organic soil layers or the mineral layers immediately below, which are the soil layers containing more nutrients but also the most exposed to environmental changes.

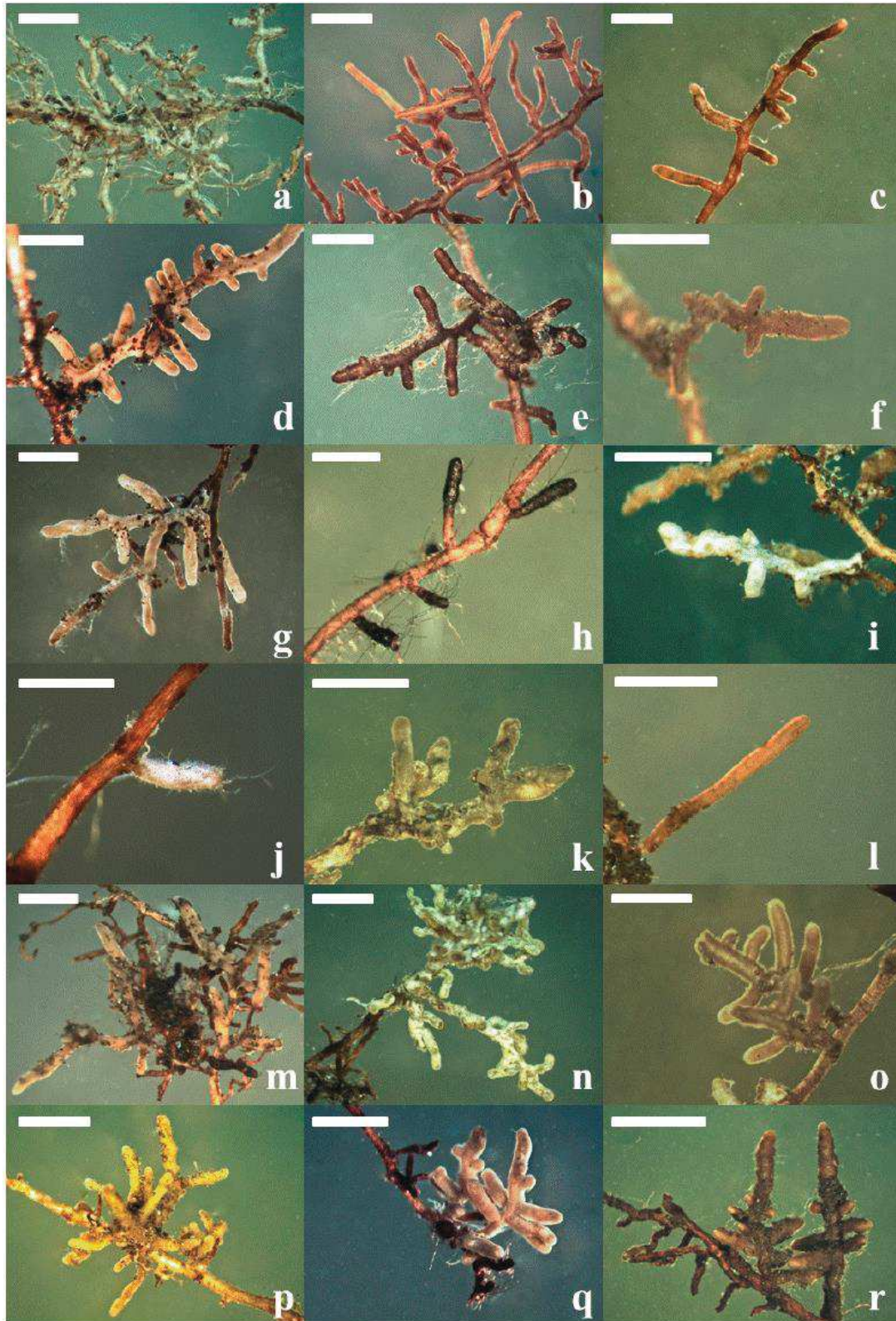


Figure 1.4. Diversity of the beautiful ectomycorrhizas colonizing *Quercus robur* trees. (a) *Paxillus involutus*, (b) *Lactarius quietus*, (c) *Russula ochroleuca*, (d) *Lactarius chrysorrheus*, (e) *Tomentella sublilacina*, (f) *Tuber* sp., (g) *Amanita citrina*, (h) *Cenococcum*-like, (i) *Scleroderma citrinum*, (j) *Boletus badius*, (k) *Thelephora terrestris*, (l) *Pachyphlodes nemoralis*, (m) *Russula fragilis*, (n) *Xerocomus subtomentosus*, (o) *Inocybe assimilata*, (p) *Russula lepida*, (q) *Xerocomus pruinatus*, (r) *Hydnotrya tulasnei*. Bars 1 mm. Photo extracted from Bzdyk et al., 2019.

Most of ECM fungi are known to come from saprotrophic ancestors (94.7% of the resolved cases; (Shah et al., 2016; Tedersoo et al., 2010)), thus explaining why some of have preserved their saprotrophic function. Indeed, some ECM have a dual life style combining an ability to degrade organic matter through the excretion of hydrolytic enzymes and to form symbiosis with plants that assimilate carbon for them. However, they have lost the ability to degrade plant cell wall polysaccharides, and contrary to endomycorrhizas, cannot penetrate plant root cells.

The establishment, performance and survival of seedlings are known to be improved by ectomycorrhizal colonization (Bingham and Simard, 2012; Rincón et al., 2007), particularly by the integration into common ectomycorrhizal mycelium networks (CMNs) (Nara, 2006; Selosse et al., 2006). Indeed, most ECM species have the ability to be interconnected by local CMNs and/or ECM diversity across landscapes (Taudiere et al., 2015). CMNs can facilitate seedling establishment by providing carbohydrates, nutrients and fungal partners from the canopy individuals to the seedlings. Being connected to CMNs can thus be particularly beneficial for young seedlings with shallow root systems, by enhancing survival during summer drought of their first year of life, which is known to be one of the major cause of seedling mortality in the Mediterranean (Gómez-Aparicio et al., 2004; Richard et al., 2009).

Ectomycorrhizas are characterized by three structural components: (i) the mantle that encloses the root, (ii) the Hartig net which is an inward growth of hyphae that penetrate plant cells between the epidermal and cortical cells, and (iii) the extraradical mycelium which is an outward growth of hyphae from the mantle to the soil which represents an extension and an hyper-development of the root system to enhance capture and mobilization of nutrients. The proportion of extraradical mycelium over fungal biomass is about 60-80%, and can account for 32% of the total microbial biomass in forests soils (Högberg and Högberg, 2002). (Agerer, 2001) described four ECM exploration strategies according to different anatomy and morphology of the extramatrical mycelium radiating into the soil: contact exploration, short-distance, medium-distance and long-distance. Longer extramatrical mycelia are able to take up nutrients and water from further distances thereby increasing drought resistance in hosts, and these longer morphotypes (i.e. Morpho-anatomies of ECM apices) are known to be favoured under drought conditions (Bakker et al., 2006).

1.5 Forest recruitment under increased droughts

The increasing frequency and severity of droughts is expected to impact seedling establishment and survival, thereby threatening the natural regeneration and future compositions of forests (Carón et al., 2015; Lloret et al., 2004). In this section, I present the potential adaptive responses to drought (mid-term drought legacies) that can influence tree recruitment in a drier world. First I introduce the responses of soil fungal communities because of their role in facilitating seedling recruitment, and second the responses in seed-bearing adult trees (mother of seedlings) that could potentially induce transgenerational adaptive responses to offspring.

1.5.1 Drought legacies on soil fungal communities

Nutrient cycling in forest ecosystems, in particular, nutrient mobilization and mineralization processes are expected to be impacted by climate change (Swaty et al., 2004). Changes in nutrient availability can compromise fungal communities that are known to be very sensitive to environmental changes (soil moisture) and nutrient availability.

From the fungal community perspective, shifts in fungal community composition are expected to occur as a consequence of climate change, and in particular reductions in diversity with an increase on stress resistant species (Courty et al., 2010; Gehring et al., 2014). Many studies have shown shifts in composition but not in species richness in response to increased CO₂ (Fransson et al., 2007), or decreases in diversity and changes in community composition in response to increasing drought and warming (Deslippe et al., 2011; Herzog et al., 2013; Shi et al., 2002).

The symbiotic interactions between fungi and plants are also expected to be impacted by climate changes. Warming and precipitation reduction can alter the mycorrhizal symbiosis in three ways: (1) by influencing the host plant through impacts on its physiological activity such as C assimilation, (2) by indirectly influencing the fungus through changes in C allocation from the host and (3) by directly influencing the fungus through changes in its enzymatic activities (van der Heijden and Sanders, 2003) (Figure 1.5).

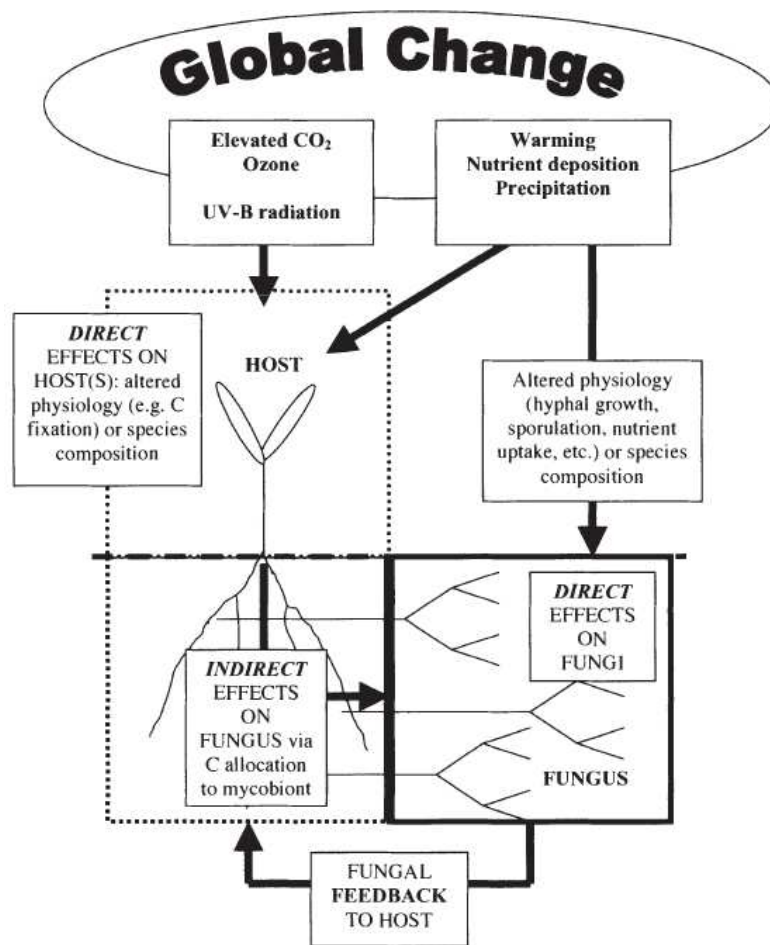


Figure 1.5. Representation of the effects of global changes on the mycorrhizal symbiosis. Figure extracted from Heijden & Sanders (2003).

The impacts of climate change on fungal communities has thus the potential to compromise the natural regeneration in forest ecosystems through effects on the nutrition status of the seed bearing trees and seedlings. Studies investigating how increasing droughts affect the interplay between fungal communities and tree recruitment are thus necessary to anticipate the future forest dynamics and to develop adapted forest management practices.

1.5.2 Drought legacies on seed-bearing trees

In response to the ongoing climate change, trees may (i) develop plastic changes in their development and physiology to become more stress resistant (phenotypic plasticity), (ii) adapt to the new conditions (genetic adaptation), or (iii) migrate to other regions where they are better adapted, leading to local extinctions. Among these three options, phenotypic plasticity seems to be the more rapid and promising response for tree survival (Nicotra et al.,

2010). Phenotypic plasticity can be defined as the capacity of a single genotype to produce different phenotypes depending on the environment. This phenotypic plasticity is adaptive when it increases the fitness of the genotype, so when the modifications of the phenotype provide better performance to survive and reproduce. Phenotypic plasticity can be heritable and thus potentially important for species evolution. A particular case of heritable phenotypic plasticity are the epigenetic modifications, which are explained in the next section.

1.6 Epigenetic modifications as a potential mechanism for adaptive plasticity

Epigenetic modifications (changes in the gene expression but not in DNA sequence) induced by environmental stresses are proposed as a mechanism that influences phenotype variation and short-term acclimation responses (Bossdorf et al., 2007). When these epigenetics modifications are heritable, they can influence in adaptation and microevolution, playing a role in the evolution of numerous plants and animals (Suter et al., 2013). Because epigenetic changes can happen much more rapidly than genetic mutations, they may play a key role in the adaptive responses to the ongoing rapid climate changes. The role of epigenetic modifications in stress acclimation would be even more important in the case of trees which are sessile organisms (obligated to adapt to the environmental conditions where they live), with long life spans and slow generations turn-over.

There are several epigenetic mechanisms that can alter gene expression: (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 (Richards, 2006). The mobility of transposable elements along the genome influences gene expression and is regulated by small interfering RNAs and by DNA methylation (Slotkin and Martienssen, 2007). The most studied and stable epigenetic mark is DNA methylation, which is the incorporation of a methyl group (CH_3) to a specific nucleotide (Law and Jacobsen, 2010). The establishment of DNA methylation (*de novo* methylation) and maintenance of DNA methylation during DNA replication is achieved by specific enzymes “DNA methyltransferases” (Edwards et al., 2017). The functions of DNA methylation are known to maintain genome stability by silencing transposable elements, or by influencing the formation of heterochromatin and thus the regulation of gene expression.

1.6.1 Epigenetics for ecologists

Epigenetics have been studied mainly by molecular biologists, until ecologists noted its potential ecological relevance (Kilvitis et al., 2014). So far, the majority of epigenetic studies have been done using model species such as *Arabidopsis thaliana* or *Drosophila sp.* under highly artificial controlled conditions lacking ecological realism. However, ecological epigenetics, which is the investigation of the consequences of epigenetics in ecology and evolution, has become a new field of research and challenge for researchers¹. Ecological epigenetics studies the relationship between epigenetic variation and ecologically relevant phenotypic variation (Schrey et al., 2013). Studies on natural populations are thus essential to evaluate the possible consequences of epigenetic processes in realistic scenarios with genetically and phenotypically heterogeneous populations, towards assessing the potential significance of epigenetic variation in adaptation and microevolution (Richards et al., 2017).

Figure 1.6 depicts the emerging field of ecological epigenetics that tries to incorporate the work of molecular biologists into the work of ecologists, but many challenges related to methodological and bioinformatics analyses still remain. Working with model species (as molecular biologist do) has plenty of advantages since the high availability of genomic resources enables to do experiments disentangling the effects of methylation and genetic variation on the phenotype. For example, the work with clones submitted to different stresses enables to control the genetic diversity and allows detecting changes in phenotype that are only due to epigenetic marks. In terms of methodology, the availability of a sequenced reference genome is also essential to investigate the consequences of DNA methylation. As an example, whole genome bisulfite sequencing (WGBS) is a method that requires a sequenced reference genome in order to detect where differences in methylation occur in the genome, and therefore the impact of these differences in methylation on the expression of the nearby genes or transposable elements. However, when the reference genome is lacking (the case of

¹ During my thesis I had the opportunity to work in two laboratories among ecologists in Centre d'Ecologie Fonctionnelle et Evolutive (CEFE, Montpellier) and among molecular biologists in Laboratoire Génome et Développement des Plants (LGDP, Perpignan). I noted how far and disconnected were the visions of researchers from the two fields, even when collaborating on the same project. Whereas ones described my work "as the study of methylomes in stressed and non-stressed plants" the others described it as "the potential link between epigenetics and the physiological responses to drought". My vision preferentially corresponded to the second one. But it was very enriching to work with researchers from different fields and with different interests, trying to link them and getting the best part of each to study ecological epigenetics.

non-model species in ecology studies), other methods like Methylation-Sensitive Amplification Length Polymorphism (MS-AFLP) are used, but with substantial drawbacks because the methylation is assessed in anonymous loci, which prevents understanding its local impact on nearby genes. Nevertheless, the MS-AFLP method is much more affordable than WGBS and it allows screening for changes in methylation patterns in a higher number of individuals from several populations, which is a key scientific questions in ecological studies. In this thesis, I did an ecological epigenetics study with a trade-off between methods and techniques from both disciplines molecular biology and ecology. I analysed DNA methylation in a natural population of a non-model long lived tree species (*Q.ilex*) using WGBS method, without having the genomic resources of holm oak, but using *Quercus robur* sequenced genome.

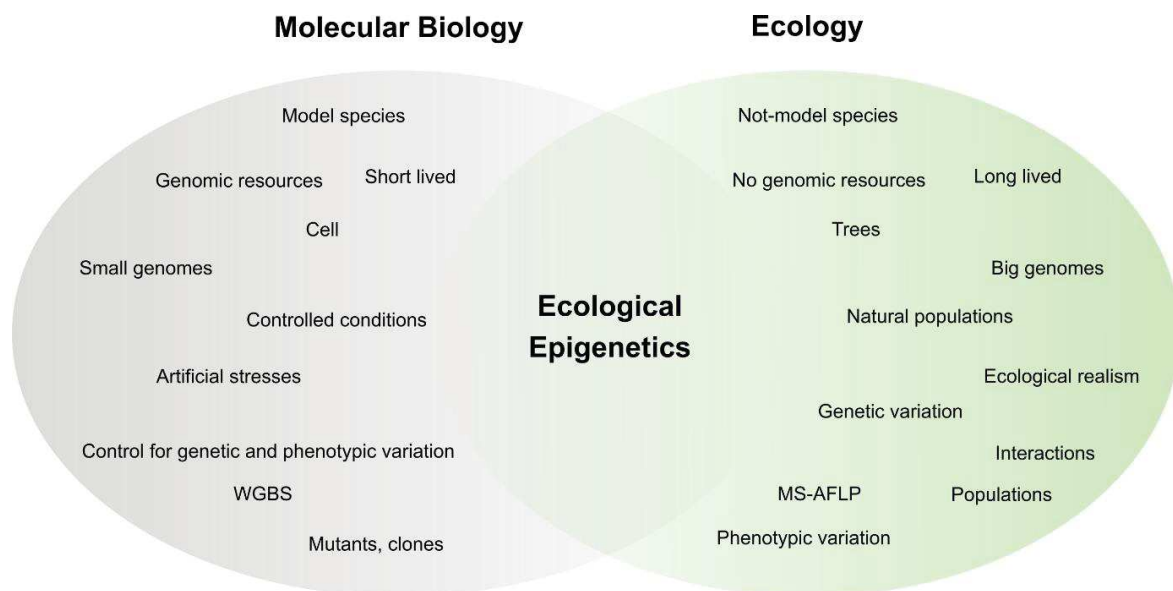


Figure 1.6. Ecological epigenetics emerging field of research and challenges from its origin fields molecular biology and ecology. WGBS: Whole Genome Bisulfite treatment; MS-AFLP methylation sensitive –AFLP

1.7 Mediterranean evergreen forests

Mediterranean evergreen forests are a dominant vegetation type in Mediterranean regions where they occupy an intermediate position along the aridity gradient between deciduous temperate forests and shrublands (garrigue, maquis, and chaparrals). Holm oak (*Quercus ilex* ssp.) is the dominant species in the Western part of the Mediterranean, where it forms evergreen forests present in the thermo-, meso-, and upper- Mediterranean altitudinal zonation and on the semiarid to humid Mediterranean climates (Rivas-Martínez, 2011), even

though it was not always the case (Box 3). Typical holm oak forests developed on calcareous soils in southern France are dense coppices where the species is dominant in the overstorey, in response to the past management practices, with frequent clear-cutting for the production of fire wood and wood charcoal.

Mediterranean vegetation has to cope simultaneously with two selective pressures (i) a double environmental constraint by cold temperature in winters and droughts in summer that determined morphological and ecophysiological evolutive responses, and (ii) an important human activity that has impacted the flora and favoured the actual vegetation (Terradas, 1999). Mediterranean forests as transition zones (ecotones) between forest and shrubs are considered to be very sensitive to global changes, and particularly by the increasing aridity and human activities that reduced forest cover in the South of the Mediterranean basin. The coppice management of holm oak forests for centuries has resulted in high-density stands with multi-stem stools that create an important competition between trees.

From the biological perspective, holm oak is a monoecious species, wind-pollinated that can get up to 15 m in height, and exceptionally 30 meters on acidic soils (Panaïotis et al., 1997). It is a hardwood tree, with small evergreen sclerophyll leaves with a thick cuticle that allows to maintain a low cuticular transpiration and high resistance to desiccation (Terradas, 1999). Leaf lifespan can be between 2 and 4 years. Vegetative growth, leaf shedding and leaf and flower emergence occur mainly in spring in May-June. Growth ceases with the onset of drought, generally in July, and may restart in early autumn after the first rains and the recovery from drought stress. Evergreenness in holm oak has the advantage of allowing photosynthesis in any season, and thus to compensate in part the summer drought. Holm oak is considered a shade-tolerant species and is dominant in late successional stages. It has a deep root system, and even young seedlings can have taproots down to 80 cm in the first year. The stomatal regulation strategy of holm oak is anisohydric as it tolerates important decreases of water potential during drought in spite of a strong stomatal regulation of transpiration (Limousin et al., 2009). Therefore, holm oak has a drought tolerant strategy that can maintain some level of gas-exchange and positive carbon balance even during mild drought (Lempereur et al., 2015).

Box 3: Origin of Mediterranean drought and vegetation

During the Pliocene (5.3 to 2.6 million of years ago), the vegetation of the Mediterranean region was mainly subtropical dominated by *Taxodiaceae*, *Myrica*, *Symplocos*, *Nyssa taxas* (Suc, 1984). A great change in climate occurred approximately 3.2 myr ago (beginning of the Pliacenzian stage), when fluctuations in soil moisture increased and seasonal summer droughts started to appear. In consequence, the structure and composition of Mediterranean sclerophyllous forests changed. Subtropical species requiring year-long available moisture declined or disappeared, whereas xerophytic species (*Quercus ilex*, *Phillyrea*, *Olea*, *Cistus*, *Pistacea*) increased in frequencies until the Mediterranean flora adapted to these new conditions. In addition, the beginning of human presence and activity had considerable effects on that flora, especially with more frequent fire episodes.

1.8 PhD objectives and structure

The overarching goal of this PhD thesis is to investigate the potential carry-over drought effects that may affect the establishment, performance and survival of holm oak (*Quercus ilex* L.) seedlings in a context of increasing aridity as expected with the on-going climate change. To do so, I have investigated:

- (i) The effects of microhabitat conditions and ectomycorrhizal colonization on the establishment, performance and survival of seedlings under drought.
- (ii) The effects of drought on the diversity and composition of fungal communities and the subsequent consequences on seedling survival.
- (iii) The impact of drought on the methylome changes and physiological responses manifested in mother trees, which have the potential to be adaptive and enhance seedling survival in the next generation.

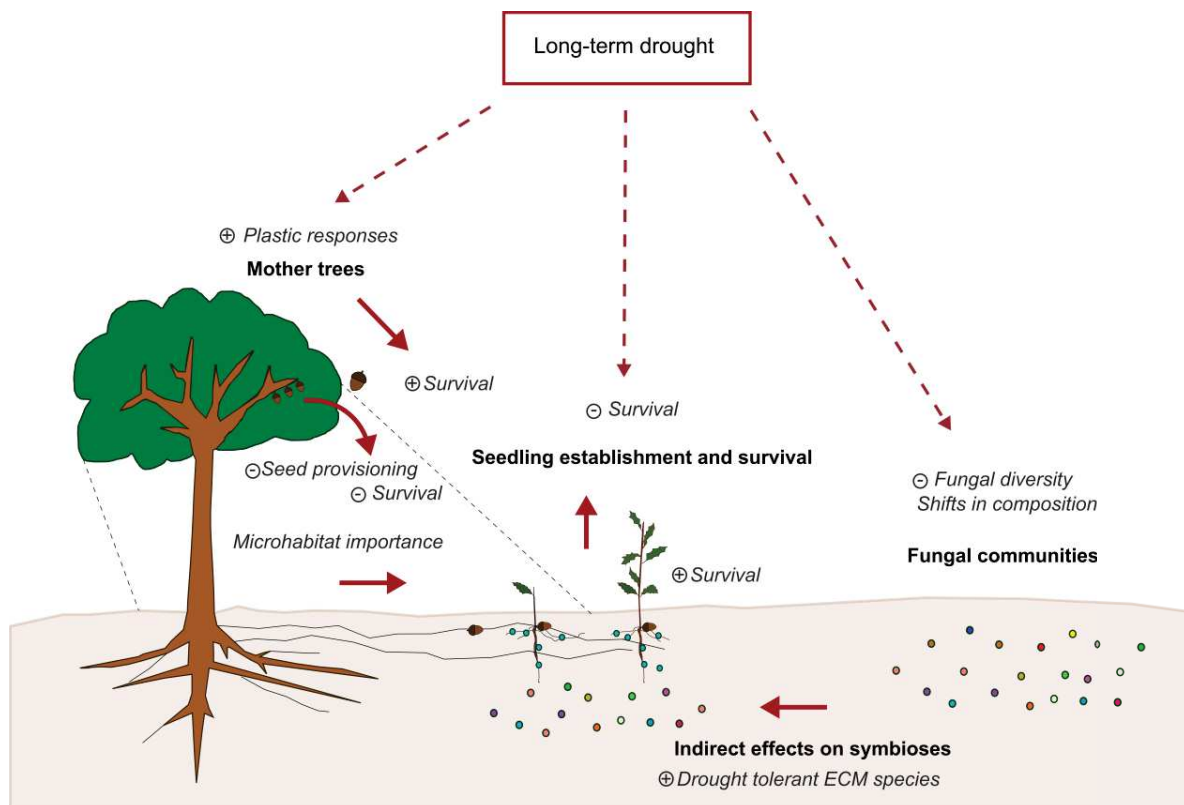


Figure 1.7. Summary of the overarching goal and hypothesis of the PhD thesis. This thesis reflects an interdisciplinary approach combining epigenetics, biotic interactions and environmental effects in a long-term in situ rainfall exclusion experiment (see study site). The hypothesis of the study are in grey with the symbols + and – meaning a positive and negative expected effect respectively.

This PhD thesis is organized in three chapters as follows:

Chapter 1 - The effects of microhabitat conditions and ectomycorrhizal colonization on the establishment and survival of seedlings under increased drought. In this chapter, I present the results from a field experimental study that investigated the role of light availability, soil moisture, ectomycorrhizae (ECM) exploration types and maternal effects (acorn mass), on acorn germination, seedling recruitment and growth.

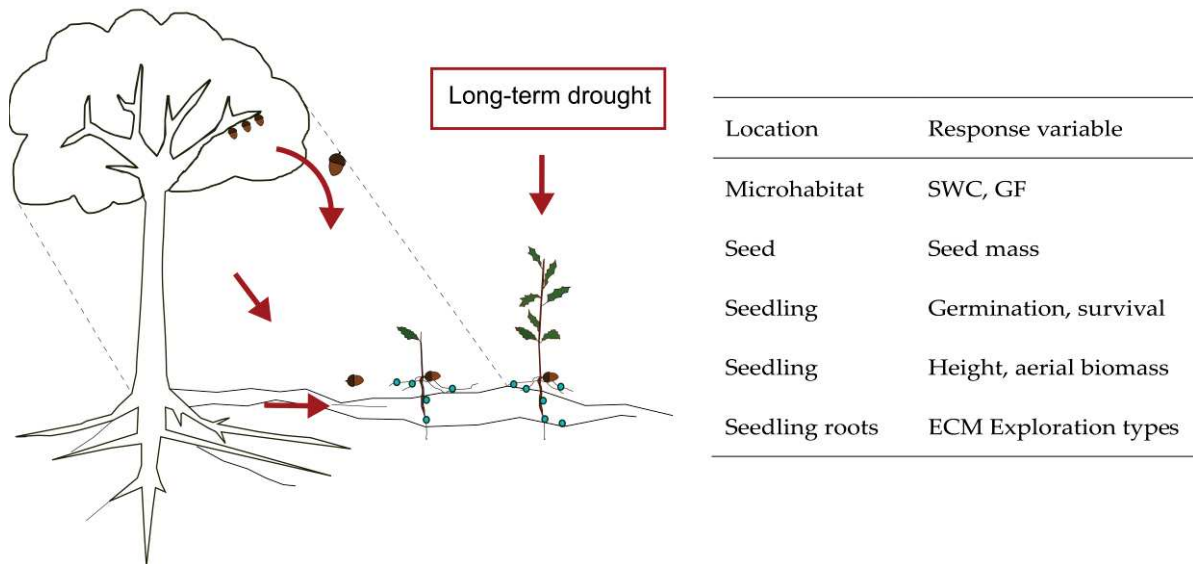


Figure 1.8. Scheme of the chapter 1, with the response variables. SWC= soil water content, GF=gap fraction

In this first chapter, it is hypothesized that increased drought will decrease seedling survival, with microhabitat conditions-dependent effects, and will favour the colonization by ectomycorrhizas with longer extramatricial mycelium.

Chapter 2 - Long-term drought effects on the ectomycorrhizal community composition present in the soil and associated to seedling root tips. In this chapter, I focus on quantifying the impact of 15 years of increased drought on soil fungal community composition and diversity, as well as on the existence of potential links between the fungal diversity, and seedling survival and growth, with a special focus on the ectomycorrhizal guild.

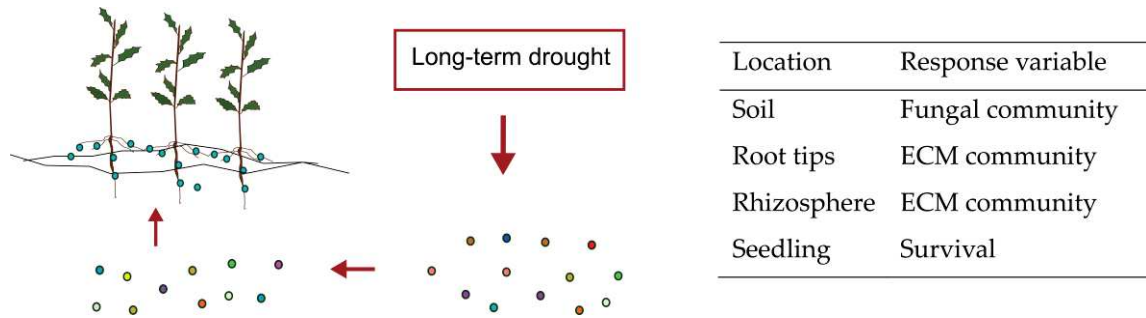


Figure 1.9. Scheme of the chapter 2, with the response variables

In this second chapter, it is hypothesized that increased drought will reduce the diversity of fungal communities, and will induce changes in community compositions towards ectomycorrhizal species more adapted to drought that will increase seedling survival.

Chapter 3 - Long-term drought effects on the physiological and epigenetic (DNA methylation) responses of seed-bearing mother trees, with potential intergenerational transmission. In this chapter, I present the results of a field study that assessed both leaf physiological responses to drought as well as whole genome methylation changes in several adult individuals of *Q. ilex*, with the aim of testing for potential links between physiological responses to drought and differentially methylated regions (DMRs) of the genome.

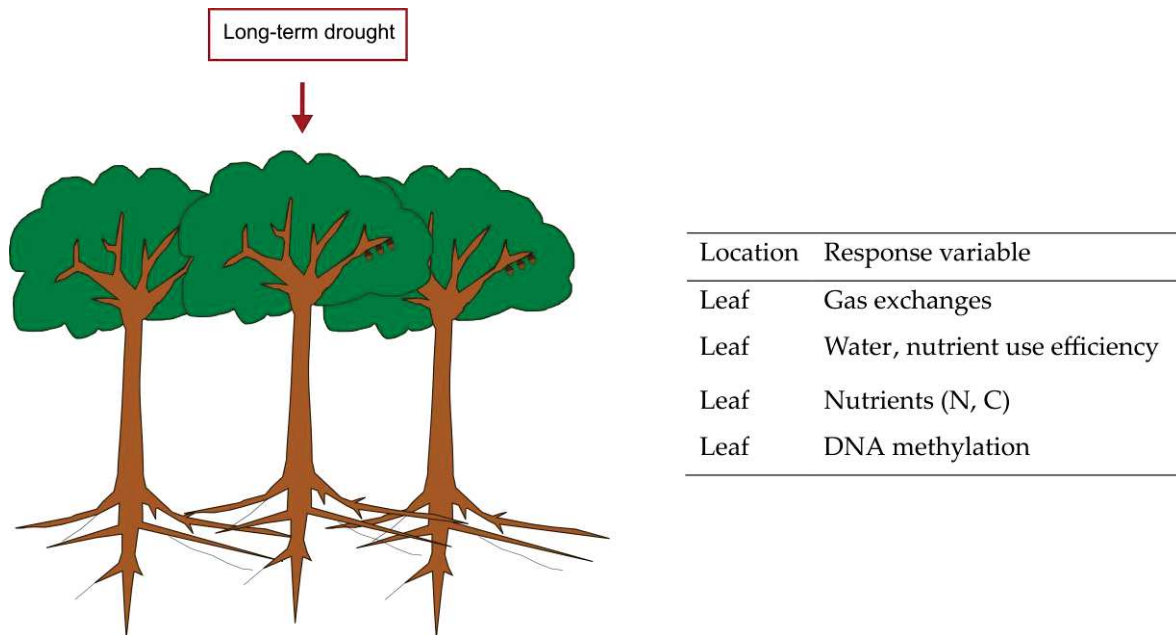


Figure 1.10. Scheme of chapter 3, with the response variables.

In this third chapter, it is hypothesized that mother trees will exhibit changes in the methylomes in response to increased drought, which will be correlated to changes in their physiological acclimation responses.

1.9 Study site

All the experiments were carried out in the CNRS experimental platform of Puéchabon which is located 35 km northwest of Montpellier (southern France) (Figure 1.11). The forest overstory is largely dominated by the evergreen holm oak (*Quercus ilex*) which accounts for more than 90% of the tree canopy cover, and the understory forms a sparse shrubby layer with a percent cover lower than 25%. This forest has been managed as a coppice for centuries, but the last clear cut was performed in 1942 and the tree density is now around 5100 stems ha⁻¹ (Rodríguez-Calcerrada et al., 2011). The climate is Mediterranean, with rainfall occurring mainly during autumn and winter, with about 80% between September and April. Mean annual precipitation ranges from 550 mm to 1549 mm (mean of 901 mm over the past 28 years), and mean annual temperature varies from 11.5°C to 14.1°C (mean of 13.1°C over the same period).



Figure 1.11. Aerial picture of the Puéchabon experimental site

The Puéchabon experimental site includes a rainfall exclusion experiment that continuously reduces the net input of precipitation to the soil by ~29% compared to the control treatment since March 2003 (Limousin et al. 2008). The rainfall exclusion experiment is replicated on three blocks (R1, R2, Sc) approximately 100 m apart from each other, and situated on a flat plateau with no lateral flow. In each replicate, two 100 m² plots are subjected to different precipitation regimes: one is a control treatment receiving natural precipitation and

the second is subjected to partial rainfall exclusion. Rainfall exclusion is achieved by using PVC gutters hung below the canopy and covering ~33% of the ground area. In the control treatment the same gutters are hung upside-down to even the microclimate effect on the understory in the two treatments (Figure 1.12).

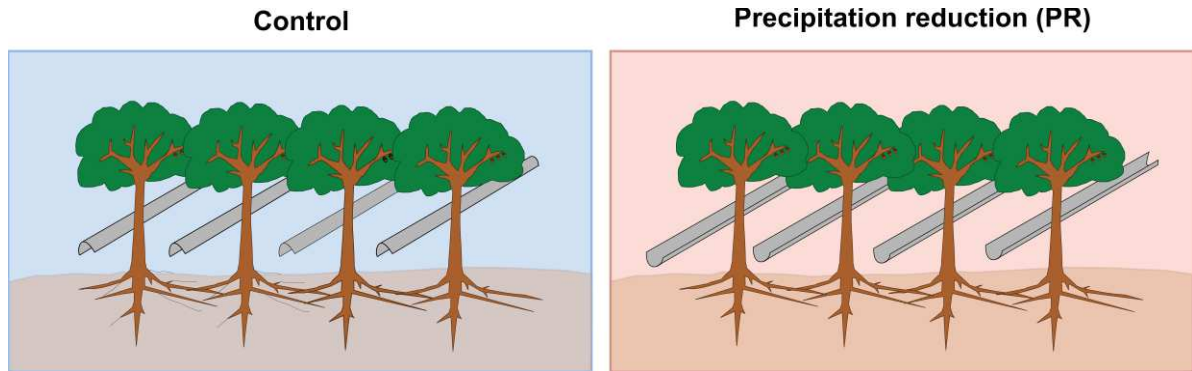
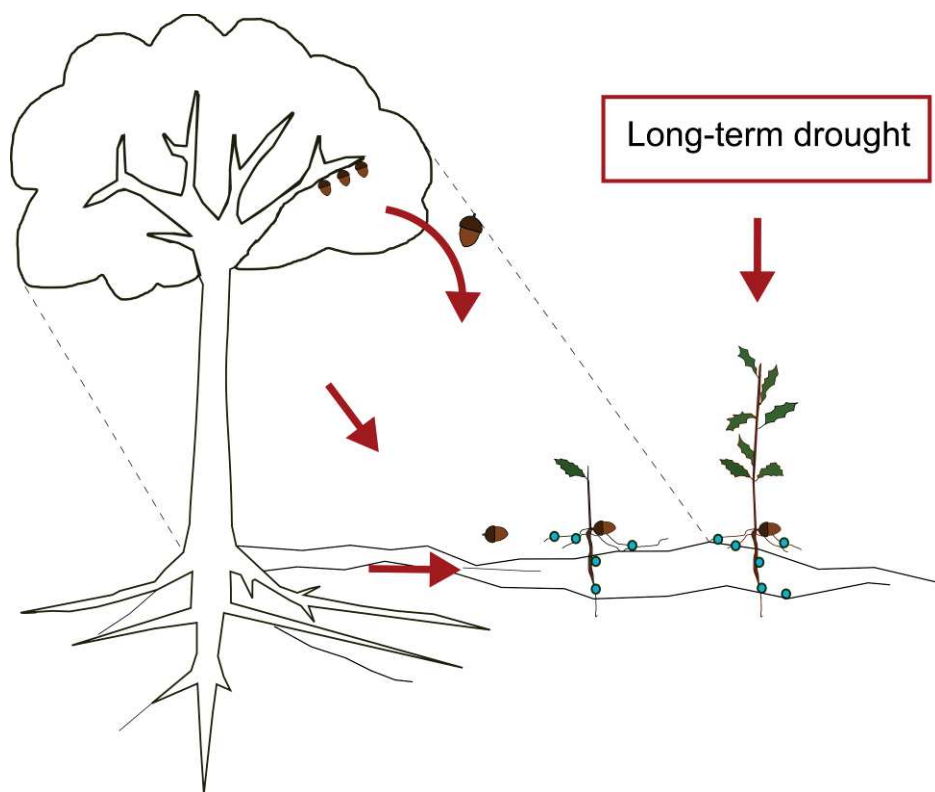


Figure 1.12. Rainfall exclusion experiment replicated on three blocks with control and precipitation reduction treatment per block. PVC gutters below canopy cover 33% of the soil area, and collect ~29% of annual precipitation in the precipitation reduction treatment, whereas in the control gutters are set upside down to allow water infiltration in the soil.

According to previous results from the Puéchabon experimental site, the main drought acclimation mechanism at both the tree and the ecosystem scales is the reduction of the leaf area by approximately 20% (Limousin et al., 2012), which reduces the canopy transpiration (Limousin et al., 2009), the ecosystem primary production (Misson et al., 2009), and the organic matter input to the soil as litterfall (Gavinet et al., 2019) compared to the control treatment. The effect of rainfall exclusion on tree diameter growth is, however, not significant (Rodríguez-Calcerrada et al., 2011) and the mortality rate compared to the control treatment only started to increase after 7 years of treatment (Gavinet et al. 2020), thus demonstrating the importance of long-term experiments for studying mature ecosystems. The rainfall exclusion also affects tree reproduction by reducing the production of sound acorns and increasing the proportion of aborted acorns, compared to the control (Pérez-Ramos et al., 2010; Le Roncé et al., in rev.). However, the impact of the rainfall exclusion experiment on seedling recruitment is complex, with the drought treatment decreasing the probability of seedling survival but increasing the probability of seedling emergence (Pérez-Ramos et al., 2013). Finally, the experimental rainfall reduction has resulted in significant shifts in ECM community composition compared to the control, but with no effect on the global richness of the community (Richard et al., 2011). Taken collectively, these results point out to potentially

interesting interactions between medium-term acclimation of mother trees and changes in soil biological properties that could both have drought legacy effects on seedling establishment, aspects that, to date, never been considered in such a rainfall manipulation experiment.



Microhabitat and ectomycorrhizal effects on the establishment, growth and survival of *Quercus ilex* L. seedlings under drought

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Keywords: Holm oak, long-term drought, recruitment, ectomycorrhizae, Mediterranean forest, climate change, pathogens.

2.1 Abstract

The success of tree recruitment in Mediterranean holm oak (*Quercus ilex*) forests is threatened by the increasing intensity, duration and frequency of drought periods. Seedling germination and growth are modulated by complex interactions between abiotic (microhabitat conditions) and biotic factors (mycorrhiza association) that may mitigate the impacts of climate change on tree recruitment. To better understand and anticipate these effects, we conducted a germination experiment in a long-term precipitation reduction (PR) field experiment where we monitored seedling establishment and survival, micro-habitat conditions and ectomycorrhizal (ECM) colonization by different mycelia exploration types during the first year of seedling growth. We hypothesized that (i) the PR treatment decreases seedling survival relative to the control with ambient conditions, (ii) microhabitat conditions of water and light availability are better predictors of seedling survival than the PR treatment, (iii) the PR treatment will favour the development of ECM exploration types with drought-resistance traits such as differentiated rhizomorphs. Contrary to our first hypothesis, seedling survival was lower in control plots with overall higher soil moisture. Micro-habitat light and

soil moisture conditions were better predictors of seedling survival and growth than the plot-level PR treatment, confirming our second hypothesis. Furthermore, in line with our third hypothesis, we found that ECM with longer extramatrical mycelia were more abundant in the PR treatment plots and were positively correlated to survival, which suggests a potential role of this ECM exploration type in seedling survival and recruitment. Although summer drought was the main cause of seedling mortality, our study indicates that drier conditions in spring can increase seedling survival, presumably through a synergistic effect of drought adapted ECM species and less favourable conditions for root pathogens.

2.2 Introduction

The success of seedling establishment is one of the most critical processes determining forest regeneration dynamics and long-term persistence of tree species. In Mediterranean forests, where summer drought is already a major cause of mortality of woody species during early life stages (Gómez-Aparicio et al., 2005; Herrera et al., 1994), the success of tree establishment is expected to deteriorate as a consequence of the ongoing climate change. Climate models for the Mediterranean region predict longer and more severe drought episodes in the near future (Giorgi and Lionello, 2008) and the first signs of climate change impacts on forests are already documented as tree dieback and crown defoliation (Carnicer et al., 2011; Gentilesca et al., 2017). As Mediterranean forests are, for the most part, naturally regenerated, a better understanding and quantification of drought effects on tree recruitment processes is needed to better mitigate the impacts of climate change on forest ecosystems.

In addition to the climatic effects, the success of tree recruitment under a forest cover is also highly dependent on microhabitat conditions (Castro et al., 2004; Gavinet et al., 2016; Gómez-Aparicio et al., 2004) and biotic interactions, especially with mycorrhizal fungi (Lehto and Zwiazek, 2011; Simard et al., 2012). The canopy cover and structure generate different microhabitats, for example through different light availabilities that further modulate local soil moisture and temperature conditions, and thus, the overall effect of drought. The canopy can act as shelter in the dry season by protecting against radiative heating, reducing soil temperature, and evaporative demands (Valiente-Banuet, A., & Ezcurra, E., 1991). Conversely, the tree canopy can also reduce the soil moisture available to seedlings due to foliar interception of rainfall, and an increased competition for soil water by the adult trees (David

et al., 2006; Franco and Nobel, 1988). In addition to these subtle differences that tree canopies can cause on water availability of understorey seedlings, canopy cover also has a direct effect on light availability. Two studies carried out with *Quercus pyrenaica* Willd. in different forest ecosystems of Spain with different water availabilities revealed higher seedling survival under open canopies in the wet sites (Rodríguez-Calcerrada et al., 2010), and under closed canopies in the dry sites (Gómez-Aparicio et al., 2008). This suggests a trade-off between light availability and soil water status, which is in line with the morphological trade-offs between plant tolerance to drought and shade (Niinemets and Valladares, 2006). However some species, such as *Quercus ilex*, are known to tolerate both shade and drought, with the shade contributing to expand their drought tolerance (Sack et al., 2003). These species might be favoured in Mediterranean ecosystems where light and water limitations co-occur, especially in the case of seedlings emerging under a dense tree canopy. In theory, the most suitable microhabitat for seedling establishment and growth is a site with enough light to maintain a positive carbon balance, and with sufficient soil moisture to avoid water deficit during the drought seasons. However, the impact of water availability on seedling recruitment is complex and not yet fully understood, with opposite effects of rainfall exclusion being documented for seedling emergence (increased in drier conditions) and seedling survival (decreased in drier conditions) (Pérez-Ramos et al., 2013).

There is also increasing evidence that the filamentous fungi known to form plant-fungal chimeras, the so-called mycorrhizae, are critical not only for nutrient uptake and defence against soil pathogens but also for helping plants to cope with the detrimental effects of soil water deficit (Egerton-Warburton et al., 2007; Lehto and Zwiazek, 2011; Simard et al., 2012). Sheltering adult trees may also facilitate seedling establishment by acting as a fungal inoculum for seedlings and providing a common mycelium network which can be beneficial for the performance of younger generations of plants (Bingham and Simard, 2012; Querejeta et al., 2003; Richard et al., 2009). The widespread Mediterranean tree species *Q. ilex* is associated with ectomycorrhizal fungi (ECM). In forest ecosystems, these ECM fungi are assembled in highly diverse communities (Tedersoo et al., 2014) made of patchily distributed species (Genney et al., 2006). Species composition of these communities are influenced by water availability, and have been shown to respond to precipitation reduction (Richard et al., 2011). Depending on fungal species, ECM vary in the anatomy and morphology of the mycelia

radiating into the soil and these morphological variations have been putatively related to ecological functions and types of foraging exploration (Agerer, 2001). According to (Agerer, 2001), four different exploration types can be distinguished based on the amount of emanating hyphae: contact, short, medium-distance and long-distance. It has been suggested that long exploration-type ECM root tips with hydrophobic mantles and highly differentiated extensive external mycelium (i.e., the so-called rhizomorphs, developed by species in *Boletus* and *Cortinarius* genera), are resistant to drought and effective at water transport and uptake for their associated hosts (Bakker et al., 2006; Shahin et al., 2013). However, to the best of our knowledge, the potential interactive effects of microhabitat environmental factors with ECM colonization and exploration types on seed germination and seedling establishment have been little investigated, especially under field conditions. Most studies on seedling ECM colonization have been done in highly artificial conditions (potted plants in greenhouses, applied fungal inoculums, simplified soil substrates) which may be prone to being affected by lab-specific environmental factors (Milcu et al., 2016) and thus, their relevance for natural field conditions should be interpreted with caution.

In this study, we investigated the effects of increasing drought conditions on the germination, growth and survival of *Q. ilex* seedlings in a long-term field experiment with precipitation reduction (PR) of 29% compared to the unaltered control since 2003. We conducted a germination experiment in the field and monitored seedling establishment and survival, soil humidity and light micro-conditions and ECM colonization of roots during the first year of seedling growth. We hypothesized that: (i) the PR treatment decreases seedling survival relative to the control with ambient conditions (Pérez-Ramos et al., 2013), (ii) microhabitat conditions of water and light availability are better predictors of seedling survival than the PR treatment, (iii) the PR treatment will favour the development of ECM fungi with drought-resistance traits such as differentiated rhizomorphs with longer external mycelium.

2.3 Materials and Methods

2.3.1 Experimental site

The study site is located 35 km northwest of Montpellier (southern France) in the Puéchabon State Forest, on a flat plateau (43°44'29'' N, 3°35'45'' E, elevation 270 m). 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 shade-tolerant evergreen oak *Q. ilex*, with a top canopy height of about 5.5 m and a stand density of *c.* 6000 stems ha⁻¹. The understorey is composed of a sparse shrubby layer with the evergreen species, *Buxus sempervirens*, *Phyllirea latifolia*, *Pistacia terebinthus* and *Juniperus oxycedrus*, a percent cover of approximately 25% at 2 m.

The area has a Mediterranean-type climate. Rainfall mainly occurs during autumn and winter with about 80% of total annual precipitation occurring between September and April. The mean annual precipitation is 920 mm with a range of 550–1549 mm (1984–2017). Mean annual temperature is 13.3°C (on-site meteorological station, 1984–2017), the coldest month being January (5.5°C) and the hottest July (22.9°C). The soil is extremely rocky from hard Jurassic limestone origin. 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). Seed germination in *Q. ilex* mostly occurs in early winter and shoot emergence in spring. The below-ground establishment occurs from germination to shoot emergence, when plants invest in their taproot and when the first associations between roots and below-ground microbial communities (ECMs and/or pathogens) take place.

2.3.2 Rainfall exclusion

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. Each replication was composed of one throughfall exclusion treatment (henceforth, precipitation reduction, PR) 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 plots, identical gutters are set up upside down so that the albedo and the understorey microclimate are as close as possible in the two treatments. However, gutters in the PR treatment are slightly tilted down in order to let the water drop out of the plots, different from the Controls where gutters are horizontally placed.

2.3.3 Sowing, establishment and seedling growth

Acorns from 10 different maternal trees were collected in November 2017 in the surroundings of the experimental site, outside of the precipitation reduction and control treatments. All acorns were submitted to a flotation test in order to discard the ones aborted or damaged by insects or fungi. All the viable acorns were weighted, numbered with a marker pen for identification, and kept in a fridge at 4°C in vermiculite substrate humidified with water for 3 weeks. Sowing was done in December 2017, placing acorns 1 cm deep into the soil in the understorey of the experimental site. In order to prevent acorn predation, sowed acorns were enclosed in wire cages of 40 × 30 × 30 cm. One acorn from each maternal tree was sowed in each cage, thus totalling 10 acorns par cage. A total of 60 cages were randomly installed in areas not occupied by stones (30 cages per treatment, 20 per block), so 600 acorns in total were used in the study.

Recruitment stages (germination, stem emergence and survival) were monitored monthly from January 2018 to the end of November 2018 in all seedlings from all cages. Two metrics of survival, and hence mortality, were considered in the study: survival from germination to the collection of the seedlings after 11 months was considered as ‘total survival’ (S_T), and survival from stem emergence to the collection of seedlings after 11 months was considered as ‘survival of emerged plants’ (S_{EP}). Stem height was measured in all seedlings from all cages at the end of the experiment in November 2018. At this time, all seedlings from half of the cages randomly chosen (15 cages per treatment, 10 per block) were pulled out from the soil for ECM analysis and biomass assessments. Aerial and belowground seedling parts were separated, the aerial parts were oven-dried at 60° C until constant mass for biomass measurements, and belowground parts were used for ECM assessments.

2.3.4 Microenvironmental conditions

Soil water content and light availability were measured six and two times respectively during the one-year experiment for each of the cages/microhabitats. Two measurements per cage were taken at two different positions of the cage that were averaged to get the final value for both soil water content and light measurements at each measuring time. Volumetric soil water content (SWC) was measured six times in the year in the first 10 cm of soil with a portable TDR (Delta-T SM150 Soil Moisture kit, Cambridge, UK), and light availability was measured in Spring and Autumn with a LAI-2200 Plant Canopy Analyser (LI-COR Biosciences, Lincoln, NE, USA). As the plant or leaf area index over the cages could not be measured because of the shading from the gutters installed in the plots, the canopy gap-fraction (GF) around a zenith angle of 38° was used as an integrative proxy of the local light availability. Simultaneous measurements of incident light above and below the canopy were taken with the LAI-2200, and the GF was computed using the FV2200 2.1.1 software (LI-COR Biosciences). GF was preferred to a simple ratio between the light below and above the canopy so as to avoid the confounding effects due to differences of the solar angle or of the fraction of direct and diffuse light at the time of measurement.

2.3.5 ECM quantification

At the end of November 2018, eleven-month old seedlings (alive and dead) from half of the cages of both treatments and blocks (30 cages and 129 plants analysed) were carefully dug up by hand with the aim to collect the largest part of the root systems down to 15 cm depth. Due to the very rocky nature of the soil, it was not possible to harvest roots that were growing deeper than 15 cm. As it was not possible to extract intact root systems, the root biomass variable was omitted from the ECM analysis. Seedlings were brought to the lab and kept in the fridge for a week before ECM quantification under a dissecting microscope. The collected root system was kept in water in trays for several hours in order to remove organic and mineral soil particles from the roots. On the extracted root systems, the percentage of root colonization by ectomycorrhizal fungi was quantified under the microscope, by counting the number of colonized and non-colonized root tips. As the assessment of the ECM colonization was feasible irrespective of the plant status (alive or dead), we quantified it in both alive and dead seedlings. For dead seedlings this measurement corresponds to the status of ECM colonization at the moment of their death and it constitutes a valuable information for

assessing the role of ECM in seedling survival since most *Q. ilex* seedlings die during their first year, particularly under adult trees (Gómez-Aparicio et al., 2004; Richard et al., 2009). Each ECM root tip was classified according to the different exploration types defined by (Agerer, 2001) based on the amount, organization and length of emanating hyphae (contact, short, and medium-distance). Proportions of each exploration type were calculated by dividing the number of ectomycorrhizae of each exploration type respective by the total number of root tips of the root system (Equation 1 and 2).

$$ECMc = \frac{n^{\circ} \text{ contact root tips}}{n^{\circ} \text{ total of root tips}} \quad (1)$$

$$ECMsm = \frac{n^{\circ} \text{ short+medium root tips}}{n^{\circ} \text{ total of root tips}} \quad (2)$$

With ECMc being the proportion of contact exploration type root tips and ECMsm the proportion of short and medium exploration type root tips. In line with the study of (Shahin et al., 2013) that was carried out at the same experimental site, we did not find any long-distance ECM associated to the root tips of *Q. ilex* seedlings, thus explaining the absence of this exploration type in our dataset.

2.3.6 Statistical analyses

All statistical analyses were performed with the R software version R 3.4.1 (R Core Team, 2013). Acorn germination, survival and growth-related response variables were analysed with generalized linear mixed-effects models with the PR treatment as a fixed factor and soil water content (SWC), canopy gap fraction (GF) and acorn mass as covariables, with the random effects being the block, the cage and the mother tree using *glmer* function from 'lme4' package version 1.1-21 (Bates et al., 2014). The SWC and GF covariables were measured at the cage level, whereas the acorn mass covariable was measured at the individual level. An example of the syntax for a model looking at germination success is as follows "*model<- glmer (Germination ~ PR_treat + SWC * GF * acorn_mass + (1|Block) + (1|Cage) + (1|ID_mother_tree), family=binomial)*". Binomial variables such as germination and survival were modelled with binomial distribution, and proportion data such as ECM colonization with betabinomial distribution. The seedling status at the time of harvest (dead or alive) was also added as an additional explanatory variable in models of ECM colonization. Minimal adequate models with the lowest AIC were obtained following the guidelines of (Zuur, 2009) with the help of the "buildmer" package version 1.4 (Voeten, 2019). Interaction between ECM colonization and

environmental variables were displayed with the “interactions” package version 1.1.1 (Long, 2019), and the survival curves with the “survival” package version 3.1-8 (Therneau, 2015). The overall effects of the PR treatment on SWC and GF across all sampling campaigns were analysed using linear mixed-effects models with PR treatment and measurement campaign (sampling date) as fixed factors and cage as random effects, whereas for the models testing the effect of PR treatment for each sampling campaign/date only the block was used as random effect.

2.4 Results

2.4.1 Effects of precipitation reduction treatment on microhabitat conditions

Volumetric soil water content measured in the upper soil layer (10 cm) at the microhabitat/cage level were significantly lower (-11%) in the precipitation reduction treatment (PR) relative to Control over all six measurement campaigns ($P < 0.002$; Figure 2.1). The same trend was found also in all individual measurement campaigns (Fig 2.1). The lowest soil water content values were observed in August and in September (2.1% in Control and 1.2% in PR in September) during the peak of the seasonal drought, thus confirming that 2018 had a typical summer drought for the Mediterranean region. The highest soil water content values were observed in April, with 27% in Control and 26% in PR treatment. There was a large variability in SWC among cages with a strong overlap of values between treatments, irrespective of the measurement period.

Similar to SWC, we observed a large variability and an important overlap of cage level GF values between treatments, indicating highly different light conditions among microhabitats (Figure 2.2). Over the two sampling campaigns, the PR treatment, in which gutters are slightly tilted down and therefore closer to cages, showed an overall lower but not significant GF value compared to the Control (7% and 8.8%, respectively) (Figure 2.2). However, the effect of PR treatment on GF was not significant in April when *Q. ilex* only supported its old leaf cohorts, but was significantly lower in September (Figure 2.2).

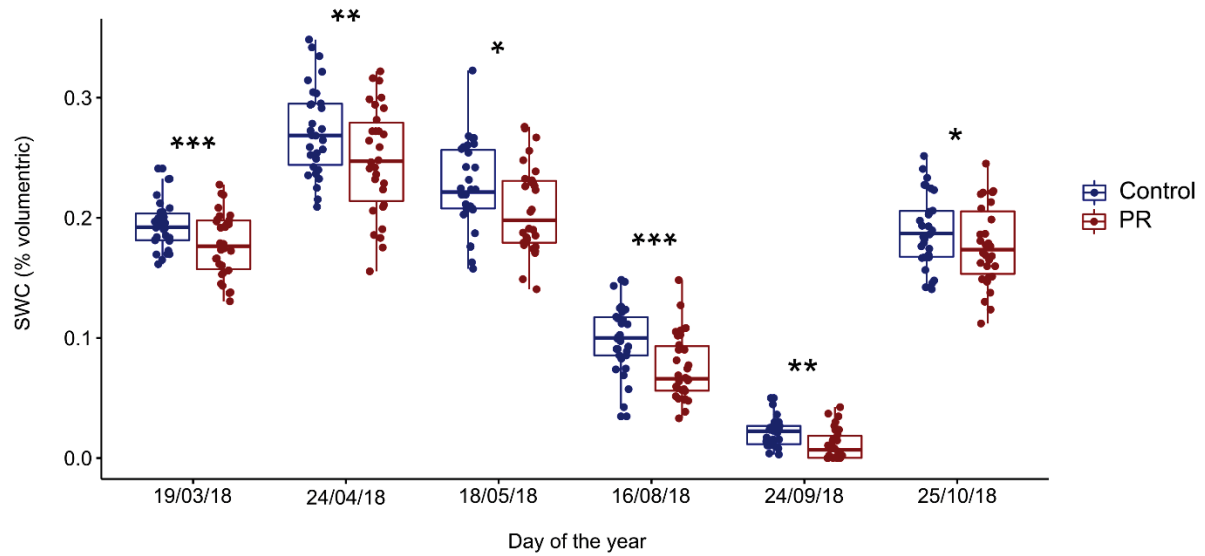


Figure 2.1. Volumetric soil water content (SWC % volumetric) per treatment measured from March to October 2018. Precipitation reduction treatment (PR, in red) and control (in blue). Each dot indicates the average SWC value in one of the 60 cages. *** for $P < 0.001$, ** for $P < 0.01$, * for $P < 0.05$, + for $P < 0.07$

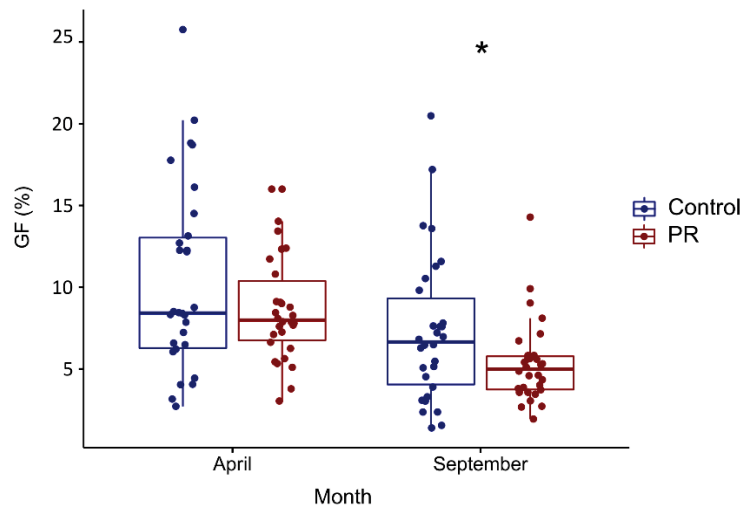


Figure 2.2. Gap fraction (%) per treatment measured in April and September 2018. Precipitation reduction treatment (PR, in red) and control (in blue). Each dot indicates one microhabitat at each of the 60 cages. *** for $P < 0.001$, ** for $P < 0.01$, * for $P < 0.05$, + for $P < 0.07$

2.4.2 Germination and survival

The proportion of germinated acorns increased with the initial seed resources (acorn fresh biomass) (Table 2.1) but was not significantly affected by the precipitation reduction treatment (PR), nor by any of the measured micro-environmental variables.

Table 2.1. Fitted estimates and significance levels of linear mixed models for seedling germination, survival and growth. Minimal adequate model outputs for the effects of precipitation reduction (PR) treatment, acorn fresh biomass (Acorn), gap fraction (GF), soil water content (SWC) and their respective interactions on germination, total survival (S_T), survival of emerged plants (S_{EP}), height, shoot biomass, root biomass of the extracted root systems, contact ectomycorrhizal colonization (ECM_c) and short + medium ectomycorrhizal colonization (ECM_{sm}). R^2_m represents the variance explained by fixed factors and R^2_c represents the variance explained by fixed and random factors. NA = non applicable, as R^2_c cannot be computed for beta binomial distributions. *** for $P < 0.001$, ** for $P < 0.01$, * for $P < 0.05$, + for $P < 0.07$. Blanks indicate that the respective variables was not retained in the minimal adequate models, whereas the estimates with a lack of symbol (star or plus) are not statistically significant, but the predictor was retained in the minimal adequate model.

Variable/ Predictor	PR	Acorn	GF	SWC	Acorn×GF	GF×SWC	R^2_m	R^2_c
Germination		0.231 ⁺	0.1672				0.02	0.29
Total survival (S_T)	0.482 [*]	0.272 ^{**}	0.211 [*]				0.04	0.11
Survival emerged plants (S_{EP})		0.238 [*]		-0.203 [*]			0.03	0.03
Height	-1.468 [*]	1.233 ^{***}					0.15	0.27
Shoot biomass		0.042 ^{***}		-0.016 [*]			0.22	0.22
Root biomass		0.047 ^{***}					0.23	0.23
ECM _c			-0.231 [*]	0.148 ⁺		0.496 ^{***}	0.15	NA
ECM _{sm}	0.536 ^{***}		-0.192 [*]		-0.263 ^{**}		0.12	NA

Total survival (S_T) of seedlings after eleven months was significantly higher (29%) in the PR treatment relative to Control (22%) (Figure 2.3 and 2.4B), and was higher in seedlings from bigger acorns and growing under more open canopies (Table 2.1). The survival of emerged plants (S_{EP}) (i.e. the survival of seedlings that already succeeded germination and stem emergence) also increased with seed resources (acorn biomass), and it was significantly lower in the wetter microhabitats (Table 2.1; Figure S2.1), with no influence of light. Seedling mortality during germination was similar in seedlings from different drought treatments (25.7% Control vs. 23.7% in PR, respectively). Mortality in spring when below-ground establishment and stem emergence occurred was slightly higher in the Control than in PR treatment (15.6% Control vs. 12% PR respectively, difference not significant). The highest seedling mortality was observed during the summer drought season and was again slightly higher in the Control than in the PR treatment (33.7% Control vs. 31.3% PR respectively, difference not significant). Finally, mortality in autumn during the drought recovery was modest in comparison and similar between treatments (3.3% in Control vs. 3.7% in PR treatment).

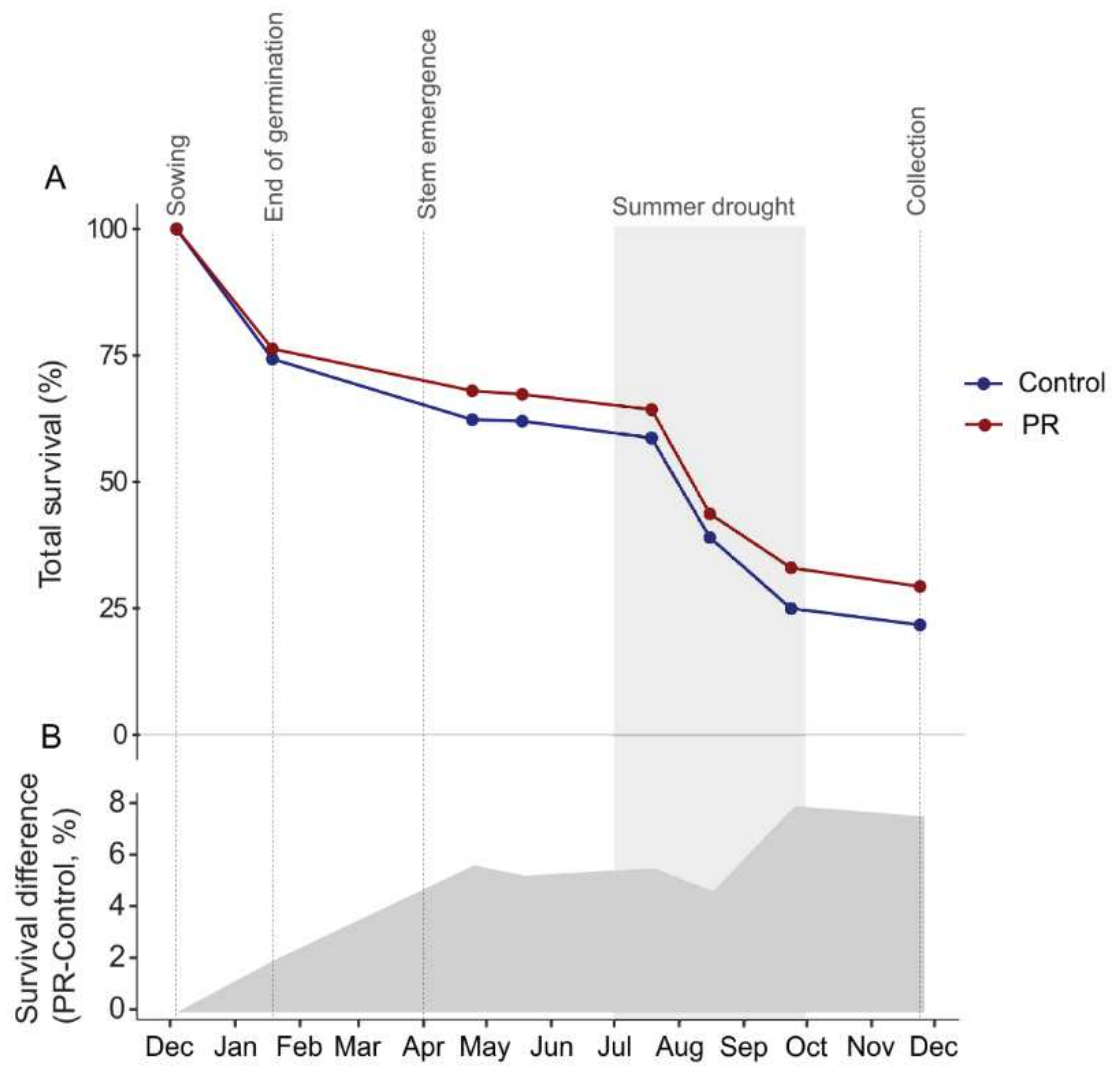


Figure 2.3. Survival of seedlings per treatment during the experiment. (A) Survival curves in the precipitation reduction treatment (PR, in red), and in the control (in blue). Values at “end of germination” corresponds to germination percentage in Fig 4A, and values at “Collection” corresponds to total survival (ST) in Fig 4B. (B) Grey polygon represents the cumulative difference between the two survival curves, total survival in PR minus total survival in Control. The drought period of the year 2018 based on SWC data is highlighted in clear grey.

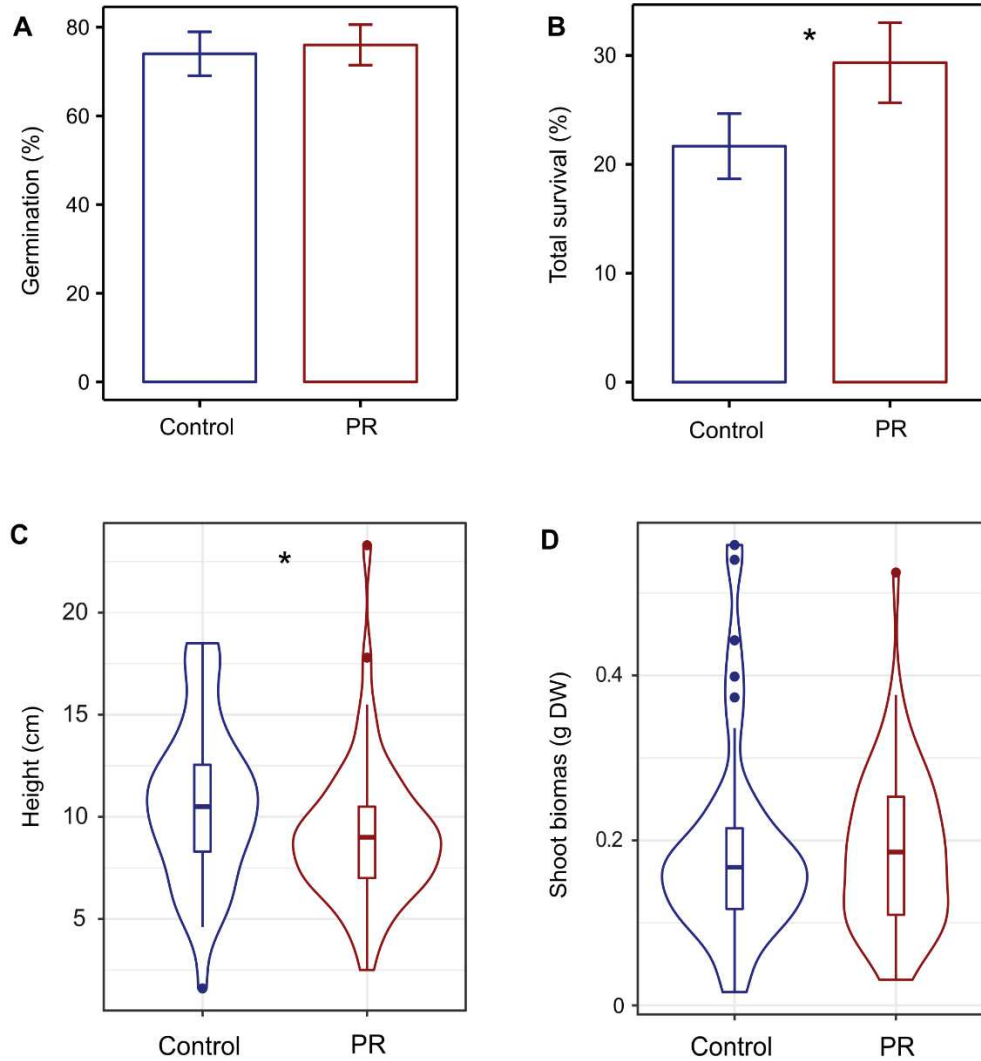


Figure 2.4. Effect of precipitation reduction treatment on germination, survival, height and shoot biomass of seedlings. (A) Germination and (B) Total survival (ST), per cage $n = 60$. (C) Height measured at the end of the experiment (November 2018) per individual $n = 153$ (alive plants from all cages) and (D) shoot biomass per individual $n = 96$ (alive plants from half of the cages). *** $P < 0.001$, ** $0.001 < P < 0.01$, * $0.01 < P < 0.05$, + $0.05 < P < 0.07$.

2.4.3 Growth

The height of the surviving seedlings was lower (-10%) in the PR treatment compared to Control (Table 2.1; Figure 2.4C). Heavier acorns with more initial seed resources were observed to produce taller and heavier seedlings in both treatments (Table 2.1). Shoot biomass was negatively influenced by the soil moisture of the micro-sites, but it was not significantly impacted by the PR treatment (Table 2.1; Figure 2.4D). Root biomass of the extracted root systems was positively influenced by acorn biomass (Table 2.1). Contrary to soil humidity, light availability at the microsite level did not have any influence on seedling growth.

2.4.4 Ectomycorrhizal colonization and correlations with seedling performance

Ectomycorrhizal colonization assessed as the proportion of mycorrhizal root tips per seedling ranged from 61 to 100%. Overall, the proportion of contact exploration type (ECMc) was slightly but not significantly lower in the PR treatment (-6.5%; Figure 2.5A) relative to the Control, whereas the proportion of short and medium exploration types (ECMsm) was significantly larger in the PR treatment compared to the Control (+59.7%; Figure 2.5B). The status of seedlings (dead or alive) as covariable in the models was not significant for any of the ECM exploration types. In terms of micro-environmental conditions, the colonization with ECMc was affected by the interaction between soil moisture and light availability (Table 2.1; Figure 2.6A); the proportion of ECMc increased under more optimal conditions with high SWC and light availability, but decreased with light availability when SWC was low. The colonization of root tips with ECMsm was affected by the interaction between acorn biomass and light availability (Table 2.1; Figure 2.6B). ECMsm was high for seedlings with low seed biomass, with a slight increase of proportions of ECMsm with increasing light availability. In contrast, in seedlings originating from heavy acorns, ECMsm decreased with increasing light availability. In other words, seedlings originating from heavy seeds and growing with relatively favourable light availability, they recruited less ECMsm.

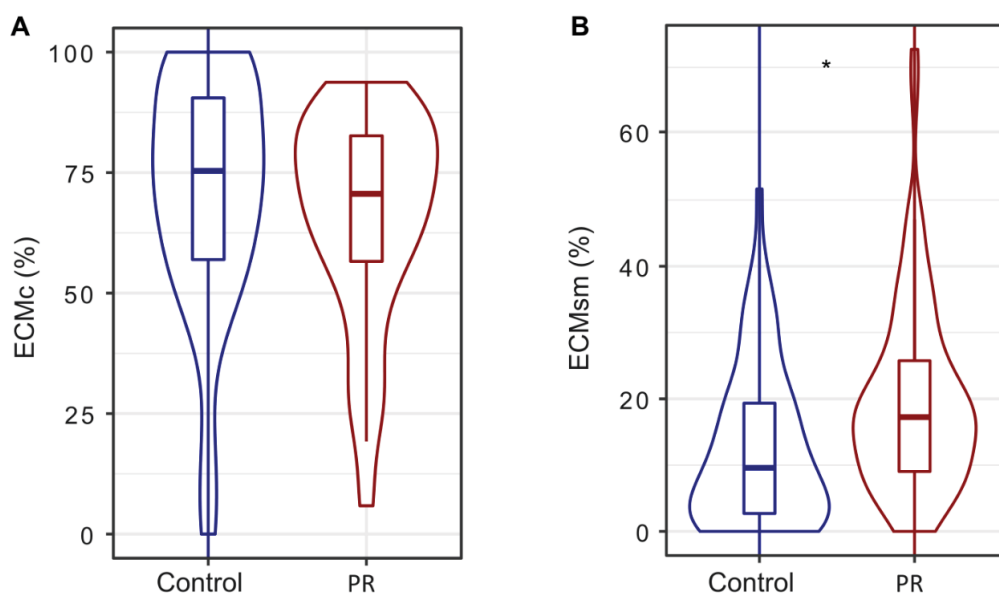


Figure 2.5. Proportion of different ECM exploration types per total number of root tips. (A) Contact ectomycorrhizal exploration type ECMc and (B) short and medium ectomycorrhizal exploration type ECMsm per individual $n=129$ (all plants from half of the cages). *** $P<0.001$, ** $0.001<P<0.01$, * $0.01<P<0.05$, + $0.05<P<0.07$.

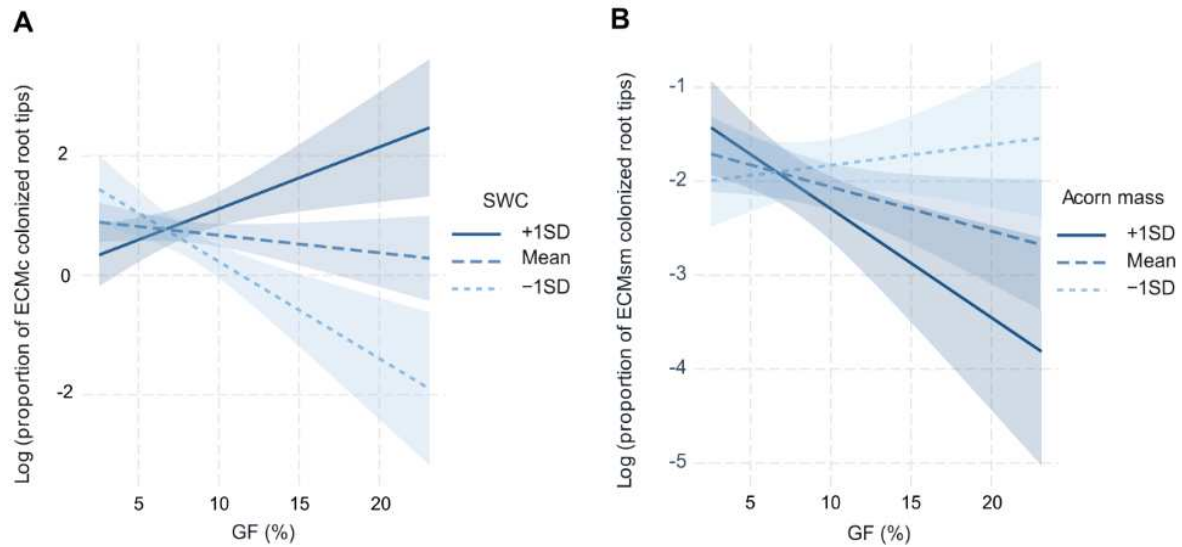


Figure 2.6. Significant interactions from mixed linear models of ECM colonization (Table 2.1). Canopy gap fraction (GF) interacts with soil water content (SWC) or acorn biomass to affect the proportion of mycorrhized root tips with (A) contact (ECMc) and (B) short and medium ectomycorrhizal (ECMsm) exploration types, per individual $n=129$ (all plants from half of the cages). The three-way interactions present the relationship at three levels of the moderator variable (-1SD standard deviation, mean and +1SD standard deviation), which is SWC in (A) and acorn biomass in (B).

When looking at the relationship between ECM colonization and seedling performance and growth of emerged plants, we found that the proportion of ECMsm colonized root tips was positively correlated with seedling survival and stem height, while the proportion of ECMc was negatively correlated with shoot biomass (Table 2.2).

Table 2.2. Model estimates and significance levels from linear mixed-effect models of ECM root tip colonization. The impact of contact ectomycorrhizal exploration type (ECMc) and short + medium ectomycorrhizal exploration type (ECMsm) on seedling survival, height and aerial biomass. R^2_m represents the variance explained by the fixed factors and R^2_c the variance explained by random and fix factors. *** $P<0.001$, ** $0.001<P<0.01$, * $0.01<P<0.05$, + $0.05<P<0.07$.

Variable	ECMc	ECMsm	R^2_m	R^2_c
Survival emerged plants (SEP)	1.334	3.675*	0.05	0.08
Height survived		4.776*	0.03	0.28
Shoot biomass survived	-0.063*		0.03	0.13

2.5 Discussion

In this study we combined a long term precipitation reduction experiment established for 15 years with an acorn germination experiment to get further insights into the factors

controlling germination, seedling establishment, growth and the links with ectomycorrhizal colonization in holm oak, a tree species structuring Mediterranean landscapes at low elevation in the western part of the Mediterranean basin.

2.5.1 The impact of the precipitation reduction treatment on survival

Edaphic drought due to precipitation reduction is known to prevent or limit the success of *Q. ilex* seedling recruitment at two important stages. First, acorns being desiccation-sensitive seeds, they may lose their ability to germinate if they dehydrate during winter dry spells (Joët et al., 2016). Second, summer drought episodes that are characteristic of the Mediterranean climate, impose severe plant water stress that can be readily lethal to young seedlings (Gómez-Aparicio et al., 2008). In our experiment, the results showed that summer drought was the most important cause of mortality (Figure 2.3), thereby confirming the critical role of water stress in determining the success of seedling establishment. Failure to germinate was the second cause of mortality (Figure 2.3), being most likely not caused by winter seed dehydration since the winter of 2018 was relatively wet (49% of the mean annual precipitation was already fallen from January to March) and we kept the acorns in humid conditions before sowing in order to maximize their chances of germinating. However, in contrast to our first hypothesis, the total seedling survival (S_T) after 11 months was higher in the PR treatment than in the Control with ambient conditions (Figure 2.4B). Additionally, the survival of emerged plants (S_{EP}) was higher for seedlings growing in soil microhabitats with lower soil moisture. These two results indicate that, although summer drought was the main cause of mortality in both treatments, the partial rain exclusion treatment did not further increase seedling mortality. Instead, we found higher mortality in control plots during spring and at the end of the summer drought. Although surprising, negative effects of higher soil moisture conditions on seedling survival have been previously reported (Matías et al., 2012; Urbieto et al., 2008), and were explained by sporadic water logging hampering radicle development and respiration. Waterlogging does not readily occur at our study site because the rocky soil of the experimental site (that includes over 70% calcareous stones), allows for a very fast water infiltration into the deeper soil horizons even during very heavy rainfall events. Therefore, we argue that the higher seedling mortality under the wetter soil conditions of the Control treatment may have been rather related to biotic factors such as soil pathogens. In particular,

root infection by Oomycetes is favoured by high soil moisture needed for sporulation and zoospore dispersion (Garrett et al., 2006). Plantations and natural forests of *Q. ilex* have indeed been severely damaged by the exotic and highly destructive Oomycete root pathogen *Phytophthora cinnamomi* (Brasier et al., 1993; Sanchez et al., 2002), which has been proposed as the main factor for oak decline in Spain and Portugal; and the high mortality of *Q. ilex* seedlings growing in *P. cinnamomi* infested soils has been observed after waterlogging combined with subsequent water deprivation (Corcobado et al., 2014). Relatedly, important root damage was observed at higher levels of soil moisture in *Quercus suber* seedlings growing in soils inoculated with *P. cinnamomi* (Homet et al., 2019). Therefore, one possible explanation that is in line with the mortality patterns observed in Figure 2.3 as well as the higher mortality in the Control is that the wetter soil conditions increased the likelihood of root infection with oomycetes. This presumably started during root system establishment in spring before the soil started drying (Figure 2.3). Thereafter, during the summer drought, a slightly higher soil moisture could have been even more detrimental as it might still allow the maintenance of oomycetes, thereby explaining the higher mortality found in the Control compared to PR. Furthermore, plants infected with oomycetes-damaged root tips could then also be more vulnerable to drought in late summer because they may be less colonized with drought-alleviating root mutualists such as ECM. This emerging conjecture needs, however, to be further tested with specific analyses of the pathogen presence and abundance in our experimental site as well as the presence of lesions on plant tissues.

2.5.2 Effects of microhabitat environmental conditions on survival and growth

Consistent with our second hypothesis, microhabitat conditions were found to be important drivers of early establishment success. Light and soil moisture conditions measured at the microhabitat (cage) level were retained as significant predictors in 7 out of the 8 response variables we assessed (Table 2.1). In contrast, the PR treatment was retained for only 3 out of 8 variables. This clearly indicates the importance of including the actual microhabitat conditions in studies aiming to understand germination and seedling survival. Our results show that light affected positively the total survival of seedlings (from germination to collection of seedlings) but not the survival of the already emerged plants (from stem emergence to collection of seedlings). This indicates that the positive effect of light takes

mainly place in the first stages of life (from germination to stem emergence and shoot development), but once stems emerged and have to deal with the summer drought the effect of higher light exposure can be detrimental as it can increase seedling water stress. These results agree partially with earlier studies (Pérez-Ramos et al., 2013) reporting that seedling germination and emergence increased linearly with light availability, but that seedling survival decreased because the light availability increases the risk of desiccation of seedlings in drier sites (Gómez-Aparicio et al., 2008). Pathogen infection was also presumably responsible for the negative effect of soil moisture on plant aerial biomass, since the infection could reduce growth.

Another finding of this study is that acorn biomass, here interpreted as the initial resources present in the acorns, is one of the most consistent predictors of germination, survival and growth. This is in line with several studies performed with *Quercus* sp. (González-Rodríguez et al., 2011b; Khan and Shankar, 2001; Quero et al., 2007) and confirms earlier evidence that seed size is one of the most important traits influencing the early phases of the plant's life cycle. During the first year of their life, young *Q. ilex* seedlings have been shown to depend more markedly on their seed reserves than on the environmental conditions where they grow (González-Rodríguez et al., 2011). It is likely that once these reserves are consumed, microhabitat conditions will gain in importance during later stages of seedling growth and survival beyond the first year after germination.

2.5.3 Effects of mycorrhiza exploration types on survival

The PR treatment favoured the root colonization of young seedlings with ECM exploration types that have longer mycelia (short and medium exploration types, ECMsm) and decreased the colonization with contact ECM (ECMc, Figure 2.5). This is in line with our third hypothesis stating that seedlings growing in more water limited conditions develop typical drought-adapted ECM morphotypes with differentiated rhizomorphs because these have a better capacity to forage nutrients and water from further distances (Agerer, 2001; di Pietro et al., 2007; Shahin et al., 2013). Apart from their function in increasing nutrient foraging, ECM can also act as biological deterrents to root pathogens as there is evidence that extended extramatrical mycelia and denser mycelia mantles can also act as physical barriers to pathogen infection. In fact, (Blom et al., 2009) found lower abundance of extramatrical hyphae and less dense mantles in *Phytophthora cambivora* infected and declining chestnut stands, compared to

non-infected chestnut stands. Similarly, (Bzdyk et al., 2019) found that declining *Quercus robur* stands were associated with less long-distance and more short-distance exploration types (contact, short and medium) than what they found in healthier *Q. robur* stands. This potential role of ECM species with longer mycelia in reducing pathogen infection could help to understand the positive relation between ECMsm and seedling survival and height found in our experiment (Table 2.2). Furthermore, some ECM species have the ability to decrease the virulence of *Phytophthora* through exudation of antibiotics or terpenes with deleterious effects on pathogens (Marx, 1973). Several studies comparing the declining status of *Q. ilex* stands revealed a negative correlation between ECM abundances and the abundances of pathogens and saprobes (Ruiz Gómez et al., 2019). In the same vein, a decrease in vital root tips (ectomycorrhized with well-developed mantles) combined with an increase of non-vital root tips (non-mycorrhized and deteriorated roots) that are more vulnerable to pathogen infection was found in declined *Q. ilex* stands (Montecchio et al., 2004). At our study site, significant changes in ECM community composition, but not in total species richness, were found in the PR treatment compared to the Control (Richard et al., 2011). It could be hypothesized that the ectomycorrhizal community in the PR treatment is the result of the selective pressure exerted by the long-term reduced water availability that favoured ECM species with abundant/more extramatrical mycelium, which may improve seedling persistence under drought conditions even if they require more carbon investment by the host seedlings. These drought-adapted ECM species may increase seedling survival in PR treatment through higher forage capacity, but also by being physical barriers against root pathogens. However, such potential mechanisms of how different ECM species may affect seedling performance needs more detailed studies, and our data does not allow to infer any causal relationships. The same for the negative correlation between shoot biomass and contact ECM exploration type (Table 2.2), the biological mechanisms involved are not clear and should be investigated in controlled experiments testing the composition and richness of contact morphotypes affecting growth.

In terms of micro-environmental conditions, light availability was found to modulate ECM colonization in interaction with soil moisture or initial seed mass depending on the type of mycorrhiza exploration (Figure 2.6). Light had a negative effect on ECMc in drier microsites, but a positive effect in more humid microsites. This is consistent with the hypothesis that light modulates the soil drying through the evaporative demand, so that being under shadow in

drier soils could be beneficial to maintain soil humidity and thereby increase the chances of being colonised by contact mycorrhiza that are more adapted to more humid soils. Besides, the interactive effect of seed mass and light on ECMsm suggests that even root colonization by mycorrhizae could be affected by the initial acorn resources, which is an aspect that, to the best of our knowledge, has not been reported previously.

2.6 Conclusions

Although summer drought was the main cause of seedling mortality, our study indicates that drier conditions in spring can increase seedling survival, presumably through a synergistic effect of drought adapted ECM species and less favourable conditions for root pathogens infection. However, it is important to put this finding in context as the particularly wet spring and autumn periods in 2018 (with frequent rain events) combined with a typical severe Mediterranean drought period in summer, all of which represent favourable conditions for pathogen infection in *Q. ilex* (Corcobado et al., 2014). Notably, acorn biomass was one of the most consistent variables positively affecting germination, survival and growth, and it even influenced ECM colonization. The regeneration of *Q. ilex* stands could thus be threatened if the more intense and longer summer droughts expected in the near future, reduce the acorn biomass by increasing the water stress experienced by acorn bearing trees (Gavinet et al., 2019; Pérez-Ramos et al., 2010; Sanchez-Humanes and Espelta, 2011).

2.7 Acknowledgements

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2.8 Supporting information

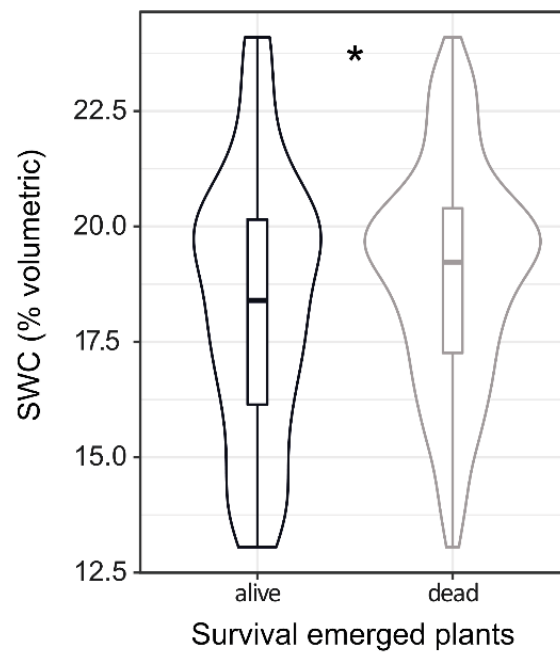
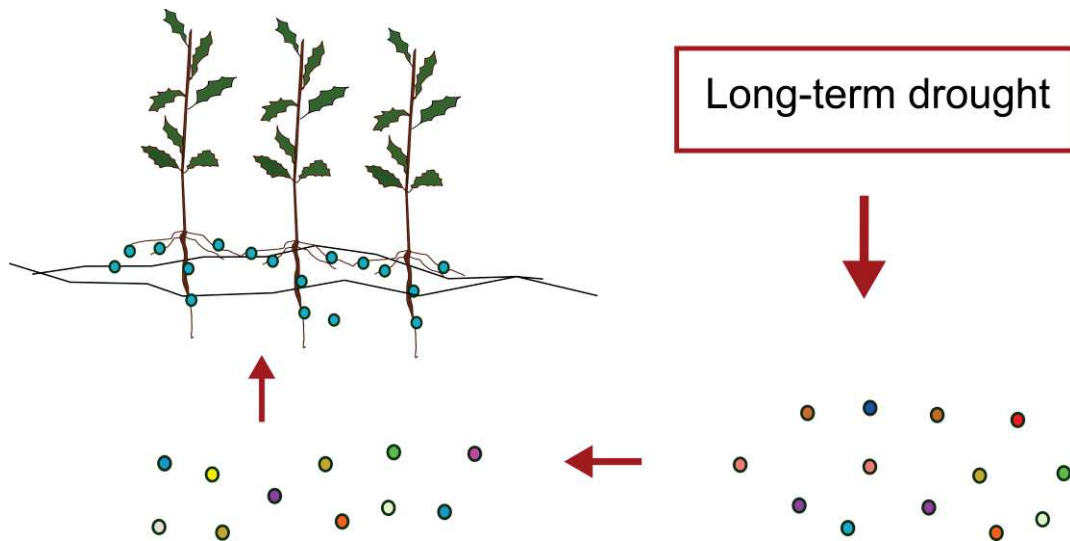


Figure S2.1. The effect of SWC (% volumetric) per cage in survival of emerged plants per individual. $n = 391$.

CHAPTER 2

Long-term drought effects on the fungal community
composition present in the soil and associated to
seedling root tips



Increased drought induce higher ectomycorrhizal diversity and changes in survival patterns of seedlings in *Quercus ilex* L. Mediterranean forests

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3.1 Abstract

The ongoing changes in climate influencing soil biochemical cycles is expected to affect microbial composition and functioning. Understanding how fungal communities are affected by increases of drought severity are essential for plant nutrition and for predict global forests responses to incoming conditions. We metabarcoded the root systems of *Quercus ilex* seedlings and its surrounding soil to investigate (i) the effect of long-term drought on the diversity and the composition of fungal assemblies (ii) the response of *Q. ilex* seedlings to these shifts in ectomycorrhizal (ECM) communities, by evaluating their survival and performance, and (iii) the influence of drought on the typology of common ectomycorrhizal mycelium networks, by assessing the sharing patterns among co-occurring *Q. ilex* seedlings. Increased drought promoted higher fungal diversity in all studied fungal guilds (ectomycorrhizal, saprotroph, and pathogenic) compared to control conditions. The increases on ectomycorrhizal richness associated to *Q. ilex* roots was positive correlated to seedling survival, and this effect was specially found in two genotypes of *Cenococcum geophilum* influencing directly on seedling survival. In addition, the higher ECM richness increased the sharing of fungal species linking co-occurring seedlings. Our findings confirms the remarkable resilience to drought of the

fungal Mediterranean species which constraints in climate may have favoured the emergence of long-term adaptation legacies. The unpredictable and highly environmental fluctuations of the Mediterranean region favoured soil fungal diversity by inducing species turnover and reducing species dominance enhancing diversity and seedling survival.

3.2 Introduction

Ongoing climate change conditions are expected to affect the composition and dynamics of terrestrial ecosystems, through impacted nutrient cycling, carbon dynamics and plant performance (Swaty et al., 2004). In the Mediterranean, global warming induced increased durations and intensity of droughts, with consequences on tree survival (Coma et al., 2009), and modified rates of soil biochemical cycling, especially N and C mineralization (Rodríguez et al., 2019). The changes in nutrients cycling due to reductions in soil moisture are known to affect soil micro-organisms activity, in particular enzyme production by filamentous fungi (Courty et al., 2016; Sardans and Peñuelas, 2005). As a consequence, understanding how soil micro-organisms communities will respond to increased ongoing climate change, and especially to drought, is crucial to understand the ability of forest ecosystem functions to be maintained in the future (Bardgett et al., 2008).

Soil fungi play a key role in terrestrial ecosystem functioning through the decomposition of dead organic matter by fungal saprotrophs, and by maintaining the hydro-mineral equilibrium of plants by fungal mutualists forming mycorrhizae with most autotrophs (Smith and Read, 2009). In particular, mycorrhizal fungi are involved in the resistance of forest to environmental stresses, including summer drought, through established belowground networks of hyphae which translocate water to their host (Barea et al., 2011; Lehto and Zwiazek, 2011; Querejeta et al., 2003). In this study we aim at investigating the effects of increased drought on fungal communities with a special focus on ectomycorrhizal (ECM) fungi due to their dominance on roots of trees in dry and poor-nutrient Mediterranean forests (Drigo et al., 2008; Richard et al., 2005).

Because ECM fungal species vary in their tolerance to soil moisture conditions (Lehto and Zwiazek, 2011; Shi et al., 2002), increased drought episodes lead to shifts in the composition of ECM community, towards species that perform better under drought (Gehring et al., 2014; Richard et al., 2011). The high diversity of ECM fungi show a wide variety of adaptations to

xeric conditions, including i) morphological/anatomical structures such as developed extramatricial mycelia that enhance their foraging performance (Agerer, 2001; Bakker et al., 2006; García de Jalón et al., 2020; Nickel et al., 2018), and ii) physiological traits, such as the ability to accumulate sugar alcohols (arabitol, mannitol) in cells to resist osmotic stress (Shi et al., 2002), or to shape extracellular enzymatic activities. Among drought-tolerant ECM species, the Ascomycete *Cenococcum geophilum* is the most emblematic, but also the most abundant one in Mediterranean soils (de Román and de Miguel, 2005; Richard et al., 2004). *C. geophilum* is known to resist xeric conditions and desiccation through i) the production of dormant vegetative propagules called sclerotia, and ii) thick walled hyphae which form a rigid ECM mantle (Agerer, 2001). From the community perspective, many studies revealed changes in ECM diversity and shifts in ECM community composition in response to drought in both temperate (Herzog et al., 2013) and Mediterranean forests (Cavender-Bares et al., 2009; Richard et al., 2005), and in heathlands (Toberman et al., 2008). In addition, the observed forest die-off following drought stress arising in the Mediterranean is known to alter the soil stoichiometric balances (García-Angulo et al., 2020). In turn, these losses in nutrients and the accumulation of mobile N and P compounds consecutive to Mediterranean forest die-off, induced a reduction of fungal richness and changes in the composition of rhizospheric fungal communities (Hopkins et al., 2018). In these ecosystems, the specific response of ECM fungi, which have the ability to mobilize water from host through hydraulic-lift to attenuate soil water deficit (Querejeta et al., 2003) is still largely unknown.

Quercus ilex dominates the low-elevation landscapes in the western part of the Mediterranean basin (Quezel and Médail, 2003). This ECM tree species hosts highly diversified fungal communities (Richard et al., 2005; Taudiere et al., 2015). During secondary succession after clear-cut and/or land abandonment, *Q. ilex* suffers drastic recruitment limitation, mainly due to high seedling mortality during the first summer drought (Bran et al., 1990; Gómez-Aparicio et al., 2004; Panaiotis et al., 1997; Richard et al., 2009). From an ECM host perspective, plant individual may respond to drought by increasing the allocation of carbohydrates to roots, in order to maintain the activity of its ECM symbionts (Williams and Vries, 2020): thus, in *Quercus ilex* seedlings submitted to drought, the amount of exudates per unit root area increased up to +21% as compared to the seedlings in control (Preece et al., 2018). Under this mechanism, maintaining mycorrhizal activity in plants during drought may be essential to

beneficiate the effects of increasing water uptake, especially for young seedlings with swallow root systems.

In Mediterranean ecosystems, the ECM symbiosis is the basis for underground links that i) mediate plant – plant interaction among co-occurring individuals (Selosse et al., 2006; Simard and Durall, 2004) and ii) drive the kinetics and the direction of secondary successions (Taudiere et al., 2015). From a mechanistic perspective, the integration of new emerging seedlings to belowground common mycelial networks (CMNs) has been shown to be involved in seedling survival (Dickie et al., 2002), by allowing transfers of resources through shared fungal symbionts among established and emerging plants (Nara, 2006). While CMNs are considered as major drivers of terrestrial ecosystems, there is little knowledge about their response to climate change.

The Puéchabon state forest has been used since 2003 as a permanent study site to investigate the long-term effects of increased drought in the Mediterranean. In this pure *Q. ilex* ecosystem, an *in situ* experiment simulates a decrease of 29% rainfall (Limousin et al., 2008). A previous *Q. ilex* acorn germination experiment, used as bait plants for ECM fungi in the present study, showed that survival patterns was changed under drought, with an significant increase of 24% after the first summer drought (García de Jalón et al., 2020). In this study, we metabarcoded the root systems of these seedlings and the surrounding soil to investigate (i) the effect of long-term drought on the diversity and the composition of fungal assemblies in this ecosystem, with particular attention paid to the ECM guild, on both established trees and one-year old seedlings, (ii) the response of *Quercus ilex* seedlings to these shifts in ECM communities, by evaluating their survival and performance, and (iii) the influence of drought on the typology of CMNs, by assessing the sharing patterns among co-occurring *Q. ilex* seedlings. We specifically addressed three hypothesis:

- **H1.:** Increasing drought reduces the diversity of fungal communities, particularly of ECM communities of established (soil, including tree roots) and emerging (seedling roots) plant individuals, and induces changes in the community composition towards ECM fungal species more adapted to drought.
- **H2.:** These changes in diversity and composition of ECM communities influence positively seedling survival. Specially, the abundance of the dominant drought-tolerant symbiont *Cenococcum geophilum* positively influences seedling survival.

- **H3.:** Sharing patterns of ECM species among co-occurring seedlings is affected by drought, with sharing rate being positively correlated with seedling survival.

3.3 Materials and Methods

3.3.1 Experimental site

The study site is located 35 km northwest of Montpellier (southern France) in the Puéchabon State Forest, on a flat plateau (43°44'29" N, 3°35'45" E, elevation 270m). This forest has been managed as a monospecific coppice for centuries. In the experimental plots, the last clear cut was performed in 1942. At the experimental plot, the current vegetation is largely dominated by the shade-tolerant evergreen oak *Q. ilex*, which exclusively constitutes a top canopy height of about 5.5 m, with a density of 6000 stems.ha⁻¹. The understorey is composed of a diffuse shrubby layer with the evergreen species, *Buxus sempervirens*, *Phyllirea latifolia*, *Pistacia terebinthus* and *Juniperus oxycedrus*, with a percent cover of approximately 25% at 2 m.

The climate is of Mediterranean-type, with 80% of total annual precipitation occurring between September and April. The mean annual precipitation is 920 mm with a range of 550–1549 mm (1984–2017). Mean annual temperature is 13.3°C (on-site meteorological station, 1984–2017), the coldest month being January (5.5°C) and the hottest July (22.9°C). The soil is extremely rocky from hard Jurassic limestone origin. 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).

3.3.2 Rainfall exclusion

In March 2003, a long-term 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. Each replication was composed of one throughfall exclusion treatment (henceforth, precipitation reduction, PR) 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 to partially intercept rainfall before it reaches the soil surface. 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 plots, identical gutters are set up upside down so that the albedo and the understorey microclimate are as close as possible in the two treatments. However, gutters in the PR treatment are slightly tilted down in order to let the water drop out of the plots, different from the Controls where gutters are horizontally placed.

3.3.3 Acorn collection, sowing and seedling performance

Seed germination in *Q. ilex* mostly occurs in early winter. The development of the germinating seedling starts with the allocation of most biomass to the edification of the taproot, and continues with the emission of secondary roots, before the first associations with below-ground microbial communities (ECMs and/or pathogens) takes place in early summer (Richard et al., 2009).

Acorns from 10 different maternal trees were collected in November 2017 at Puéchabon site, in the surrounding of the experimental plots. All acorns were submitted to a flotation test in order to discard the ones aborted or damaged by insects or fungi. All the viable acorns were weighted, numbered with a marker pen for identification, and kept in a refrigerator at 4°C in vermiculite substrate humidified with water for 3 weeks. Sowing was done in December 2017, placing acorns 1 cm deep into the soil of the experimental plots. In order to prevent acorn predation by rodents and wild boars, sowed acorns were enclosed in wire cages of 40 × 30 × 30 cm. One acorn from each maternal tree was sowed in each cage at a distance of 5 cm from each other, thus totalling 10 acorns per cage. In each experimental plot, 10 cages were installed at a minimum distance of 2 m between each other, to limit pseudo-replication. In total, 60 cages were installed in the field, including 30 cages per treatment (20 per block). For each seedling, survival of both aerial and underground parts was estimated by visual examination under dissecting microscope, and stem height was measured. In the laboratory, aerial and belowground seedling parts were separated, the aerial parts were oven-dried at 60° C until constant mass for biomass measurements, and belowground parts were used for ECM root tips assessments and molecular analyses.

3.3.4 Soil and root tips sampling for fungal community analysis

In December 2017 (after sowing), soil sampling was performed as follows: eighty four 5 x 5 x 3 cm deep soil cores were collected, including i) one core at the centre of each of the 60 cages and ii) four additional cores located at the corners of one cage randomly designated in each of the six plots, to assess the intra-cage variability of fungal communities. In three cages of each plot, the organo-mineral (3-6 cm) was also collected, totalling 18 samples (6 samples per block). Soil samples were brought to the laboratory, and oven dried at 45°C for 3 days in sealed coffee filters. Dried samples were then roughly sieved to remove roots and rocks by performing holes at the bottom of sealed filters using a sterilized sharp metal pointer, to collect fine particles within another filter placed underneath. Sieved soil was transferred to 15mL tubes and stored at -20°C for DNA extraction.

In November 2018, emerged seedlings (alive and dead) from half of the cages were carefully dug up by hand with the aim to collect the largest part of the root systems. Due to the very rocky nature of the soil, it was not possible to harvest roots that were growing deeper than 15 cm. As it was not possible to extract intact root systems, the root biomass variable was omitted from the ECM analysis. Seedlings were brought to the lab and kept in the fridge. Before handling root tips, each collected seedling was kept in distilled water in trays overnight in order to remove adhering organic and mineral soil particles from the roots. Each extracted root system was analysed under a dissecting microscope, and ECM root tips were counted, assigned to morphotypes and related explorations types, exhaustively picked, placed in Eppendorf tubes and stored at -20°C until DNA extraction. Because most seedling death events occurred less than two months before picking (*i.e.* at the end of summer drought), the assessment of the ECM mycorrhization rate was feasible irrespective of the plant status (alive *vs.* dead) and then quantified for all collected seedlings. For dead seedlings, this measurement corresponds to ECM mycorrhization rate at death time and constitutes a valuable data about early colonization events since a majority of *Q. ilex* seedlings die during the first summer drought (Gómez-Aparicio et al., 2004))

3.3.5 Molecular identification and bio informatics analysis

Fungal DNA from soil samples and root tips was analysed as in (Taudière et al., 2018). Briefly, fungal DNA was extracted using FastDNA SPIN kit for soil (MP Biomedicals. Irvine. CA). The internal transcribed spacer (ITS) region of the extracted rDNA was amplified by

polymerase chain reaction (PCR) using the primer pair ITS3-ITS4 (ITS3: GC ATC GAT GAA GAA CGC AGC; ITS4: TCC TCC GCT TAT TGA TAT GC). Before sequencing, amplicons were tested on agarose gel 2%, and sent to “Grand plateau technique regional de génotypage” (GPTR) GenSeq UMR ISEM (Montpellier) for analysis with Illumina MiSeq.

All bioinformatic and ecological subsequent analysis were run under R 3.6.3 (R Core Team, 2013) mostly using dada2 (Callahan et al., 2016) and phyloseq packages (McMurdie and Holmes, 2013). The detailed code of the bioinformatic pipeline is available in <https://github.com/adrientaudiere/MiscMetabar/tree/master/R>. Briefly, we merged paired-end reads, discarded low-quality reads (parameter *--average_qual* set to 20) and trimmed primers using fastp (Chen et al., 2018). Following recommendation from (Pauvert et al., 2019), we used the dada2 R package to compute an amplicon sequence variant (ASV) table, as a complement to operational taxonomic unit (OTU) table. ASV table is a higher-resolution analogue of the widely used OTU table, and records the number of times each exact amplicon sequence variant was observed in each sample (Callahan et al., 2016). Chimeras were removed using the *removeBimeraDenovo* function from dada2 package and filtered out ASVs with less than 250 bp. We then assigned taxonomy comparing ASVs sequences to Unite database (sh release dynamic 04.02.2020; Nilsson et al., 2019) thanks to the *assignTaxonomy* function from dada2. Finally, Funguild software (v1.1; Nguyen et al., 2016) was used to attribute an ecological trophic strategy (*i.e.* mycorrhizal *vs.* saprotroph *vs.* parasite) to ASVs/OTUs taxonomically assigned at the genus level. Especially, guild information allows computation of separate analysis for ectomycorrhizal ASVs/OTUs only.

We build two different ASV/OTU tables for the colonization of ectomycorrhiza in the roots; the rhizosphere (remnant mycelia adhering the ECM tips plus the root tips) and the root tips alone. For each seedling, a rank – abundance distribution curve was plotted (using number of reads per ASVs/OTUs as a proxy). On all distributions, a visual analysis was systematically performed to detect a clear slope discontinuity between abundant ASVs/OTUs (assigned to ECM root tips) and rare ones (assigned to remnant mycelia still adhering the ECM tips after root cleaning). The database rhizosphere was the sum of the root tips and the remnant mycelia. For all distributions, the result of this selection was compared to a contingency table assembled at picking step and detailing all picked ECM tips with precision of their morphotype (García de Jalón et al., 2020).

3.3.6 Bipartite network analysis

The analysis of the topology of bipartite networks linking *Q. ilex* seedlings and ECM fungi was performed following methods developed in (Taudiere et al., 2015). At each cage, we built a binary matrix of association between all alive *Q. ilex* seedlings at harvesting date and all ECM fungal ASVs/OTUs identified on their root system, and filled at position (i, j) with the numbers of reads n_{ij} for each pair of seedling (i) and ECM fungal ASV/OTU (j).

For each seedling in each cage, we calculated the degree k_p as the number of ECM fungal ASVs/OTUs associated with a given seedling individual. For each cage, we defined (k_{cage}) as the total number of ECM fungal ASVs/OTUs hosted by all seedlings, and (k_{mean}) as the averaged degree of seedlings within a cage. Under facilitation process, we postulated that the higher the values of k_{cage} and k_{mean} , the higher the survival rate of *Q. ilex* seedlings in the cage. In each cage, we calculated the projected degree of *Q. ilex* seedlings, l_{cage} as the number of seedling individuals that are linked through shared fungal ASVs/OTUs. This metric was used as a proxy to evaluate below-ground interaction among co-occurring individuals through CMN inter-connection. Under facilitation process, we postulated that the higher the values of l_{cage} , the higher the survival rate of *Q. ilex* seedlings in the cage

3.3.7 Statistical analysis

All statistical analyses were performed with R software version R 3.4.1 (R Core Team, 2013). The effect of PR treatment on Hill numbers diversity of the fungal community was analysed with linear mixed-effects models with “PR treatment” as fixed factor and the “Block” as random factor using “nlme” package version 3.1 (Pinheiro et al., 2017). The values of Hill numbers were averaged at the cage level, for both soil fungal diversity (i.e. averaging values of the 4 samples when 4 cores were collected at a given cage) and seedling ECM diversity (i.e. averaging values of all seedlings per cage).

The effect of PR treatment on the structure of fungal communities was tested using a Chi-squared test. The abundances (using the number of reads as a proxy) of ASVs were classified by ranges of abundances in a series 2^{n+1} . The effect of PR treatment on the richness and abundance of fungal families was tested using a Wilcoxon rank sum test. The effect of PR treatment on the abundance of ASVs (number of reads per ASV) was tested using a log Fold₂ Change ratio calculated with “DESEQ2” package version 1.24. (Love et al., 2019).

The effect of fungal diversity on seedling survival per cage was assessed with linear mixed models and with the regression coefficient R^2 of the relationship. The choice to compute averaged values per cage and not the sum per cage was made to limit the effect of sampling size induced by unequal numbers of seedlings depending of sampled cage. In addition, we analysed the effect of richness (Hill $q=0$) per seedling on the survival and growth (height, aerial biomass) using linear mixed models with Hill $q=0$ as fixed factor and “Block” as random effect using “nlme” package version 3.1 (Pinheiro et al., 2017). The effect of PR treatment on the bipartial network analysis was also done using mixed linear models with the “sharing of ASVs” as fixed factor and “Block” as random effect.

3.4 Results

3.4.1 Description of the three data sets

The metabarcoding of the 84 soil samples collected in December 2017 generated 257 successfully identified fungal ECM amplicon sequencing variants (ASVs), 469 fungal saprotrophs ASVs and 136 fungal pathogen ASVs (Table 3.1). For ECM guild, 23.47 ASV and 597 reads were identified in average per cage in the 0-3 cm depth of soil, whereas in the 3-6 cm soil depth, 39% more of ECM ASV per cage were found compared to the 0-3 cm soil depth with 37.82 ASV and 4912 reads in average (Table 3.1).

The analysis of ECM root tips from 155 seedlings collected one year after the soil samples collection (December 2018), provided 741 ASVs (427 OTUs) in the rhizosphere (+65% more ASV compared to the soil 0-3 cm), and 334 ASVs forming root tips (+23% more ASVs compared to the soil 0-3cm). At the cage level, the analysis provided in average 82.07 ECM ASVs (49.26 OTUs) in the rhizosphere, for 6579 reads (6579 OTUs reads), and 7.32 ECM ASVs forming root tips, for 5635 reads.

Table 3.1. Molecular fungal diversity detected in soil samples at depth 0-3 and 3-6 cm deep, and on *Quercus ilex* seedlings forming ECM root tips, and ECM rhizospheric mycelia. Values in brackets for rhizosphere are in OTUs for rhizosphere.

Data set	Guild	#samples	#ASV (mean/cage)	#reads (mean/cage)	Date of collection
Soil 0-3 cm	ECM	102	23.47	597.6	December 2017
	Saprotroph	102	47.11	1502.9	
	Pathogen	102	15.01	302.6	
Soil 3-6 cm	ECM	17	37.82	4912	December 2017
	Saprotroph	17	61.76	3300	
	Pathogen	17	16.35	687.2	

Roots	Rhizosphere	155	82.07 (OTU 49.26)	6579 (OTU 6583)	December 2018
	Root tips	155	7.32	5635	

3.4.2 Effect of drought on fungal communities

3.4.2.1 Distribution of abundances

For all guilds, the distribution of ASV abundances was not significantly affected by precipitation reduction in the first three centimeters of soil (Figure 3.1), and on *Q. ilex* seedling roots (Chi2-test not significant).

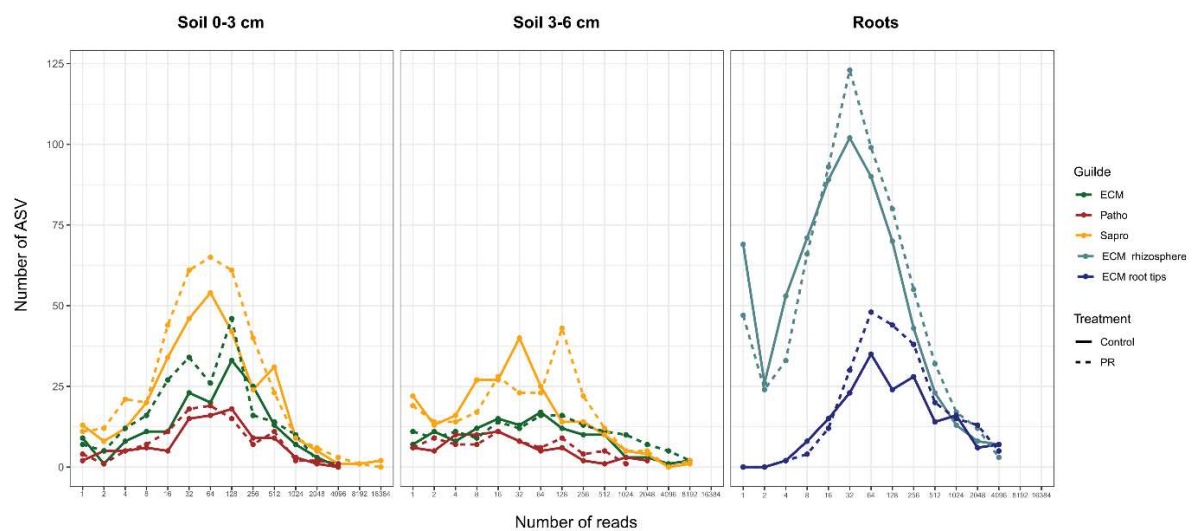


Figure 3.1. Abundance of individual ASV plotted against rank order (number of reads) per guild used in the study. The effect of drought tested with Chi2 test was not significant for any fungal guild.

The abundances of all species together (number of reads of each guild per cage) were not significantly affected by drought in the soil for any fungal guilds (Table S3.1), excepting from the ectomycorrhizal ASV abundance associated to roots that was significantly increased by drought 28% in the rhizosphere ($P=0.07$; Wilcoxon-test) and by 19% in the roots ($P=0.05$; Wilcoxon-test).

3.4.2.2 α -Diversity

The species richness of fungal communities associated to roots of *Q.ilex* seedlings and present in soil either adhering to ECM root tips (rhizospheric ECM diversity) or located in the surrounding fraction of soil was positively affected by drought (Table 3.2; Figure 23.). First, ECM richness (Hill $q=0$) was increased by 21% and 34% in PR treatment plots as compared to control plots at 0-3 cm and 3-6 cm soil depths, respectively ($P=0.08$ and 0.04). For saprotrophs, Hill $q=0$ was increased by 25% and 15% at both soil depths respectively ($P=0.02$ and 0.43 NS),

and for pathogens by 28% and 15% at both soil depths respectively (Figure 2; $P=0.006$ and 0.55 NS). On Seedling roots, drought increased ectomycorrhizal ASV richness by 16% in the rhizosphere and by 8% established ECM root tips ($P=0.04$ and 0.14 NS) (Table 3.2; Figure 23.). The OTU analysis for the rhizosphere revealed the same tendance with a significant increase of ectomycorrhizal OTUs richness of 15% ($P=0.04$; Figure S3.1 left).

Table 3.2. Effect of PR treatment on local diversity of fungal communities, evaluated by Hill numbers calculated for 0-3 cm and 3-6 cm of soil depths and for the ECM rhizosphere and ECM root tips of *Quercus ilex* seedlings. Estimates of the mixed linear models, with P -values* for $0.01 < P < 0.05$ and + for $P < 0.06$.

	Soil 0-3 cm n=60			Soil 3-6 cm n=17			Root tips n=30	
	ECM	Sapro.	Patho.	ECM	Sapro.	Patho.	ECM rhizosphere	ECM root tips
Hill $q=0$	5.47+	13.26*	4.75**	13.97*	ns	ns	0.88*	ns
Hill $q=1$	ns	6.70*	3.38**	ns	ns	ns	ns	ns
Hill $q=2$	ns	4.21*	2.37**	ns	ns	ns	ns	ns

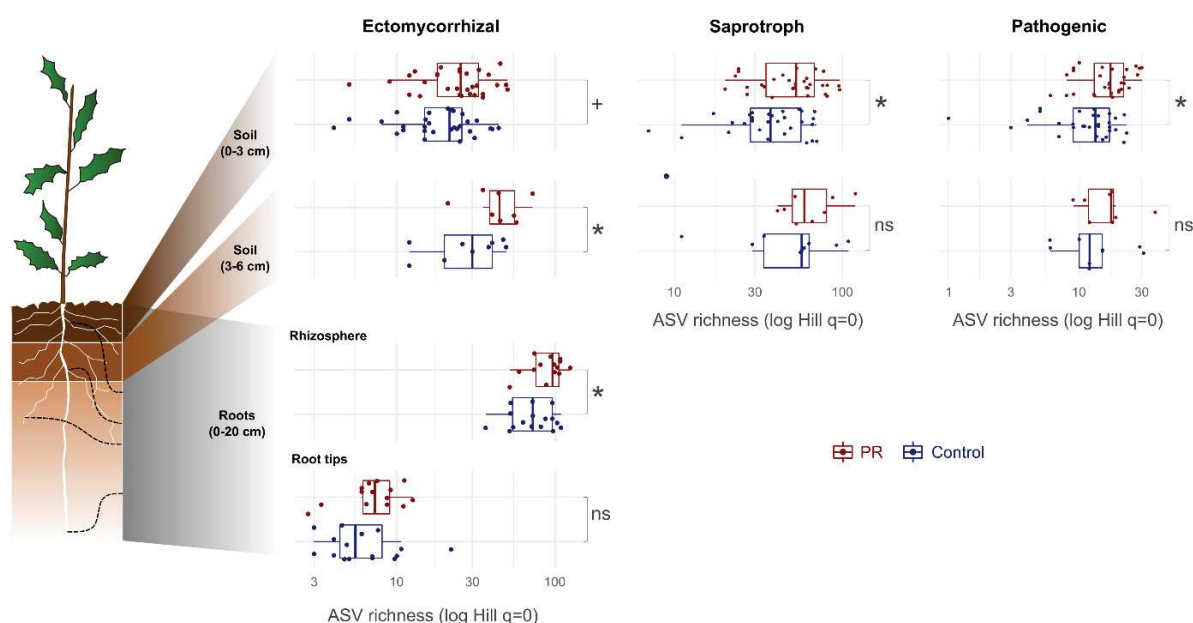


Figure 3.2. Effect of PR treatment on the richness of fungal communities in *Quercus ilex* forest soil and on root tips of *Quercus ilex* seedlings. PR = forest plots with rainfall reduced by 29% , Control: forest plots without rainfall reduction

3.4.2.3 β -Diversity

Permanova tests confirmed significant different community compositions depending on treatment (precipitation reduction vs. control) and on soil layer (0-3c m vs 3-6 cm depth) for ECM, saprotroph and pathogenic fungi (Table 3.3).

Table 3.3. Effect of PR treatment and soil layer on the composition of communities of ectomycorrhizal, saprotroph and pathogenic fungi in *Quercus ilex* soils, and on the diversity of ectomycorrhizal fungi established on *Q. ilex* seedling roots or present in the rhizosphere. In bold significant P-values.

	ECM		Pathogen		Saprotroph		Rhizosphere		Root tips	
	R ²	P	R ²	P	R ²	P	R ²	P	R ²	P
Treatment	0,014	0,025	0,011	0,166	0,013	0,021	0,048	0,03	0,045	0,06
Soil layer	0,030	0,001	0,029	0,001	0,031	0,001	-	-	-	-
Treat:Layer	0,010	0,212	0,006	0,963	0,008	0,724	-	-	-	-

3.4.2.4 Composition

Precipitation reduction increased significantly the species richness (Figure 3.3) and the abundance (Figure S3.2) of some fungal families- For soil ECM fungi, *Gloniaceaea* (+38%) and *Boletaceae* (+100%) in the soil depth 0-3cm and *Tricholomataceae* (+41%), *Bankeraceae* (+64%), and *Clavulinaceae* (+100%) families were significantly richer in PR treatment plots as compared to control plots in 3-6 cm soil depth (Figure 3.3). On *Q. ilex* seedling roots, ASV richness significantly increased for *Inocybaceae* (+67%). In seedling rhizosphere, the number of ASVs in the family *Russulaceae* (+35%) was significantly increased with significant higher richness in the PR treatment compared to the control. The guild of saprotroph fungi showed a marked effect of drought on ASV richness: *Thelephoraceae* (+36%), *Pseudeurotiaceae* (+36%), *Lasiosphaeriaceae* (+51%), *Cephalothecaceae* (+45%), *Chaetomiaceae* (+36%), *Umbelopsidaceae* (+77%), *Irpicaceae* (+87%), in the soil depth 0-3cm and *Lophiostomataceae* (+87%), *Sporomiaceae* (+62%) in the soil depth 3-6 cm. Four families of fungal pathogens were significantly richer in PR treatment plots as compared to control plots, including *Cordycipitaceae* (+42%), *Amphisphaeriaceae* (+29%) and *Clavicipitaceae* (+41%) in the soil depth 0-3cm and *Clavicipitaceae* (+50%) in the soil depth of 3-6 cm.

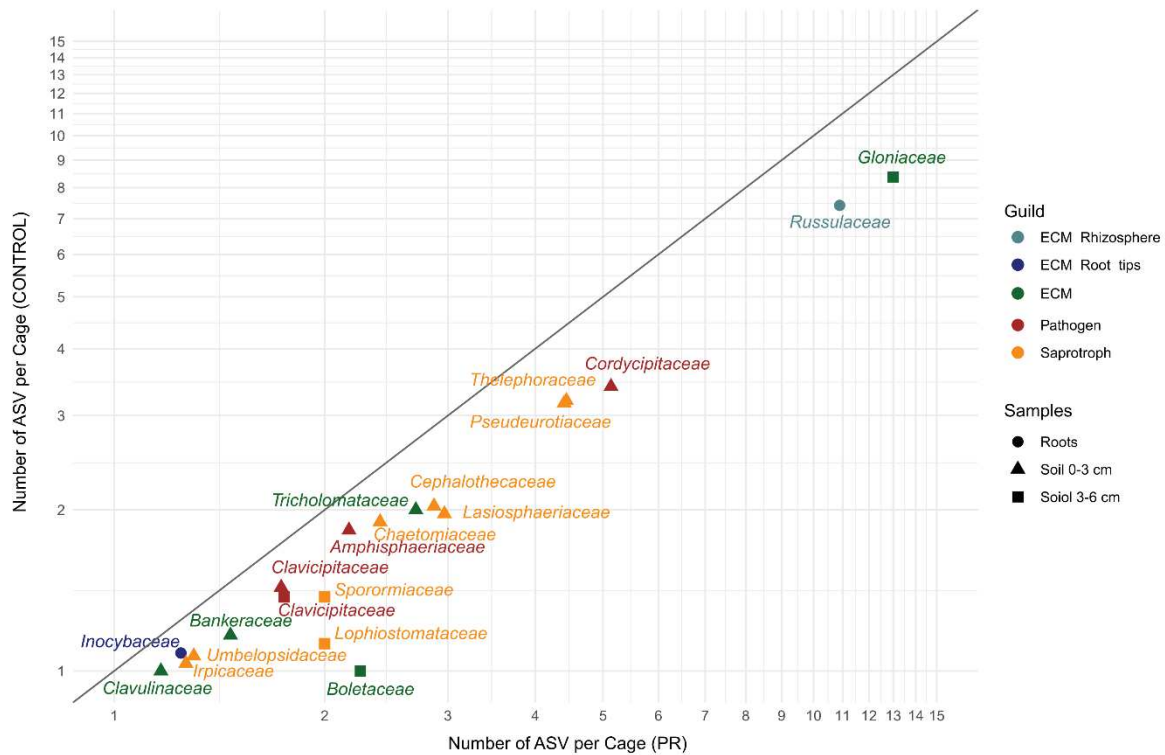


Figure 3.3. Fungal families with significant difference of number of ASVs in soil and on roots of *Q. ilex* seedlings, in response to PR treatment. Values are at the cage level. Different colours indicatedifferent fungal guilds: blue: ectomycorrhizal, red: pathogens, orange: saprotrophs.

At the species molecular variant level, the log fold change, which expresses the level of change between the abundance of ASVs in the PR treatment respective to the control, revealed significant difference in abundance between treatments for i) fifteen ECM ASVs presents to the rhizosphere, ii) 3 ECM ASVs associated to seedling root tips, and iii) nine ECM ASVs in the soil layer between 3 and 6cm deep (Figure 3.4). In the rhizosphere, there were eleven ASVs significantly more abundant in the PR treatment relative to control: three ASVs taxonomically assigned to *Russula nympharum*, between five and seven times more abundant in the PR treatment, one taxonomically unassigned ASV among the *Russulaceae*, two ASVs taxonomically assigned to *Inocybe griseolilacina*, one ASV taxonomically assigned *Tuber rufum*, five times more abundant in the PR treatment, two taxonomically unassigned ASVs among the *Thelephoraceae* , four times more abundant in the PR treatment), and two ASVs taxonomically assigned to *Cenococcum geophilum*, 2.5 more abundant in the PR). In contrast, there were four ASVs more abundant in the Control respective to the PR: one ASV taxonomically assigned to *Tuber nitidum*, ten times more abundant in the Control, one ASV taxonomically assigned to *Russula grisea*, five times more abundant in Control, one ASV

taxonomically assigned to *Inocybe rimosa*, three times more abundant in Control), and one ASV taxonomically assigned to *Cenococcum geophilum*, two times more abundant in Control).

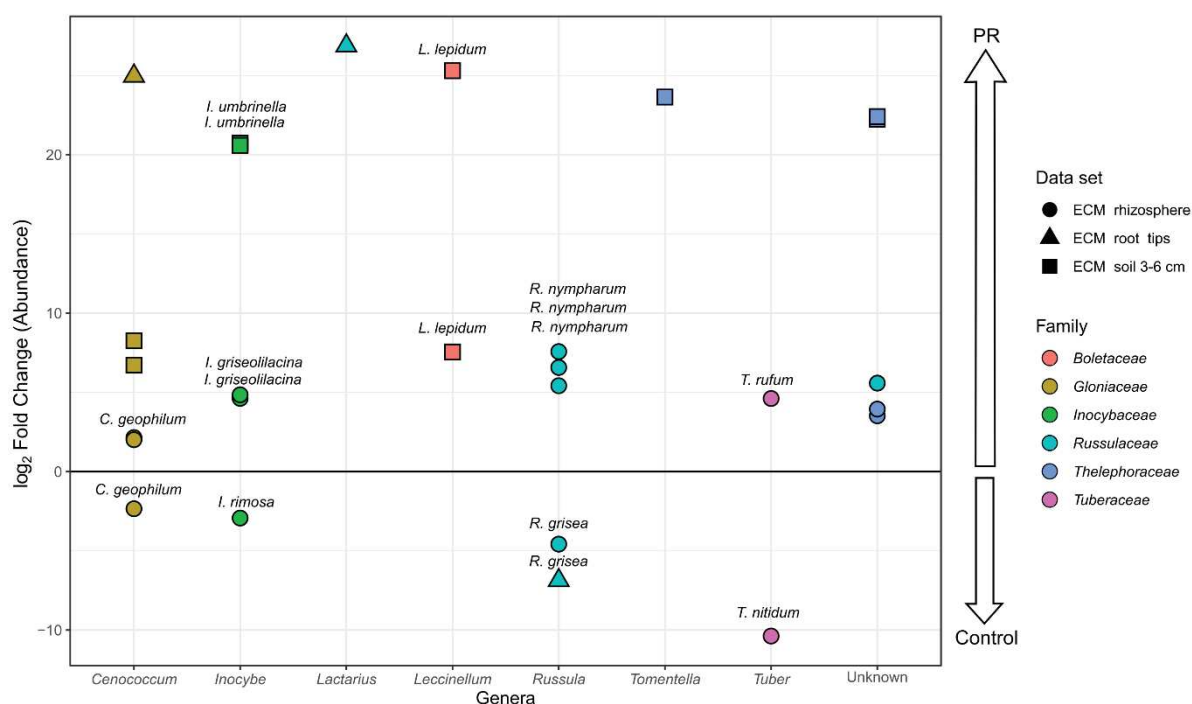


Figure 3.4. Ectomycorrhizal ASVs with significant changes in abundances between treatments expressed by log Fold Change ratio, with indication of their taxonomic assignment. .

3.4.3 Relationships between fungal diversity and survival of *Q. ilex* seedlings

At the cage level, the survival rate of eleven months old *Q. ilex* seedlings was positively correlated with the averaged richness per cage (Hill $q=0$) of both ECM fungi established on root tips and ECM mycelia and spores present in the rhizosphere (Figure 3.5 B,C for ASV; Figure S3.1 right for OTUs). Contrastingly, there was no significant correlation between survival rate and the ECM richness in soil in each of the two analyzed depths (Figure 3.5 A). In contrast, the diversity of ECM community (Hill $q=1$, $q=2$), and the richness and diversity of saprotroph and pathogenic fungi were not significantly correlated with seedling survival (data not shown).

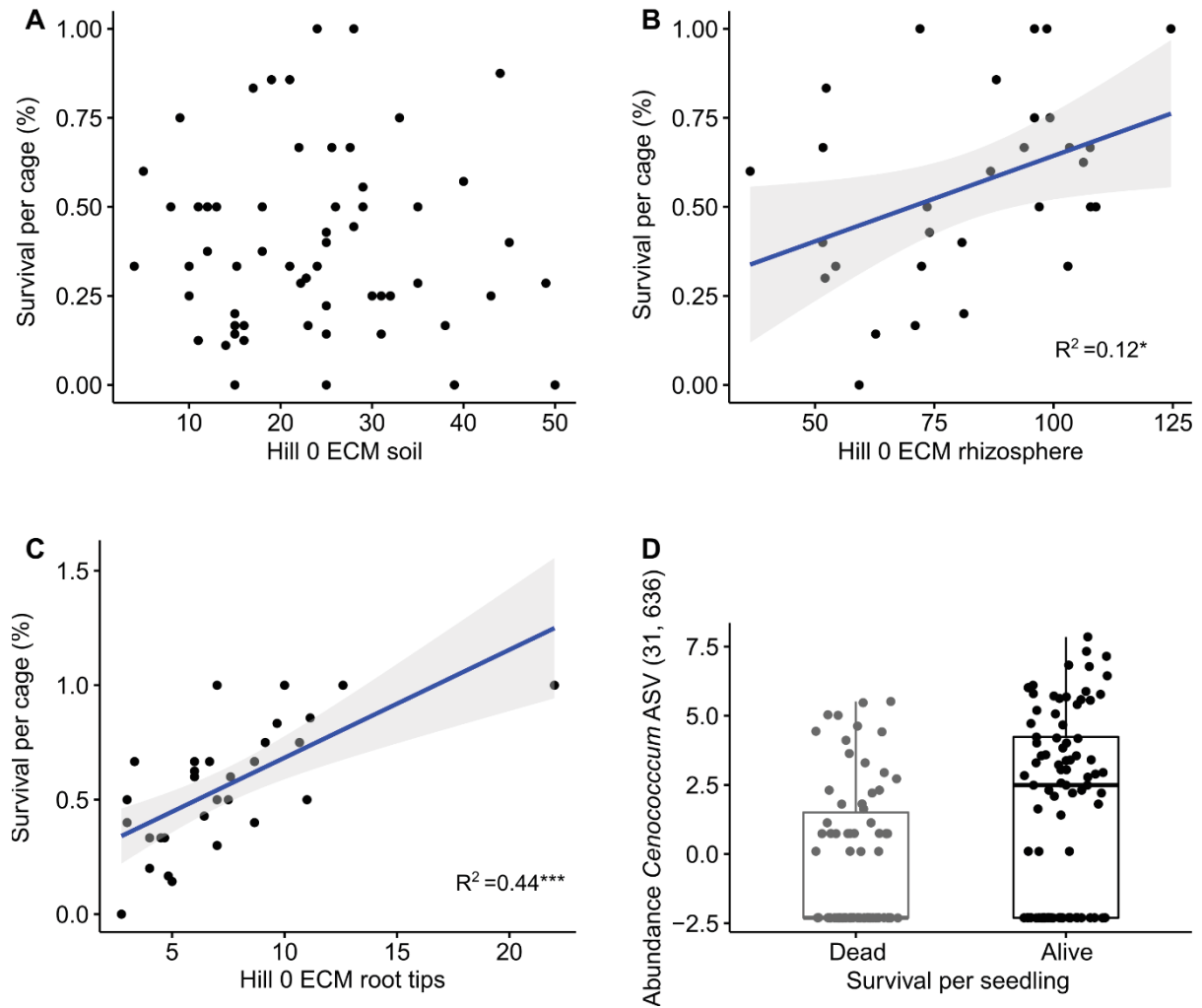


Figure 3.5. Relationships between seedling survival and the richness of ECM community (A) present in the soil, (B) detected in the rhizosphere of *Q.ilex* seedlings, and (C) established on the root tips of *Q.ilex* seedlings. Abundance of (D) two dominant *Cenococcum geophilum* ASVs showing contrasted patterns between PR plots and control plots (P -value=0.007). Y-axis is in logarithmic scale. X-axis is a binary variable of survival defined by 1 for alive seedlings and 0 for dead ones. In A, B, C, values are mean per cage, whereas in D values are per seedling. R^2 Regression coefficient, * for $0.01 < P$ -value < 0.05 and *** for $P < 0.001$

In order to explore the determinants of the increased diversity measured in the PR treatment on the seedling survival, the eleven ASVs significantly more abundant in drought plots compared to control plots (Figure 3.4) were tested for their relationship with seedling survival (since seedling survival was higher in PR treatment than in control; García de Jalón et al., 2020). Among them, only ASV636 and ASV31, which were taxonomically assigned to *Cenococcum geophilum* species and *Cenococcum* genera respectively revealed a positive correlation with seedling survival (Figure 3.5 D, $P=0.007$). Similar to the effects of species richness on growth, height and aerial biomass were no correlated to any of the ASV more present on the PR treatment.

The effect of the ectomycorrhizal richness (Hill $q=0$) associated to root systems in the performance of seedlings was also evaluated per seedling and it was found a positive effect of richness with seedling survival (as it was found averaging per cage), but not with seedling growth (height and aerial biomass) (Table 3.4).

Table 3.4. Effect of ECM richness, evaluated using Hill ($q=0$), on the survival and growth of *Quercus ilex* seedlings. Values are per seedling $n=155$. The output of the generalised linear model is showed.

	Predictor	Hill $q=0$	Std.Error	t/z	P-value
Rhizosphere	Survival	0.0091	0,0043	2,102	0,0356*
	Height	0.0057	0.0075	0.763	0.447
	Shoot biomass	2.95 E-5	2.27E -4	0.13	0.897
Root tips	Survival	0.003	0.001	2.49	0.01*
	Height	4,905E-05	0,000215	0,22794	0,8201
	Shoot biomass	0,006945	0,00853	0,81421	0,4171

3.4.4 Effect of drought on the ECM networks

The cage degree (*i.e.*, the total number of ASVs per cage) varied between 4 and 35 (mean 14.4) in cages positioned in control plots, between 8 and 59 (mean 25) in cages positioned and drought plots (Table S3.2), and was significantly higher in drought than in control ($P=0,007$ by mixed linear model). Similarly, the averaged degree of seedlings (*i.e.*, the total number of ASVs per seedling) ranged from 4 to 22 (mean 8.43), and from 4 to 14.5 (mean 9.84) in cages positioned in control and drought plots, respectively (Table S3.2), with no significant difference between treatments. The sharing of ECM fungi between pairs of co-occurring seedlings (the total number of ASVs shared by two seedlings) averaged 11.09% and 16.61% of ECM ASVs in Control and PR respectively (accounting for 1.63 and 4.04 ASVs in average in Control and PR respectively; Figure 3.6 left), (Table S3.2). This difference was not significant (Figure 3.6 left).

In control plots, the survival rate of seedlings per cage was significantly and positively correlated with sharing between pairs of co-occurring seedlings (Figure 3.6 right; Table 3.5). The relationship was not significant in cages installed in drought plots. The precedent analysis provided similar results when using OTUs instead of ASVs (Table S3.2).

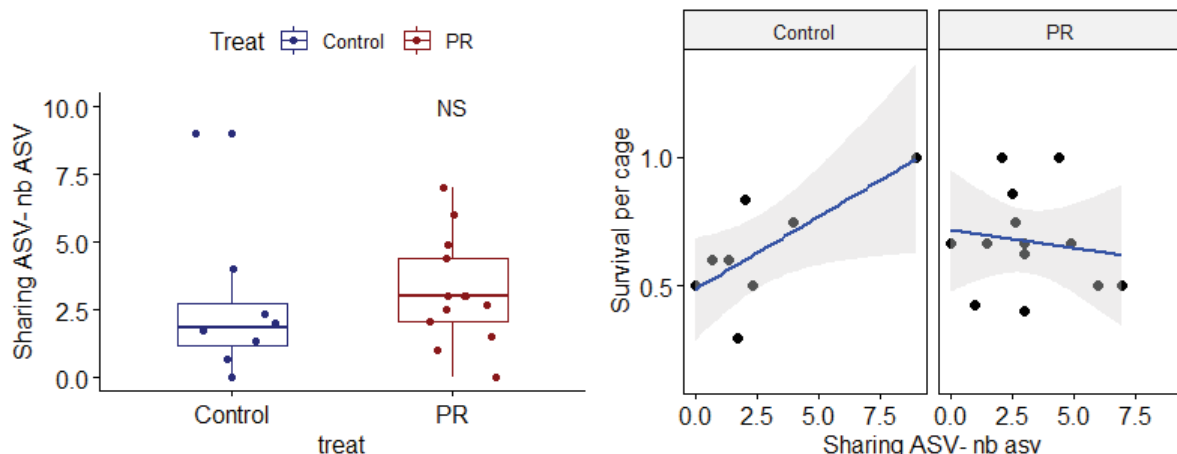


Figure 3.6. Effect of drought on sharing of ECM ASVs between pairs of co-occurring *Q. ilex* seedlings (left), correlation between sharing of ECM ASVs between pairs of co-occurring *Q. ilex* seedlings and seedling survival (right) and output of the generalised linear model testing the effect of sharing and treatment on *Q. ilex* seedling survival.

Table 3.5. Output of the mixed linear model testing the effect of seedling survival on the sharing (nb of ASV shared by two seedlings) in interaction with the PR treatment. P-values + for $0.05 < P < 0.08$, * for $0.01 < P < 0.05$ and *** for $P < 0.001$

	Estimate	Std.	t	Pr(> t)	
(Intercept)	0.48695	0.09371	5.196	7.28e-05	***
Sharing nb_ASV	0.05633	0.02508	2.246	0.0383	*
TreatPR	0.22858	0.13809	1.655	0.1162	
Sharing nb_ASV:TreatPR	-0.07031	0.03724	-1.888	0.0762	+

3.5 Discussion

In this research, we provide the first evidence of the remarkable resilience of Mediterranean soil fungal communities to drought stress, with a positive response to 15 years of experimental increment, including a gain in ECM species richness and a subsequent positive effect on *Quercus ilex* seedling survival.

3.5.1 The response of Mediterranean fungal communities to increased drought

Long-term drought significantly increased the diversity (ASV/OTU richness) of fungal communities in the soil of an old *Quercus ilex* monospecific forest and established on roots of young *Q. ilex* seedlings. These results were unexpected because they contrast with the patterns reported from North European temperate forest (Nickel et al., 2018). However, our finding is not inconsistent with previous research performed at the study site a decade ago (*i.e.* after nine

years of increased drought experimentally applied to the ecosystem) by Richard et al., (2011), who reported a maintained richness of ECM fungi under increased drought, based on a description of the ECM community associated with the old trees. The marked contrast between our results and those reported from Northern forest ecosystems suggests that 1) there is not only one but multiple responses of fungal communities to global warming, depending on the considered biomes and ecosystems (see Boddy et al., 2014 for a review on the variability of fruiting response of mushrooms to the current climate change) and 2) the under-explored Mediterranean fungal diversity may provide a valuable model system to explore the resilience of biological diversity to man-induced disturbances. Thus, the unpredictable and highly fluctuating Mediterranean climate may have favoured the emergence of long-term adaptation legacies on the organisms dominating the typical ecosystems of the region, such as *Quercus ilex* and its species-rich assembly of mutualistic fungal associates (Richard et al., 2005).

This “pre-adaptation scenario” of Mediterranean fungi to the intensity and duration of drought experimentally applied at the study site may explain the contrast between our study and previous research reported from Northern Europe. However, this hypothesis is insufficient to understand the mechanisms underlying the observed increasing of species richness exposed to increased drought. Thus, in our system the increased drought drove i) an increased richness of all fungal guilds at the site level (Table 3.2), ii) an increased ECM richness on roots of newly established seedlings (Table 3.2 & Figure 3.2) and iii) an increased richness of ECM fungi forming CMNs linking co-occurring seedlings (Figure 3.6 left). In *Quercus ilex* forest soils, ECM communities are made of an astonishing diversity of co-occurring species at the local scale (Richard et al., 2005, 2004). In the first centimetre of these soils, the environmental fluctuation favoured species co-occurrence (Table S3.2). This result suggests that the processes driving the temporal dynamics of hyper-diverse communities may be primarily environmental, as those operating to explain the nonequilibrium dynamics and the biodiversity of plankton communities (Descamps-Julien and Gonzalez, 2005; Holmgren and Scheffer, 2010; Huisman and Weissing, 1999; Hutchinson, 1961).

Our study revealed a clear response of fungal communities to long-term drought in terms of composition at both family and ASV level (Figure 3.3, 3.4). Twenty families showed significantly higher richness in drought treatment plots compared to control plots. This result suggests that drought conditions favoured a wide range of lineages, and concerned a large

spectrum of ecologies. Interestingly, the ectomycorrhizal families found in our study with significant effect of drought differ from those reported a decade ago by Richard et al., (2011). In 2011, *Cortinariaceae* were found to be more abundant under drought treatment and *Exidiaceae* to be more abundant in control plots, in the present study these two families were not significantly impacted by 15 years of drought. This difference may be explained by different sampling strategies (ECM root tips of adult trees in 2011 vs. soil and ECM root tips of seedlings in the present study), since ectomycorrhizal community compositions differ according to host age (Richard et al., 2005). In the present study, various ectomycorrhizal species (ECM ASVs) showed treatment-dependent patterns of occurrence, including the short and medium exploration types *Cenococcum geophilum*, *Inocybe griseolilacina*, *Thelephoraceae* sp. (more represented in drought treatment plots), and the contact and short exploration types *Russula nymphaeum* and *Tuber nitidum* (more represented in control plots). We are not able to determine whether species favoured in the PR treatment are more adapted to drought since we do not know their potential plasticity in the physiological mechanisms like in their enzymatic activities. Further studies analysing the physiological activities of these identified species may be interesting to investigate the reason why they are more present under drought conditions.

3.5.2 Ectomycorrhizal diversity promotes seedling survival

Increased ectomycorrhizal richness on roots positively influenced the survival of eleven month-old *Quercus ilex* seedlings (Figure 3.5). This result may reflect the well-documented links between taxonomic and functional diversities (Maherali and Klironomos, 2012), and suggests that higher ECM fungal diversity entails higher ECM functional diversity, and a facilitated access of diversified soil resources for species (Courty et al., 2016, 2005). Among all the identified species with treatments-dependent patterns of occurrence (Figure 3.4), two *Cenococcum* ASVs showed a correlation between their abundance associated to seedling rhizosphere and the seedling survival. The positive effect of drought on *C. geophilum* abundance in this study is in accordance with previous research showing its ability to resist xeric conditions. For instance, (Pigott, 1982) found that *C. geophilum* resists desiccation (up to -5.5MPa) remaining alive and inactive during drought assuming its role in seedling survival. However, to our knowledge our study is the first to reveal a positive correlation between *C. geophilum* colonization on roots and seedling survival. Interestingly, this pattern has emerged at the ASV level, and not at the species complex level, suggesting that intraspecific genetic

variability should be taken into account before interpreting distribution patterns of the hyper-abundant and genetically highly variable species *C. geophilum* (Douhan and Rizzo, 2005; Lobuglio et al., 1991). Unexpectedly, we observed one ASV of *C. geophilum* more abundant in the control than in the PR (Figure 3.4). The highly genetic variation, and the ability to grow under different environmental conditions of this species could explain this observation. We therefore hypothesize a potential divergence of *C. geophilum* species of different genotypes with different degrees of drought tolerance presented in our study site. Nevertheless, it is important to mention that the control of our study site suffers already Mediterranean seasonal drought (reaching up to -5MPa in August 2017 and -4.3Mpa in August 2018) which can explain the presence of drought tolerant species also in Control.

Interestingly, ectomycorrhizal richness in the soil of the cages was not correlated with the survival of seedlings (compared to ectomycorrhizal richness in roots) (Figure 3.5 A). This result could suggest a potential ability of the host to select ectomycorrhizal species (host filtering; Pickles et al., 2015) from the local pool to be associated may be towards species more adapted to drought.

3.6 Conclusion

In contrast to our study hypothesis, drought increased the diversity of fungal communities in the soil and on plant roots. We postulate that drought conditions may favour soil fungal diversity by inducing species turnover and reducing species dominance. Our limited current knowledge of fungal ecology does not allow testing whether these species positively responding to increased drought were more adapted to drought. However, in the case of the ECM guild, we found that higher diversity influenced positively seedling survival that was higher under drought conditions. Therefore these fungal species favoured by drought conditions could play a positive role in the survival, like the 2 ASV of *C.geophilum* that were positively correlated to survival (confirming hypothesis 2).

3.7 Supplemental material

Table S3.1: Effect of treatment on abundance of fungal communities per guild and the correspondent P-value.

	Guild	Effect PR Abundance	P-value
Soil 0-3cm	ECM	+12%	NS
	Saprotrophs	-18%	NS
	Pathogen	+31%	NS
Soim 3-6 cm	ECM	+49%	NS
	Saprotrophs	+25%	NS
	Pathogen	-36%	NS
Root	rhizosphere	+28%	0.07
	ECM root tips	+19%	0.05

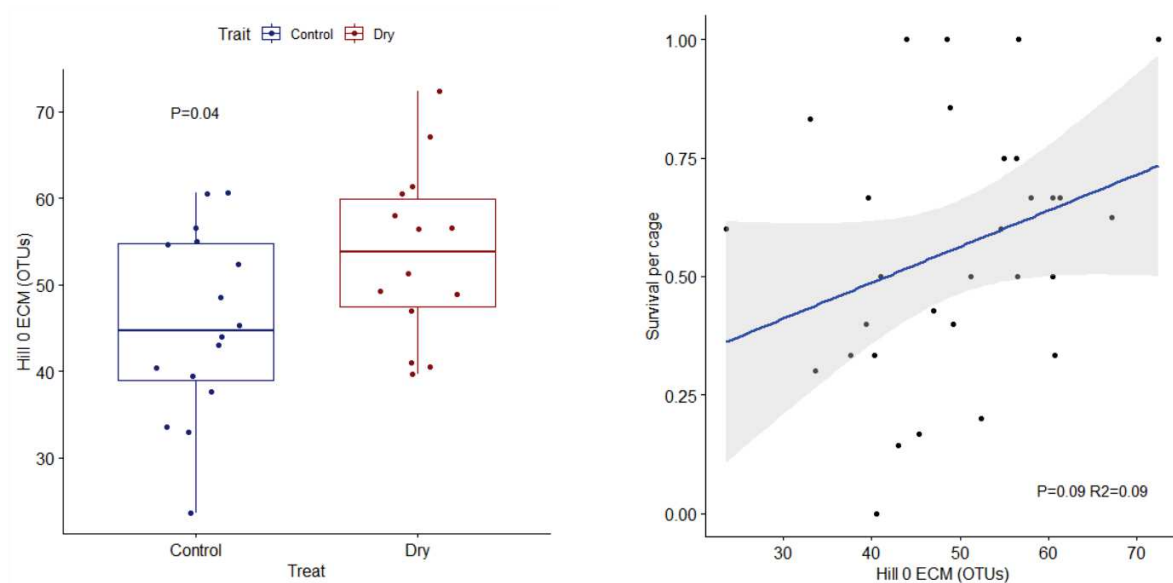


Figure S3.1. Analysis with OTUs. Effect of PR treatment on the Hill $q=0$ of the Ectomycorrhizal guild (left), and correlation of Hill $q=0$ of the ectomycorrhizal guild with survival per cage (right). P= P-value and R2= regression coefficient of the relationship

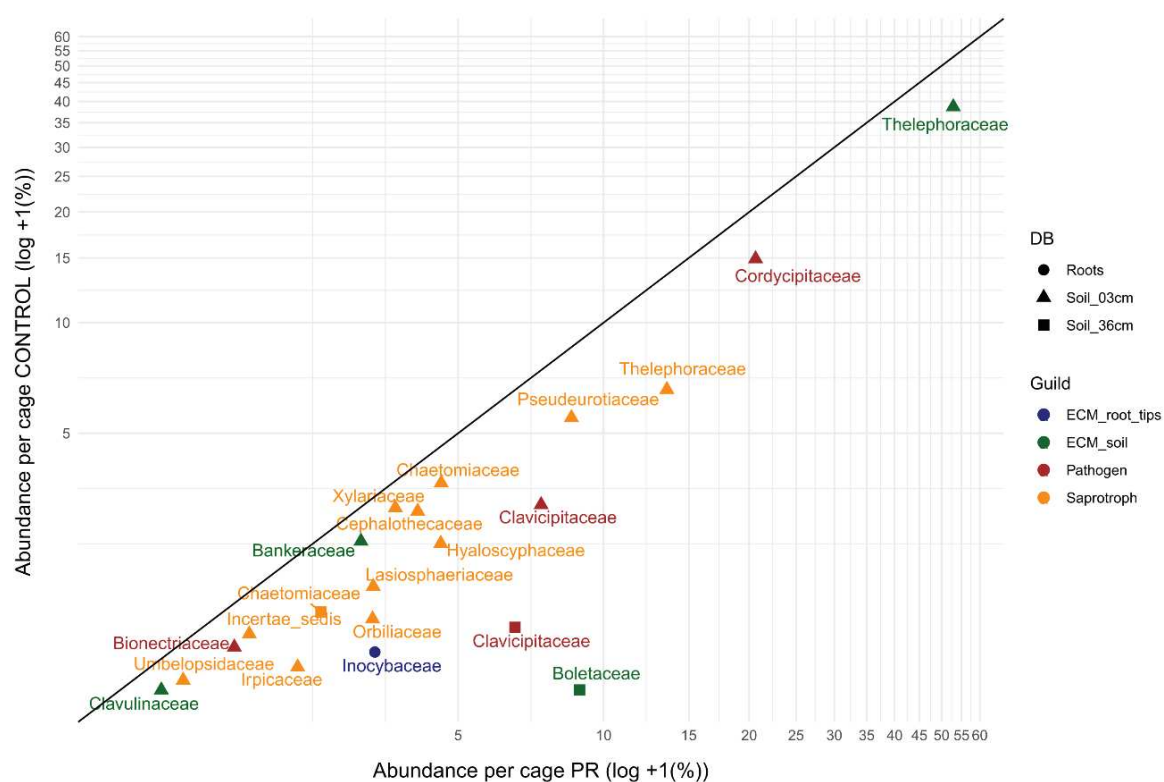


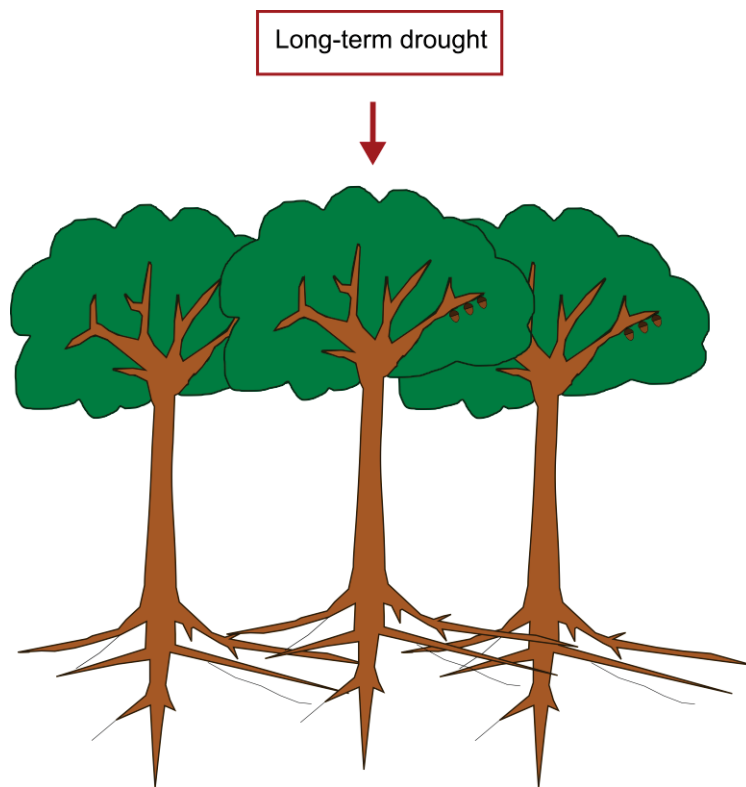
Figure S3.2. Fungal families with significant effect of PR treatment on proportional abundance of each family per cage in the roots and soil samples. Each colour represent a fungal guild.

Table S3.2. Results of the ectomycorrhizal network measured in alive seedlings. Values are mean per block per treatment.

Clustering sequences	Treat	Block	Degree (#ASV, cage)	Mean degree (#ASV, seedling)	Projected degree (%) (#seedlings connected)	Sharing (%) (#ASV shared by 2 seedlings)	Sharing (#ASV shared by 2 seedlings)	Survival rate (%)
ASV	Control	R1	12,33	8,17	66,67	10,21	1,26	53,75
		R2	16,00	10,20	94,45	17,52	2,80	75,00
		Sc	15,00	6,91	83,34	5,56	0,83	71,67
	Dry	R1	22,33	11,67	95,83	19,60	4,38	50,83
		R2	22,60	8,08	57,25	17,69	4,00	78,33
		Sc	30,00	9,78	57,89	12,53	3,76	66,71
OTU	Control	R1	9,33	5,88	83,33	20,13	1,88	53,75
		R2	8,50	6,38	44,60	51,39	4,37	75,00
		Sc	6,80	3,80	55,55	8,25	0,56	71,50
	Dry	R1	13,00	7,43	93,33	35,23	4,58	51,00
		R2	12,40	4,73	62,10	10,99	1,36	78,40
		Sc	15,80	5,37	62,00	12,39	1,96	66,00

CHAPTER 3

Long-term drought effects on the physiological and epigenetic responses of seed-bearing mother trees, with potential intergenerational transmission



Long-term drought effects on methylome and physiological responses in holm oak (*Quercus ilex* L.)

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Keywords: *Quercus ilex*, long-term drought, adaptive plasticity, DNA methylation, Mediterranean forest, climate change

4.1 Abstract

Shifts in rainfall patterns and increasing temperatures associated to climate change is causing widespread forest decline, especially due to the increase and duration of droughts. Tree species may have to adapt quickly to the incoming conditions, and epigenetic modifications are expected to play a key role in the regulation of rapid acclimation responses. In this study we measured acclimation physiological responses and methylome responses in holm oak trees submitted to 15 years of precipitation reduction treatment (PR) and in their respective controls. We address two hypothesis i) oak trees exposed to long-term precipitation reduction (PR) will exhibit different foliar traits due to carry-over phenotypic plasticity to drought, and ii) methylation levels within the drought-related different methylation regions (DMRs) may correlate with the foliar traits. Our results confirmed both hypothesis. Trees

exposed to PR treatment presented phenotypic responses in line with acclimation to drought relative to control trees. Furthermore, the methylome results revealed 84 drought related DMRs comparing trees from different precipitation treatments, where 16 DMRs were significantly correlated to phenotypic measured responses. The present study provides evidence of the role of epigenetic mechanisms regulating tree acclimation responses in natural populations of *Q.ilex* facing increased drought.

4.2 Introduction

A large body of research shows that higher tree mortality around the globe is related to human-induced climatic changes leading to warmer temperatures and increased water stress (Allen et al., 2010; Choat et al. 2012; Anderegg et al. 2016). There is, therefore, increasing concern that forecasted future rising of temperatures combined with increasing fluctuations/incertitude in water availability (especially due to changes in frequency, duration and intensity of droughts IPCC, 2014), will severely threaten the composition, structure and biogeography of the forests and their ecosystems services. As sessile organisms with low-dispersal seeds, trees are forced to adapt locally to environmental stress, but they are also particularly sensitive to rapid global changes, because their long-life span does not allow for rapid adaptation (Aitken et al., 2008; Petit and Hampe, 2006). In this context, phenotypic plasticity is assumed to be a crucial determinant of plant adaptation to stress in the short and long term (Nicotra et al., 2010). Epigenetic modifications induced by environmental stresses have been proposed as an accelerated alternative pathway for evolutionary change that contributes to local adaptation in natural populations independently from genetic variation (Bossdorf et al., 2007). Epigenetic modifications are changes in gene expression and function, but not in DNA sequence (Richards, 2006). These changes allow individuals to produce adaptive stress responses to environmental changes (Angers, et al. 2010; Richards et al. 2017), leading to phenotypes more resistant and resilient to stress (Mirouze and Paszkowski, 2011), effects that may also be inherited across generations (Hauser et al., 2011; Saze et al. 2003).

Of the several potential and non-mutually exclusive epigenetic mechanisms are (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

the most studied and stable epigenetic marker occurring in three different contexts (CHH, CHG and CpG, with H=A, C or T) (Du et al., 2015). Although the specific and functional role of DNA methylation in forest trees in response to the environment is still not well understood, there is evidence suggesting that the DNA methylation affecting 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 properties, and biotic interactions. Nevertheless, there are now several studies in natural populations that highlight the relevance of DNA methylation in creating phenotypic variation under stress conditions. For example, (Herrera and Bazaga, 2013) found differences in genome-wide DNA methylation patterns correlated to different leaf morphology following herbivory pressures in *Ilex aquifolium*. Other studies found differences in DNA methylation patterns in response to climatic variations in *Quercus sp.* (Gugger et al. 20; Rico et al., 2014) and in the alpine herb *Wahlenbergia ceracea* (Nicotra et al., 2015). Verhoeven et al., (2010) found changes in DNA methylation in response to biotic and abiotic stresses in *Taraxacum officinale*, with transmissions of DNA methylation patterns to the non-stressed progeny, suggesting the potential role of stress-induced epigenetic inheritance in adaptation and evolution. Differences in the methylation structure between and within natural populations of *Viola cazorlensis*, were suggested to be analogous to sequence-based genetic variation (Herrera and Bazaga, 2010) and to be linked to herbivory responses (Herrera and Bazaga, 2011). 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 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 differentially methylated regions of the genome (DMRs) 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). Last but not least, most epigenetical studies to date have very low replication at the individual level, which prevents their statistical power and questions the generality of their results. Taken collectively, these results 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 additional knowledge about the potential role of DNA methylation in the drought-response of a non-model tree species (*Quercus ilex*) with high ecological importance in the Mediterranean region. We used of a long-term field experiment where a precipitation reduction by 29% has been applied since 2003 in a natural *Quercus ilex* forest in the South of France (Limousin et al., 2008), and performed concurrent measurements of genome methylation and foliar traits in the two conditions after 15 years of treatment. We focused on foliar traits because leaves are the main siege of tree gas-exchange so they play a central role in tree performance and sensitivity to water stress. Besides, as new leaf cohorts are produced by the trees every growing season, 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 differentially methylated regions and different leaf physiological responses, in order to investigate the contributions of epigenetic responses to phenotypic tree acclimation to drought. We hypothesized that i) oak trees exposed to long-term precipitation reduction (PR) will exhibit different foliar traits due to carry-over phenotypic plasticity to drought, and ii) methylation levels within the drought-related DMRs may correlate with the foliar traits. Finally, we aimed at identifying the DMRs positions nearby genes and transposable elements (TEs) relevant for the tree response to drought.

4.3 Materials and Methods

4.3.1 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 (43°44'29'' N, 3°35'45'' E, elevation 270 m). 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 shade-tolerant 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 *c.* 2m. The soil is extremely rocky from hard Jurassic limestone origin. 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 area 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. Each replication was composed of one throughfall exclusion treatment (henceforth, precipitation reduction, PR) 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 plots, 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).

4.3.2 Leaf ecophysiological and physicochemical measurements

The effect of the rainfall exclusion experiment on tree water stress was verified regularly since 2003 by measuring the predawn leaf water potential (Ψ_{pd} ; Bykova et al., 2018). Measurements were carried out seven times in 2017 and six times in 2018 between the months

of June and October. Three shoots were sampled before dawn on five trees per treatment, stored in a sealed plastic bag in a dark cooler, and Ψ_{pd} 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 Ψ_{pd} between measurements and in periods in years when field measurements were not available. The relationship between measured and simulated Ψ_{pd} had an $R^2=0.85$ (RMSE=0.58 MPa) for the control treatment, and $R^2=0.89$ (RMSE=0.57 MPa) for the dry treatment.

Leaf gas exchange was measured on 6 trees per treatment during two field campaigns: one in autumn (27, 28 November, and 1st December 2017) on leaves that had experienced the severe 2017 summer drought, and one in spring on 7 trees per treatment (2,3,6 July 2018) on newly emerged leaves before the onset of the 2018 drought. We measured three different leaves per tree selected from the upper canopy and accessed with a scaffolding platform. Measurements were taken in three consecutive days with similar meteorological conditions. Leaf gas exchange measurements were performed with two 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 CO_2 concentration of $400\mu mol\ CO_2 mol^{-1} air$ and a saturating photosynthetic photon flux density of $1500\ \mu mol\ m^{-2} s^{-1}$. Light saturated net assimilation rate (A_{max}) 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 before measuring leaf dark respiration (R_d). Leaf mesophyll conductance to CO_2 from the sub stomatal cavities to the chloroplasts (g_m) was calculated from A_{max} , R_d and chlorophyll fluorescence measurements following the variable electron transport rate method of Harley et al. (1992):

$$g_m = \frac{A}{C_i - \frac{\tau^* [J_{ETR} + 8(A + R_d)]}{J_{ETR} - 4(A + R_d)}} \quad (Eq.1)$$

where τ^* is the CO_2 compensation point in the absence of mitochondrial respiration taken from Bernacchi et al. (2002). and J_{ETR} 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. Leaves for nutrient leaf content, non-structural carbohydrate and DNA methylation analyses were collected and pooled per tree (five leaves from five different branches of the measured trees). In the laboratory, we measured the projected leaf area of the collected leaves 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/>). Leaves were then oven-dried for 3 days at 60°C to determine their dry mass and calculate their leaf mass per area (LMA), before being grinded 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 ($\delta^{13}\text{C}$) were measured at the “Laboratoire de Biochimie et Physiologie Moléculaire des Plantes” (BPMP) in Montpellier using an elemental analyser (Model, Company) coupled with a mass spectrometer (Model, Company). Leaf phosphorous content (LPC) was measured at “Plateforme d’analyses chimiques en ecologie” (PACE), CEFCE CNRS in Montpellier using a colorimetric determination of phosphorous with sodium heptamolybdate which coloration is proportional of phosphorous concentration.

Intrinsic water use efficiency (WUE_i) was calculated from the $\delta^{13}\text{C}$ following the equation of Farquahar et al. (1989):

$$\text{WUE}_i = \frac{\text{Ca}}{1.6} \left(\frac{b - \Delta^{13}\text{C}}{b - a} \right) \quad (\text{Eq.2})$$

where Ca is the atmosphere CO_2 concentration taken as $400 \mu\text{mol mol}^{-1}$, a is the C isotope fractionation during diffusion through the stomata, b is the discrimination during carboxylation of the RUBISCO, and $\Delta^{13}\text{C}$ is the photosynthetic C isotope discrimination calculated following Farquhar and Richards, 1984:

$$\Delta = \frac{(\delta^{13}\text{C}_{\text{atm}} - \delta^{13}\text{C}_{\text{plant}})}{(1 + \delta^{13}\text{C}_{\text{plant}})/1000} \quad (\text{Eq. 3})$$

where $\delta^{13}\text{C}_{\text{atm}}$ is the isotopic ratio of C at the atmosphere, and $\delta^{13}\text{C}_{\text{plant}}$ is the isotopic ratio of the C in the leaf.

Photosynthetic nitrogen use efficiency (PNUE) was calculated as A_{max} divided by the amount of nitrogen per unit leaf area ($\text{PNUE} = A_{\text{max}} * 100 / \text{LNC} * \text{LMA}$). Finally, the leaf scale carbon use efficiency was approached by the ratio of dark respiration to maximum photosynthetic assimilation R_d/A_{max} .

4.3.3 Methylome and bioinformatic analysis

Leaves used for DNA methylation analyses were healthy leaves randomly selected on 7 trees per treatment by pooling the leaves from 5 different branches of the upper canopy of each tree. Leaf sampling for molecular analyses were collected in January 2018 in 12 trees and an additional leaf sampling of two different trees was done in November 2018 (totalling 7 trees per treatment). 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 manufacturer's protocol and quantified using the ND-1000 Spectrophotometer (NanoDrop Technologies) and Qubit Fluorometric Quantification. Extracted DNAs samples for 14 trees (7 trees per treatment) were sent to NOVOGEN company for whole genome bisulfite assessment. Library preparation, sequencing and data quality control was done by the company. Bisulfite conversion rate was above 99.85% for all samples. Quality control of FASTQ files provided by the company was evaluated using FastQC tool. 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 and bisulfite converted using "Bismark Bisulfite Mapper v0.22.1" (Krueger and Andrews, 2011). Bismark also performed PCR duplicates removal and methylation call for every single cytosine analysed. Bismark methylation extractor provided an output file with the position of every cytosine, the context (CpG, CHG or CHH), and the precision of methylated Cs labelled as forward reads (+), or non-methylated Cs 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 strict method (noise filter) provided by DMRcaller was used with the most strict 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) was applied for DMR detection. 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 physiological traits. Identification of DMRs was done with genetically

different individuals (7 from Control and 4 from PR treatment) after identification of clones of the measured trees (see next section Genotyping). However, methylation level at each DMR was calculated in all trees including non-genetically different individuals (7 from Control and 7 from PR) in order to have a high sample size for the correlations with physiology. The physiological variables used in correlations were from the spring campaign (and not in autumn), since the goal was to assess the role of DNA methylation induced changes in new emerged leaves without drought experience. Gene functions in promoter regions of the identified 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).

4.3.4 Genotyping

Tree genotyping at 70 single nucleotide polymorphism markers (SNPs) was used to identify potential clones among the sampled trees. Genotyping was performed by the Genome Transcriptome Facility of Bordeaux (PGTB) using MassARRAY (System d'Agena Bioscience technology) on 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 as in (Gavinet et al., 2020). The identified clones were discarded from the identification of DMRs as was commented above in the previous section.

4.3.5 Statistical analysis

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 “nlme” package version 3.1 (Pinheiro et al., 2017). An example of the syntax for a model looking at photosynthesis in spring season is as follows “`model<- lmer (Amax ~ PR_treat + (1|Tree), data=Spring)`”. Minimal adequate models with the lowest Akaike information criterion (AIC) were obtained following the guidelines of (Zuur, 2009) with the help of the “buildmer” package version 1.4 (Voeten, 2019). Correlations between methylation level within DMRs and

all physiological variables were conducted using a Pearson's coefficient correlation with a false discovery rate correction of (Benjamin and Hochberg, 1995).

4.4 Results

4.4.1 Precipitation reduction effect on tree water stress

The precipitation reduction experiment in Puéchabon has resulted in significantly more severe tree water stress during the summer drought in most of the years since experimental setup 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 dry treatment on October 11, 2017. Over the 7 Ψ_{pd} measurements performed in 2017, the five campaigns between August and October exhibited a significant treatment effect on Ψ_{pd} at $P < 0.05$ (Figure 4.1). In 2018, the drought was milder and minimum Ψ_{pd} recorded at the drought peak were characteristic of the average water stress for the site. The treatment effect on Ψ_{pd} was nevertheless significant in two of the six campaigns (in August and October, $P < 0.05$; Figure 4.1).

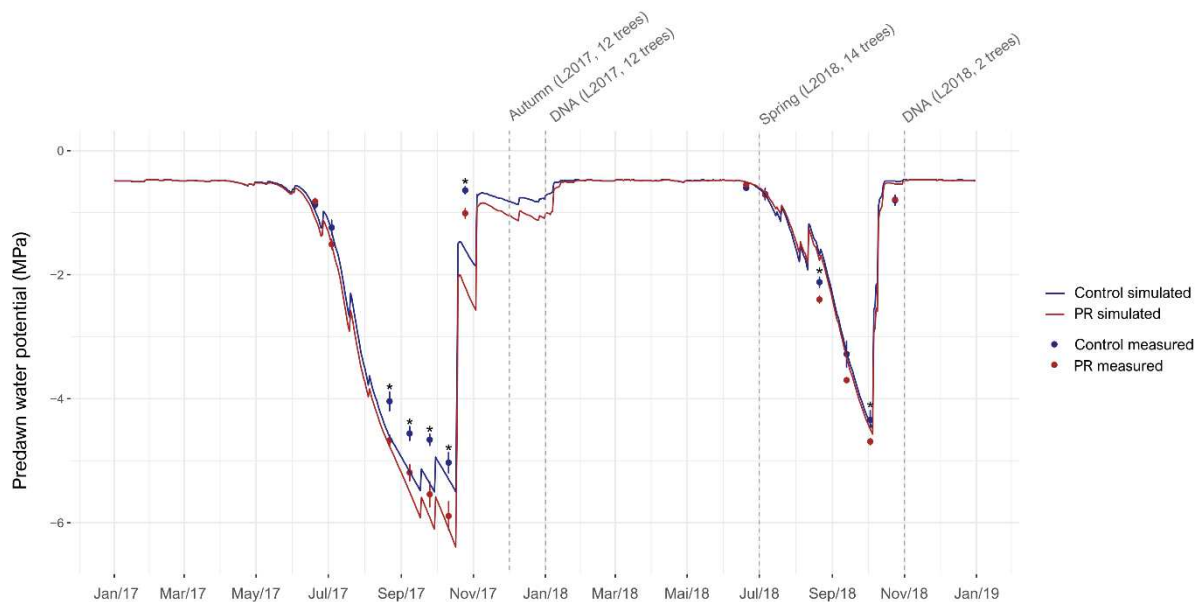


Figure 4.1. Predawn leaf water potential MPa measured in 6 trees from the Control (blue) and PR treatment (red) in 2017 and 2018. The continuous lines show the daily Ψ_{pd} simulated by the water balance model in the two treatments for an indication of the seasonal dynamic of water stress (blue line for Control, and red line for PR). The autumn and spring physiological measurement campaigns are shown in the figure, as well as the date of leaf collection for DNA analysis. Physiological and methylation measurements in 2017 were taken in leaves emerged in 2017 (L2017), and physiological measurements in spring 2018 were performed on leaves emerged in 2018 (L2018). *= P -values <0.05

4.4.2 Phenotypic and physiological responses to drought

Maximum photosynthetic rate (A_{max}) and stomatal conductance (g_s) in autumn after the summer water stress were significantly reduced by -28% and by -35%, respectively, in trees exposed to PR compared to trees from the Control (Figure 4.2). Mesophyll conductance (g_m) was also lower in the PR treatment, although not significantly. In contrast, in spring 2018 leaf gas exchange was significantly increased in trees exposed to PR compared to control ones, with increases of +27% in A_{max} , +24% in g_s and +30% in g_m . 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% in trees submitted to PR treatment compared to the Control in autumn 2017. In contrast, WUEi of newly emerged leaves was not significantly different between treatments in spring 2018, although there was a little tendency for an increased WUEi in trees from PR treatment.

Regarding the leaf chemical composition, we found no changes between treatments in leaf nitrogen, phosphorous and carbon in autumn (Figure 4.2). Conversely, we observed that trees exposed to the PR treatment 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), a morphological trait typically associated with sclerophylly and drought tolerance, was increased by +10% in the PR treatment in autumn 2017, but this trait was not different between treatments in newly produced leaves in spring 2018.

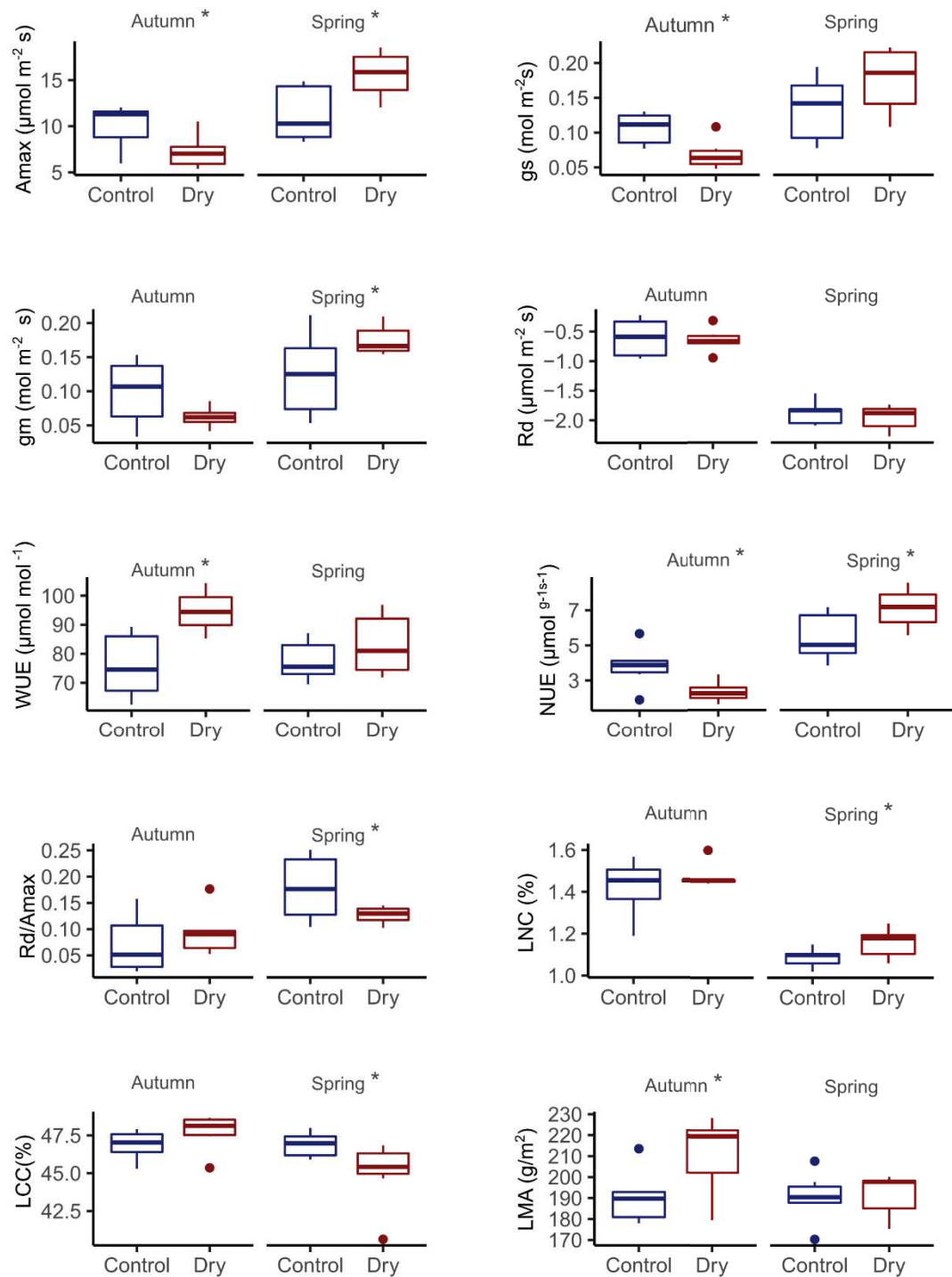


Figure 4.2. Boxplots of holm oak upper canopy leaves phenotypic responses to the precipitation reduction treatment: CO_2 maximal assimilation (Amax), stomatal conductance (gs), mesophyll conductance (gm), respiration (Rd), intrinsic water use efficiency (WUEi), nitrogen use efficiency (NUE), respiration to assimilation ratio (Rd/Amax), leaf nitrogen content (LNC), leaf phosphorous content (LPC), leaf carbon content (LCC), leaf mas area (LMA). Asterisks show the statistical significance at $P < 0.05$ ($n=12$ trees in autumn, $n=14$ tree in spring).

4.4.3 DNA methylation responses to drought

The mapping efficiency obtained after mapping bisulfite reads of *Quercus ilex* samples against the *Quercus robur* reference genome was about 25%, which means that 75% of the reads were lost, indicating that our data concern the most conserved genomic part between the two species. On average, 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 PR treatment 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 25% of mapped remaining, we found 84 regions of the genome with differently methylation regions (DMRs) between trees from the two treatments (Table 4.1). 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 PR compared to the Control) whereas one third (29%) of the DMRs were hypomethylated (lower methylation in the PR compared to the Control).

Table 4.1. Summary description of the 84 DMRs found between trees from PR and control treatments according to the cytosine context (in genetically different individuals: $n_{\text{Control}}=7$, $n_{\text{PR}}=4$). Number of DMRs with percent in brackets, hypermethylation, and number of DMRs in the proximity of ± 2000 bp of genes and TEs.

	All C contexts	CG	CHG	CHH
# DMRs	84	54 (64%)	30 (36%)	0
Hypermethylation	60 (71%)	35 (42%)	25 (30%)	0
Genes ± 2000 bp	32 (38%)	22 (26%)	10 (12%)	0
TEs ± 2000 bp	70 (83%)	43 (51%)	27 (32%)	0

In order to analyse the functionality of the DMRs we searched for gene-promoter regions and transposable elements at their proximities defined as ± 2000 base pairs (bp). Of the DMRs, 38% were found near genes (7 DMRs in CG and 1 DMR in CHG), 83% near TEs (27 DMRs in CG and 18 DMRs in CHG), and 29% near both genes and TEs (15 DMRs in CG and 9 DMRs in CHG) (Figure 4.3). These numbers make more than 100% since DMRs considered nearby both genes and TEs are also considered alone in nearby genes, and alone in nearby TEs. Genes with DMRs in their promoter regions or within their gene bodies were identified and their functions were summarized in Table S4.1. Among the DMRs associated

to genes, 56% were in the gene body, 19% downstream genes, and 25% upstream genes. Among the functions of these genes, 33% of the genes were related to the regulation of enzymes or proteins activities involved in different metabolic reactions like DNA transcription, DNA replication and chromatin remodelling. 15 % of the genes functions were related to the biosynthesis and transportation of carbohydrates of any group of organic compounds $C_x(H_2O)_y$. The remaining gene functions were linked to general biochemical reactions (18%), cell-cycle regulators like ion transportation, signal transducers, or homeostatic pathways (19% of the genes), and unknown functions (15% of the genes).

84 DMRs in total; 16 DMRs correlated to phenotype

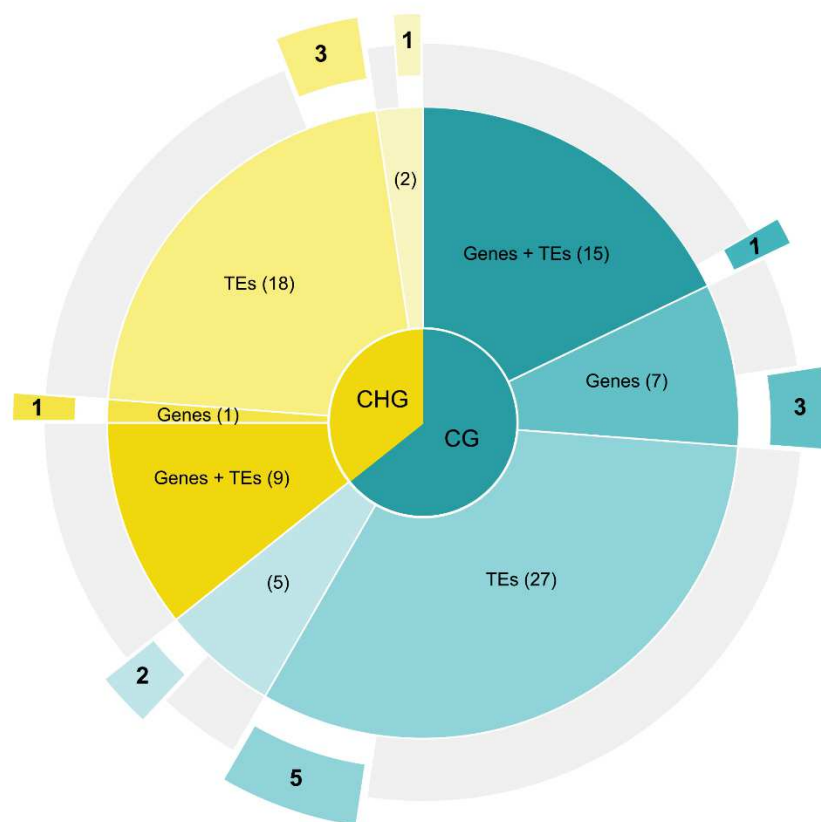
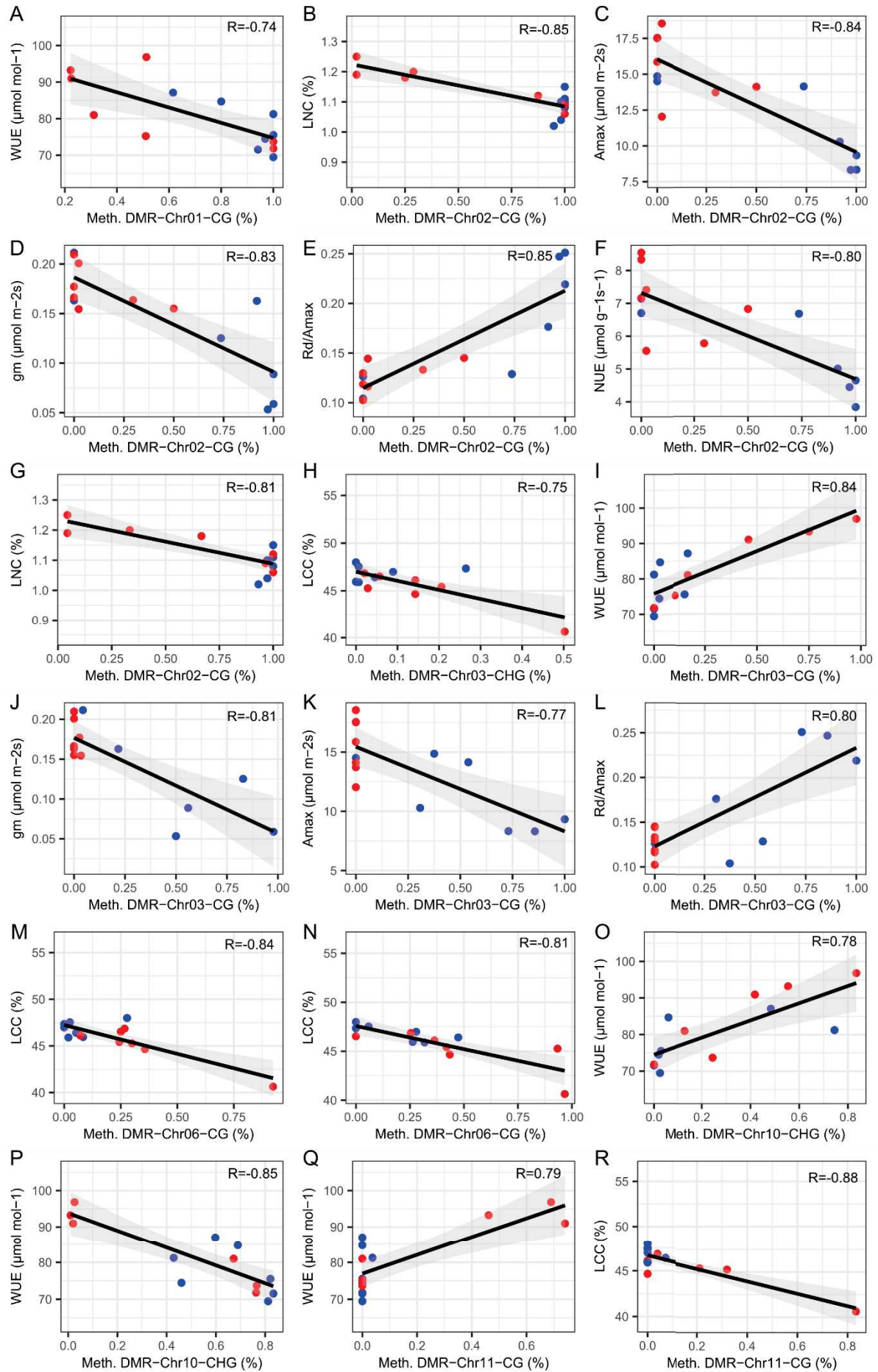


Figure 4.3. Summary of 84 drought related DMRs. The Pie-chart is divided first by cytosine context, second by DMR position in nearby regions of genes, TEs or both ± 2000 bp, third by DMR methylation proportion significant correlated to phenotype traits. Numbers indicate number of DMRs.

4.4.4 Correlations between DMRs and physiology

DNA methylation levels (number of methylated reads/ total number of reads) for each DMR of each tree was correlated to all the phenotypic variables measured in spring 2018 (Figure 4.4). Among the 84 identified DMRs, 16 DMRs (12 in CG context and 4 in CHG context) were significantly correlated to at least one measured phenotypic variable. The phenotypic variables correlated to DMRs were WUE_i, LNC, Amax, gm, Rd/Amax, NUE, LCC that were correlated to 5, 2, 2, 3, 2, 1 and 7 DMRs respectively. Among these 16 DMRs significantly correlated to phenotypic traits, 1 DMR was located in the promoter region of both genes and TEs, 4 were located in promoter regions of genes (Figures 4.4B, 4.4G, 4.4I, 4.4J), 8 were located in promoter regions of TEs, and 3 were located in other regions of the genome.

Figure 4.4. Significant correlations between methylation level (%) in each DMR and phenotypic variables. Pearson's *R* coefficient of the correlation are indicated on the figure. Each point represents a single tree *n*=14 (blue points for the Control trees, and red points for the PR treatment). The position of the DMR of chromosome and context are indicated in the x-axis.



4.5 Discussion

Trees exposed to precipitation reduction in our experiment presented phenotypical responses common to plastic responses to water stress that could induce a better acclimation to drought compared to control trees. In addition, we observed a different plasticity between seasons in the two treatments. Whereas trees in the drought (PR) treatment showed contrasting physiological and morpho-chemical traits between autumn 2017 and spring 2018, trees from the control treatment exhibited less variable values. In autumn 2017, when trees were still recovering from the severe summer drought, we found a significant reduction in A_{max} (-27%) and g_s (-35%) in PR compared to the control treatment (Figure 4.2). This reduction of leaf gas-exchange rates in the PR 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 (Figure 4.1). 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 (Munné-Bosch et al., 2001; Sainz et al., 2010), by hydraulic limitation due to the loss of hydraulic conductance in the leaf or in the branches (Sainz et al., 2010), or by the selective shedding of more drought resistant but less physiologically performant leaves. Consistently with this last hypothesis, leaves sampled in autumn in the PR treatment were significantly higher in LMA (+10%), a trait commonly associated with a better tolerance to leaf desiccation (Ogaya and Peñuelas, 2007), and presented higher intrinsic water use efficiency (+20 % WUE). Higher WUE 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 (Li et al., 2017). 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 A_{max} , g_s and g_m , respectively (Figure 4.1). This pattern is consistent with the higher leaf nitrogen content (+6%) in the new leaves of the PR treatment, although the significantly higher PNUE indicates that leaf nutrient concentration is not solely responsible for the higher gas-exchange rates. Moreover, besides being more efficient in water use and nitrogen use, we also observed that the new leaves in the PR treatment had a lower R_d/A_{max} ratio which indicates a higher carbon use efficiency (Limousin et al., 2015). These results suggest that

even without being exposed to water stress, the new leaves produced by trees in the PR treatment were more efficient in resource use than in the control treatment, which points out to a conservative resource use strategy to face water limitation as trees from the PR treatment also support a lower leaf area (Limousin et al., 2012). These results also show an increase in plasticity to respond to and recover from drought stress in the PR trees. The mechanisms involved in this increased plasticity were not analysed, but one might hypothesize a putative 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 winter-dormant shoot apical meristems of *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 precipitation reduction compared to control trees in all three cytosine contexts (+9.1% at CG, +9.4% at CHG, +9.2% at CHH), and also at a locus specific level with 71% of the identified drought-related DMRs being hypermethylated (Table 4.2). These results can be compared to those from Rico et al., (2014) who found an increased methylation of the hypermethylated loci (C^mC^mGG/GGC^mC^m) and a decreased methylation of the fully methylated loci (CC^mGG/GGC^mC) in *Quercus ilex* trees submitted to a precipitation reduction treatment for 12 years (being C^m a methylated cytosine, C a non-methylated cytosine and G a Guanine). Discrepancies 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 allows 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 climate (T_{max}) 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 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 by natural selection, in contrast to the other C contexts that were not sensitive to environmental variables (Dubin et al., 2015).

We analysed the potential role of the identified DMRs in the expression of nearby genes and TEs (Table 4.1). DNA methylation can repress transcription when methylation is found in gene promoter regions, but 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 found that 38% of the identified DMRs were located in the vicinity of genes and 83% of them in the vicinity of TEs (± 2000 bp.) (Table 2). Among the DMRs associated to genes, 56% were in the gene body, 19% downstream the genes, and 25% upstream the genes. We can make the hypothesis that the genes in the vicinities of DMRs with functions potentially involved in the increased plasticity of trees under PR could be those involved in the biosynthesis and transportation of carbohydrates or other organic compounds $C_x(H_{20})_y$. Soluble sugars possess the role of cell turgor maintenance through osmotic adjustments during drought (Silva et al., 2010). An efficient regulation in the use, degradation and transportation of carbohydrates can be crucial under drought conditions enhancing drought resistance and faster recovery after drought release (Hagedorn et al., 2016). In addition, there were 9 DMRs associated to genes involved in transcription and chromatin remodelling which could suggest different activation 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), that could be linked to the cumulated stresses suffered across their long lifespan, in our case study influenced by 15 years of PR treatment.

The present study provides evidence for an impact of long-term drought treatment on DNA methylation in natural populations of *Quercus ilex*. Moreover we have found correlations between variation in DNA methylation levels at specific loci and variation of phenotypic traits, opening new perspectives for future studies. Indeed, the methylation level for 16 drought-

related DMR was significant correlated to physiological or morpho-chemical traits per tree (Figure 4.3; Figure 4.4). Many 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.

Even if the present study is a step forward in the understanding of the role of DNA methylation in the acclimation to drought in natural populations, it possess many limitations common to studies on natural systems with low genomic resources (Bossdorf, et al., 2008; Richards et al., 2017). First, the unavailability of *Quercus ilex* reference genome forced us to map *Quercus ilex* reads to the *Quercus robur* genome and therefore to lose a significant portion of the reads. This certainly reduces the power of the study because the part of the *Quercus ilex* genome that we were unable to analyze might have contained important loci involved in drought acclimation. Indeed *Quercus robur* is a deciduous species from temperate forests, whereas *Quercus ilex* is an evergreen species from drought-prone Mediterranean forests with a long history of adaptation to drought (Urli et al., 2015). However, 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 closer 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). Nevertheless, given these above-mentioned methodological constraints, we were able to find in eleven genetically different trees 16 DMRs significantly correlated to the measured phenotypic traits (that we chose to be potentially related to drought plasticity), which strengthen the importance 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 16 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 manipulative experiments would further be 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 DMRs in altering the expression of the adjacent genes. In the case where these DMRs would be positive validated, they could be used for build an epigenetic marker for asses drought stress of tree natural populations.

4.6 Supplemental material

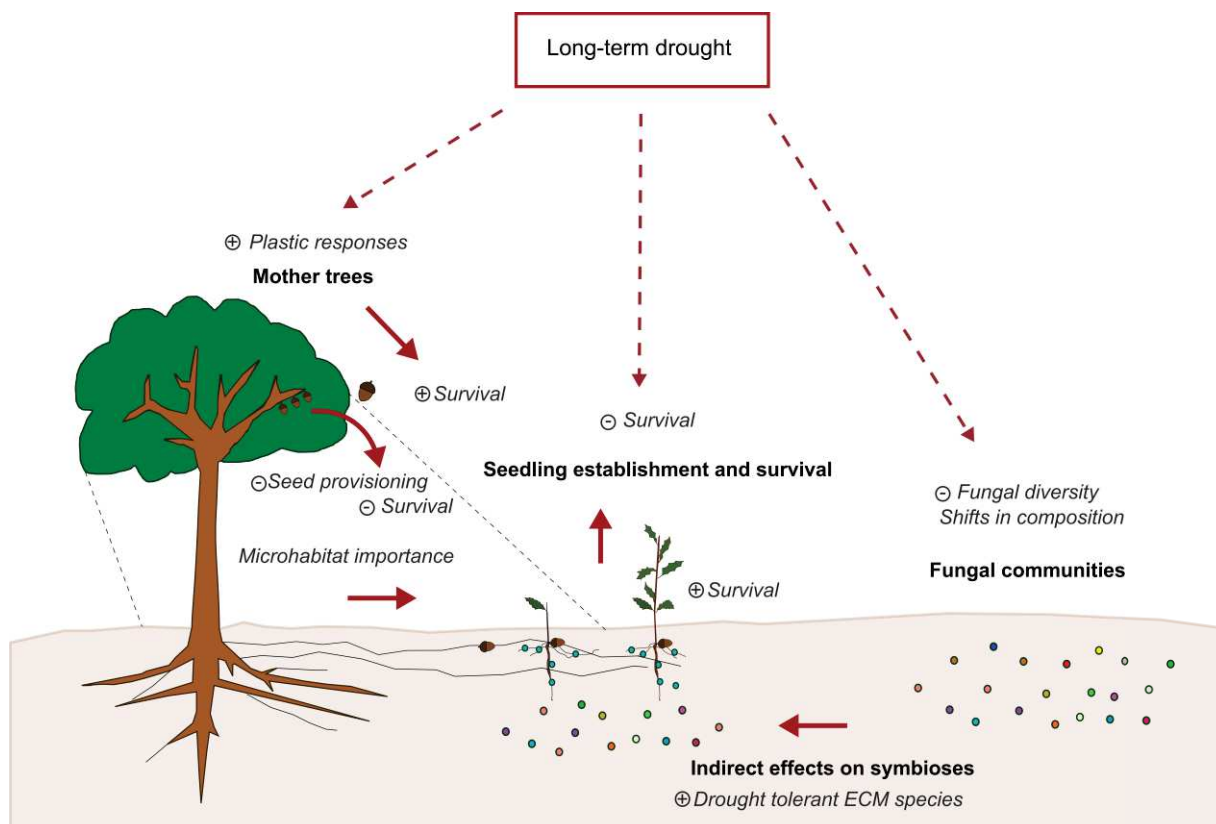
Table S4.1: Description of protein functions of the 32 drought related DRMs found in promoter regions of genes (± 2000 bp.), with their correlation information with physiology (R coefficient correlation, ***=significant correlation).

Chr. context	Name protein	Description	Position	Physio. correlation
Chr01_CHG	(M=2) K04499 - RuvB-like protein 1	Coenzyme and enzyme regulator. RuvB-like protein 1 is the proposed core component of the chromatin remodeling INO80 complex which is involved in transcriptional regulation, DNA replication and probably DNA repair.	after gene	-
Chr01_CHG	(M=2) K04499 - RuvB-like protein 1	Coenzyme and enzyme regulator. RuvB-like protein 1 is the proposed core component of the chromatin remodeling INO80 complex which is involved in transcriptional regulation, DNA replication and probably DNA repair.	after gene	-
Chr02_ CG	(M=1) PTHR23083//PTHR23083: SF- Tetratricopeptide repeat protein, TPR	Proteins containing TPRs are involved in a variety of biological processes, such as cell cycle regulation, transcriptional control, mitochondrial and peroxisomal protein transport, neurogenesis and protein folding.	before	-
Chr02_ CG	(M=1) PTHR24423//PTHR24423: SF370 - TWO-COMPONENT SENSOR HISTIDINE KINASE	Protein transmembrane, involve in homeostatic pathways. Catalysis of the phosphorylation of a histidine residue in response to detection of an extracellular signal such as a chemical ligand or change in environment, to initiate a change in cell state or activity.	in and before	-
Chr02_ CG	(M=14) 3.4.22.68 -Ulp1 peptidase.	Catalysis of the hydrolysis of peptide bonds in a polypeptide chain	after gene	LCC; R=0.63
Chr02_ CG	(M=2) 2.3.1.97 - Glycylpeptide N-tetradecanoyltransferase	Catalysis of the reaction: Adds a myristoyl group to the N-terminal glycine residue of certain cellular proteins.	after gene	LNC R=-0.84*** (Fig. 4.4B)
Chr02	(M=1) K03177 - tRNA pseudouridine synthase B [EC:5.4.99.12]	Any process involved in the conversion of one or more primary RNA transcripts into one or more mature RNA molecules.	in and after	LNC; R=-0.81** (Fig. 4.4G)
Chr02_CHG	(M=69) PF02365 - No apical meristem (NAM) protein	Any process that modulates the frequency, rate or extent of cellular DNA-templated transcription.	in after	-
Chr03_ CG	(M=110) 1.11.1.7 - Peroxidase.	Any process that results in a change in state or activity of a cell or an organism (in terms of movement, secretion, enzyme production, gene expression, etc.) as a result of oxidative stress, a state often resulting from exposure to high levels of	in	-

		reactive oxygen species, e.g. superoxide anions, hydrogen peroxide (H ₂ O ₂), and hydroxyl radicals.		
Chr03_CG	(M=2) PTHR13980 - CDC68 RELATED no information		in	-
Chr03_CG	(M=53) PTHR24177:SF10 - Ankyrin repeat-containing protein	Ankyrin repeats are tandemly repeated modules of about 33 amino acids. They occur in a large number of functionally diverse proteins mainly from eukaryotes. The repeat has been found in proteins of diverse function such as transcriptional initiators, cell-cycle regulators, cytoskeletal, ion transporters and signal transducers.	in	WUE; R=0.84*** (Fig. 4.4I)
Chr03_CG	(M=1) PTHR19321:SF3 - Anaphase spindle elongation protein	Cytokinesis: the division of the cytoplasm and the plasma membrane of a cell and its partitioning into two daughter cells.	in	Gm; R=0.81*** (Fig. 4.4J)
Chr03_CHG	(M=29) PF00560//PF08263 - Leucine Rich Repeat // Leucine rich repeat N-terminal domain	Interacting selectively and non-covalently with any protein or protein complex	before	LNC; R=0.76
Chr04_CG	(M=1) KOG2091//KOG2806 - Member of glycosyl hydrolase family 18 [Carbohydrate transport and metabolism]. // Chitinase	The chemical reactions and pathways involving carbohydrates, any of a group of organic compounds based of the general formula C _x (H ₂ O) _y . Includes the formation of carbohydrate derivatives by the addition of a carbohydrate residue to another molecule.	after gene	LCC; R = 0.66
Chr05_CG	(M=10) KOG0054//KOG0055//KOG0056//KOG0057//KOG0058//KOG0059//KOG0061//KOG0065 - Multidrug resistance-associated protein/mitoxantrone resistance protein ABC superfamily [Secondary metabolites biosynthesis transport and catabolism]. // Multidrug/pheromone exporter ABC superfamily [Secondary metabolites biosynthesis transport and catabolism]. // Heavy metal exporter HMT1 ABC superfamily [Inorganic ion transport and metabolism]. // Mitochondrial Fe/S cluster exporter ABC superfamily [Intracellular trafficking secretion and vesicular transport]. // Peptide exporter ABC superfamily [Intracellular trafficking secretion and vesicular transport]. // Lipid exporter ABCA1 and related proteins ABC superfamily [Lipid transport and metabolism]		between	-
Chr05_CG	(M=13) PTHR10024:SF201 - C2 Calcium/lipid-binding plant phosphoribosyltransferase-like protein	Interacting selectively and non-covalently with any protein or protein complex	in	Amax; R=-0.69
Chr05_CHG	(M=1) PF00562//PF04560//PF04567 - RNA polymerase Rpb2, domain 6	DNA-directed RNA polymerase activity: Catalysis of the reaction: nucleoside triphosphate + RNA(n) = diphosphate + RNA(n+1).	before	Amax; R=0.69
Chr05_CHG	(M=1) PF00562//PF04560//PF04567 - RNA polymerase Rpb2, domain 6	DNA-directed RNA polymerase activity: Catalysis of the reaction: nucleoside triphosphate + RNA(n) = diphosphate + RNA(n+1).	between	Amax; R=0.60
Chr05_CHG	(M=1) PF00562//PF04560//PF04567 - RNA polymerase Rpb2, domain 6	DNA-directed RNA polymerase activity: Catalysis of the reaction: nucleoside triphosphate + RNA(n) = diphosphate + RNA(n+1).	between	Amax; R=0.62
Chr06_CG	(M=1) K11492 - condensin-2 complex subunit G2	A membrane-bounded organelle of eukaryotic cells in which chromosomes are housed and replicated.	in	WUE; R=-0.66
Chr07_CG	(M=6) PF03759 - PRONE (Plant-specific Rop nucleotide exchanger)	Rho guanyl-nucleotide exchange factor activity GO:Stimulates the exchange of guanyl nucleotides associated with a GTPase of the Rho family	before and in	LNC; R=0.60
Chr08_CG	(M=5) PF05266 - Protein of unknown function (DUF724)		after gene	-

Chr08_ CG	(M=1) PF07557 - Shugoshin C terminus	A membrane-bounded organelle of eukaryotic cells in which chromosomes are housed and replicated.		-
Chr08_ CG	(M=1) KOG1840//KOG2002//KOG4626 - Kinesin light chain [Cytoskeleton]. // TPR-containing nuclear phosphoprotein that regulates K(+) uptake [Inorganic ion transport and metabolism]. // O-linked N-acetylglucosamine transferase OGT [Carbohydrate transport and metabolism Posttranslational modification protein turnover chaperones Signal transduction mechanisms].		before	Amax; R=-0.65
Chr08_ CHG	(M=2) K04499 - RuvB-like protein 1	Coenzyme and enzyme regulator. RuvB-like protein 1 is the proposed core component of the chromatin remodeling INO80 complex which is involved in transcriptional regulation, DNA replication and probably DNA repair.	before	-
Chr08_ CHG	(M=2) K04499 - RuvB-like protein 1	Coenzyme and enzyme regulator. RuvB-like protein 1 is the proposed core component of the chromatin remodeling INO80 complex which is involved in transcriptional regulation, DNA replication and probably DNA repair.	before	-
Chr09_ CG	(M=1) PTHR23324//PTHR23324:SF46 - SEC14 RELATED PROTEIN // SUBFAMILY NOT NAMED		between	-
Chr11_ CG	(M=3) K00703 - starch synthase [EC:2.4.1.21]	The chemical reactions and pathways resulting in the formation of substances; typically the energy-requiring part of metabolism in which simpler substances are transformed into more complex ones.	before	-
Chr11_ CG	(M=8) PF11443 - Domain of unknown function (DUF2828)		in	-
Chr11_ CG	(M=1) PTHR11689//PTHR11689: SF75 - Chloride channel // subfamily not named	The process in which a solute is transported from one side of a membrane to the other.	in	-
Chr11_ CG	(M=1) 2.4.1.101 - Alpha-1,3-mannosyl-glycoprotein 2-beta-N-acetylglucosaminyltransfe rase.	Initiates complex N-linked carbohydrate formation. Essential for the conversion of high-mannose to hybrid and complex N-glycans	before	Amax; R=0.64
Chr11_ CHG	(M=8) PF11443 - Domain of unknown function (DUF2828)		in	Amax; R=-0.64

GENERAL DISCUSSION



The accelerating climate change induced by anthropogenic activities threatens the persistence of forests ecosystems and the conservation of their biodiversity (Blowes et al., 2019; Lindner et al., 2010). The impacts are expected to differ among the Earth bioclimatic zones. Tropical, temperate and Mediterranean biomes are expected to suffer higher impacts on biodiversity than boreal and drylands biomes (Blowes et al., 2019). In the Mediterranean region, the intensified droughts have already led to tree defoliation and mortality, along with unsuccessful recruitment of the dominant tree species (Pulido et al., 2013). Under these changes, the current dominant tree species, such as holm oak, could be replaced by shrubby vegetation (Ibáñez et al., 2015). Understanding how Mediterranean forests will respond to the on-going climate change is highly important to anticipate these effects and mitigate the impacts by applying the appropriate managements.

In my PhD thesis I tried to advance our understanding of the capacity of Mediterranean holm oak forests to persist under predicted increasing droughts through the establishment of new tree cohorts. Specifically, I tried to understand the factors that will influence the establishment and survival of *Quercus ilex* seedlings under drought as well as the potential adaptive role of epigenetic changes. First, I investigated the importance of microhabitat environmental conditions and ectomycorrhizal colonization on the germination, establishment and survival of seedlings under drought (Chapter 1). Second, I studied how drought alters the soil and rhizosphere fungal community compositions, with a particular focus on the ectomycorrhizal guild, and how these changes affect the survival of seedlings (Chapter 2). And third, I investigated the plastic acclimation to drought of mother trees and the role of epigenetic modifications regulating these plastic responses that potentially may affect offspring survival and performance under drought (Chapter 3).

5.1 Forest regeneration explained by several drought-legacy effects

A global overview of the results of this PhD appears in Figure 5.1. The three dashed red arrows represent the potential three pathways by which drought influences the establishment and survival of *Quercus ilex* seedlings.

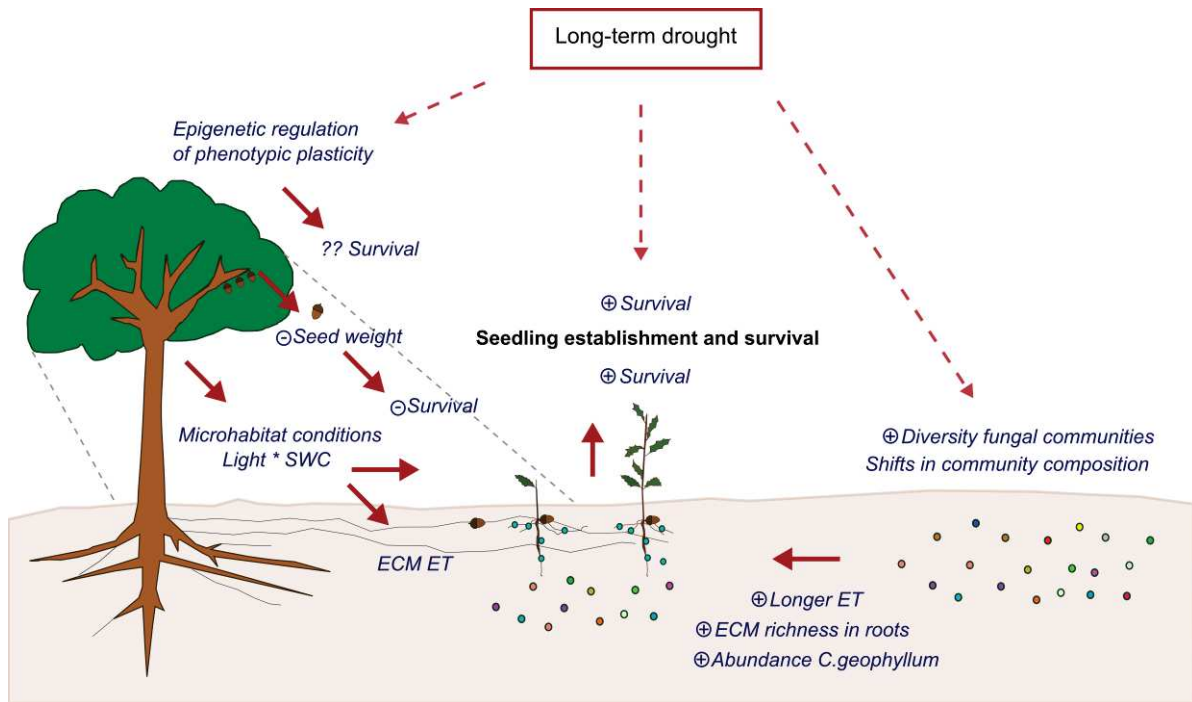


Figure 5.1. Overview of the main findings of this PhD thesis. ET=Exploration types

The first pathway that influences seedling survival corresponds to the drought effects on seedling germination, growth and survival that are also influenced by other environmental factors such as microhabitat conditions. Interacting environmental factors like light availability and soil moisture were found to modulate the effects of increased drought on survival and to influence the colonization of roots by different ectomycorrhizal fungal vegetative strategies (the so-called morphotypes *sensu* Agerer, 2001 ; Chapter 1). Specifically, light availability in spring, when soils were water saturated, was favourable to seedling emergence and growth, whereas shadow was preferable during summer drought when water started to be deficient and light increased the risk of seedling desiccation.

The second pathway that affects seedling survival is the drought effect on fungal diversity, (which increased under long-term increased drought), and the shift in the

composition of the fungal community. Specifically, changes in the ectomycorrhizal guild associated to roots impacted positively seedling survival, such as (i) an increased ECM species richness, (ii) an increased colonization by ECM species with longer-distance exploration strategies, and in particular (iii) a higher abundance of two genotypes of *Cenococcum geophilum* with positive effect on the survival of *Quercus ilex* seedlings under drier conditions (Chapter 1 and Chapter 2).

Finally, the third pathway by which drought influences seedling survival is the plastic acclimation to drought of mother seed-bearing trees. Trees exposed to recurrent droughts may plastically adjust to the drought conditions and potentially transmit the drought legacies to the next-generation of plants. Environmental stress transmission from mother trees to offspring can be through seed reserves that will influence positively the survival (Quero et al., 2007), or through epigenetic modifications (Herman and Sultan, 2016) associated to plastic drought responses that will be conferred to seedlings. (Chapter 3). The amount of seed provisioning by mother trees can be significantly reduced by drought in dry years (Pérez-Ramos et al., 2010), although it has not been observed to change significantly in response to long-term rainfall reduction in our experiment (Le Roncé et al. submitted). In my study, I observed plastic responses to drought potentially regulated by epigenetic mechanisms in adult trees, however, the potential intergenerational epigenetic transmission could not be assessed (see section Caveats in Annexs). In fact seed-bearing trees presented plastic responses to drought that are in line with acclimation capacity of this species to drought. The responses consisted in physiological and morpho-chemical modifications in their leaves that allowed them to be more efficient in the water, nitrogen and carbon use. We also observed a potential compensation of the physiological activities between autumn (after the drought season) and spring (before the onset of the drought season). While trees from the dry treatment were still impacted by carry-over effects from the previous drought in autumn, their new leaves emerging during the next spring presented, surprisingly, higher C assimilation rates and higher nitrogen and carbon use efficiencies compared to leaves from control trees. The physiological mechanisms underlying this different plasticity to drought between treatments were not analysed directly, but we observed significant correlations between tree plastic responses to drought and genome methylation in differentially methylated regions, that

suggest a potential epigenetic role in the regulation of these plastic responses and in tree drought memory.

The results of this PhD confirm that seedling establishment in a drier world will be modified by long-term drought legacy effects mediated by ectomycorrhizal communities and drought impacts on mother trees. Furthermore, the probability of an emerging seedling to survive will be dependent on the microhabitat conditions where seeds fall and germinate and on its interaction with the established ectomycorrhizal network. In particular, sufficient light and soil moisture conditions will be necessary to first, maintain seedling growth without excessive desiccation and second, to have a positive carbon budget that allows to maintain the symbiotic relationship with the rhizosphere. Even though we did not test the potential transmission of the observed plastic responses in trees to the next generation, we suggest that if the methylation patterns observed in mother trees are conserved and transmitted to offspring, they could play an important role in the survival of seedlings and in the adaptation to drought of future *Quercus ilex* forests.

5.2 Soil drought-legacy effects

The positive effects of increased drought on seedling survival found in our experiment were completely unexpected. Even though summer drought was the main cause of seedling mortality, our partial rain exclusion treatment did not increase further the seedling mortality. These results contrast with previous studies where the survival of tree species was negatively affected by increased drought treatments (Gómez-Aparicio et al., 2008; Hallett et al., 2011; López-Sánchez et al., 2019). Furthermore, in our study we found that fungal diversity was higher under increased drought conditions which also contrasts with precedent literature. Previous studies analysing the effect of drought on fungal communities found reduction of fungal diversity and shifts in community compositions (Nickel et al., 2018; Toberman et al., 2008). Other studies found that reductions in soil moisture affected the composition but not the diversity of fungal communities (Schmidt et al., 2018), including the study of Richard et al. (2011) in our study site after five years of rainfall exclusion. To the best of my knowledge, the only evidence I found of positive effects of drier conditions on diversity and abundance of fungal communities is the work of Hawkes et al., 2011. However, the treatments of this study

consisted in adding rainfall in the wetter season and compare to ambient conditions, rather than increasing drought stress on the dry season.

A potential explanation of these controversial results is the long-term drought treatment (fifteen-years of precipitation reduction) in our study site that may have led to the establishment of fungal species adapted to recurrent droughts and therefore performing better under seasonal droughts than fungal species from the control. This pattern could not be found in short-term experiments when species do not have the time to adapt under short-term experimental treatments. Fungal species are known to vary in their tolerance to water limitation and their capacity to adapt to drought (Shi et al., 2002). For instance, desiccation resistant spores or hyphae persistence during drought will allow fungal species to recover from drought episodes and remain alive. Changes in the functioning of the extramatricial mycelium can also induce drought tolerance (Bakker et al., 2006). Furthermore, (Courty et al., 2010) suggested that lower C allocation of host to the rhizosphere during drought can induce an increase in the saprotrophic function of mycorrhiza that could counteract the effects of drought experienced by the fungus. We can thus hypothesize that the long history of recurrent droughts impacting Mediterranean fungal species and plant hosts, exerted selective pressures and filtered tolerant and adapted to drought species. These drought filtered adapted fungal species seem not only to be unaffected by increasing drought, but also to be positively affected by drought with increases in richness and facilitating seedling survival. In the context of the study, this result is in line with the diversity patterns within the Mediterranean region, which high levels of biodiversity are partially explained by the constant environmental fluctuations and disturbances suffered (Cowling et al., 1996).

Another potential explanation for the higher survival rates of seedlings under drier conditions is a presumed higher root infection by soil pathogens under wetter conditions (Brasier et al., 1993). Indeed, species of the genus *Phytophthora* are among the organisms causing the most serious damages and diseases to fine roots in *Quercus ilex*, and are considered as the main factor of oak decline in Spain and Portugal (Corcobado et al., 2014; Sanchez et al., 2002). The most favourable conditions for *Phytophthora* infection are around 25 °C and soil moisture content around 30% vol. (Corcobado et al., 2010), which in our study site corresponds to April-May. At this time of the year radicle elongation is growing into the soil and can interact either with beneficial partners like mycorrhizal species that improve seedling

performance and survival or with pathogens decreasing its performance and potentially causing its death. A recent study revealed that in response to drought, host *Quercus* sp. species can increase the chemical plasticity of their roots in order to enhance mycorrhizal colonization, like by decreasing ellagitannins concentration with antimicrobial properties (Suseela et al., 2020). Reductions in tannins with antifeedant capacity could also be beneficial for pathogens, but since this occurs under drier conditions, we could imagine that pathogens are less present under these conditions. Even if we did not test for pathogen abundance in our study site, we can hypothesize that higher seedling survival observed under drier conditions may be explained through a synergistic effect of ECM adapted species that have been filtered along fifteen years of drought treatment and less favourable conditions for root pathogens.

To end this section on soil legacy effects, I would like to mention a methodological point in the choice of clustering sequences of fungal species and highlight the contribution of this method to account for intraspecific variation in our study. We chose Amplicon Sequencing Variants (ASV) over Operational Taxonomical Units (OTUs) for clustering the fungal sequences, which is arising as the most realistic estimate for assessing richness and community composition of fungi (Pauvert et al., 2019). With the ASV method, we were able to detect important changes in intraspecific variation that may be ecologically important, as intraspecific effects can sometimes be more relevant than species effect (Albert et al., 2010; Des Roches et al., 2018). Indeed ASVs allowed us to reveal a differential effect of drought treatment on the abundance of different genotypes of the species-complex *Cenococcum geophilum*, as well as the identification of two genotypes from the same species highly correlated to seedling survival. Finally, the use of ASV benefited to our analysis of the ectomycorrhizal network by linking co-occurring seedlings with same fungal ASVs genotypes and not molecular species based on OTUs. In network analysis, we assumed that two co-occurring seedlings were colonised by the same fungus individual when it was found in both plants, which overlooks the case when fungal associates from both plants were different individuals. Therefore a higher level of accuracy in detecting different genetic individuals would reduce the overestimations of shared fungal associates.

In the following two sections, I will explain the results of my PhD in the context of the stress gradient hypothesis and in the context of the Mediterranean forests.

5.3 Our results in the context of the stress-gradient hypothesis

After reading the results of this PhD thesis, one might be inclined to interpret the findings as proof that increasing droughts will be beneficial for the establishment and survival of *Q. ilex* seedlings. In fact, in our experiments the survival of seedlings as well as the diversity of ectomycorrhizal communities influencing seedling survival, were increased under drier conditions. Even the adult trees seemed to compensate the increasing droughts with plastic responses increasing the use efficiency of nutrients and water. The only negative drought effect was found in the reduced number of seeds produced by mother trees (Gavinet et al., 2019 Le Roncé et al. submitted), while the seed mass of mature acorns appeared to be maintained.

A potential explanation for the positive effects of drought in our study is the interpretation via the framework of the “stress gradient hypothesis” (Figure 5.2). The stress gradient hypothesis postulates that facilitation and competition interactions, which are key drivers in a wide of natural communities (Bruno et al., 2003), may vary inversely across gradients of stresses (Maestre et al., 2009). Facilitation increases with the harshness of stresses being maximal at mild-stresses levels and it decreases with extreme stress levels, whereas competitions increases when stress are at maximum values or when there are no stresses.

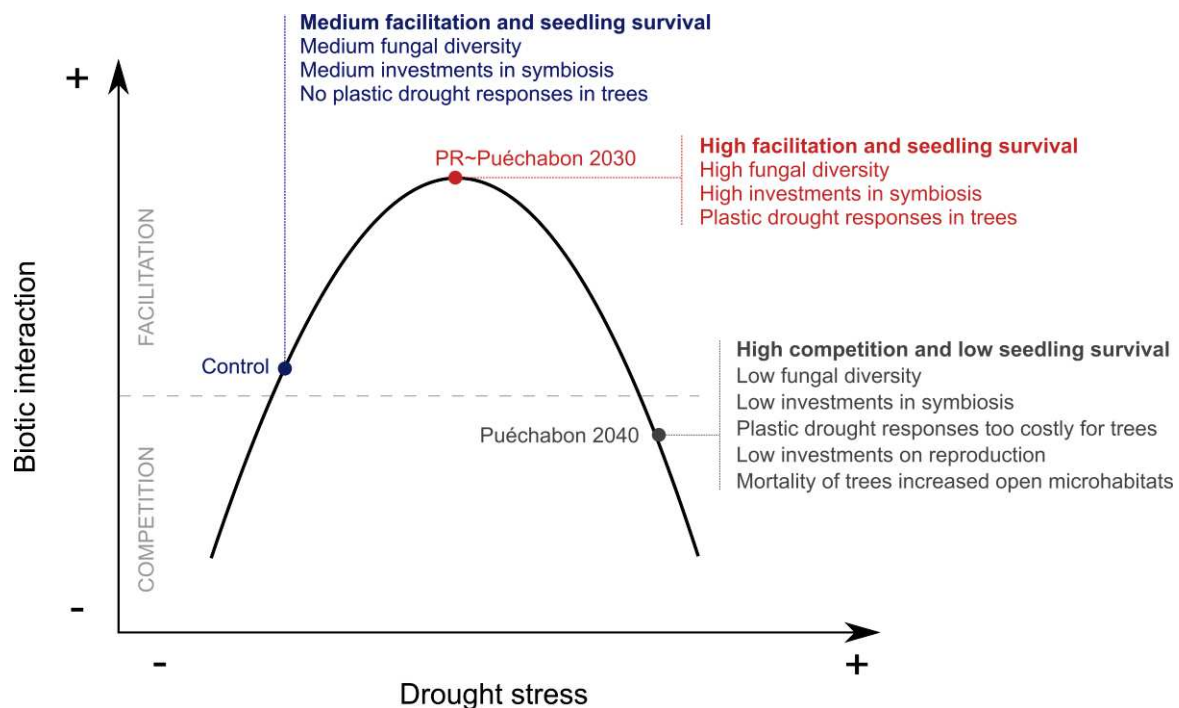


Figure 5.2. Hypothetical representation of the results of this PhD according to the stress gradient hypothesis. Figure idea extracted from Kawai and Tokeshi (2007). PR=Precipitation reduction treatment of our experiment, Puéchabon 2030 and Puéchabon 2040 are the predictions of water stress index (WSI) in our study site (see Annex).

In our experiment, seedling survival increased with higher severity of drought stress (Position PR in Figure 5.2). At this point of higher stress, the plant-fungus facilitation is higher, and could increase the ectomycorrhizal richness associated to seedlings influencing seedling survival. We hypothesize that the -29% reduction of precipitation treatment applied in our experiment is severe enough to increase facilitation between species over competition, but not too severe to exacerbate competition more than facilitation as might happen under more severe climate change scenarios. Such a framework could explain the increased species richness of the fungal communities and the associated increased survival rate of seedlings. Regarding the adult trees, we can hypothesize that long-term precipitation reduction treatment has allowed them to develop plastic responses by which they increase their use efficiency of water and nutrients and compensate the detrimental effects of more severe water stress, potentially transferring adaptive plasticity to offspring. Conversely, it can be argued that in the control treatment of our experiment where drought exposure is less severe (left part of Figure 5.2), the facilitative interactions and the drought acclimation of adult trees are lower.

However, if the drought stress in the future becomes more severe than that simulated by our actual precipitation reduction experiment (like the one expected in Puéchabon for 2040 or latter according to the tendencies of water stress index (WSI), Lempereur et al., 2017), the results of this thesis might have shown opposite effects (right side of Figure 5.2). At this point where drought stress is close to maximal physiologically tolerable plant threshold, the facilitation between species and therefore the maintenance of the symbiotic relationship may become too costly for both fungus and plant. In addition, the resources at this point are very limited and competition for resources among fungal species would dominate, impacting fungal diversity and seedling survival. In such a scenario, we can imagine that fungal communities would be impacted (i) directly by the extreme environmental conditions that reduces the soil moisture and microbial enzymatic activities (Sardans and Peñuelas, 2005), and (ii) indirectly by reductions of the symbiotic relationships due to reductions in carbon allocation from host trees (Courty et al., 2010). In the case of host trees, we can imagine that

more severe water stress could not be compensated by plastic acclimation and that the detrimental carry-over effects of previous droughts (hydraulic cavitation, defoliation, mortality of tissues) would dominate the tree plasticity and reduce durably the tree performance and carbon balance. In that case host trees may reduce their carbon investment in the mycorrhizal symbiosis which would further increase their vulnerability to drought (decreasing the facilitation strength). In particular, we could expect that the longer ECM exploration types, which are more C costly for the host but also more important for seedling survival under drought (Chapter 1), would be the most impacted under more severe water stress. Finally, one might expect that under extreme drought stress, the tree allocation patterns between reproduction and growth would change towards a lower investment in reproduction compared to growth (Bazzaz et al., 1987 ; Le Roncé et al. submitted), which will also affect negatively the chances of successful seedling establishment.

In conclusion, although our results revealed that Mediterranean species have evolved robust adaptive responses to survive drought stress, and that the regeneration will be assured under increasing droughts in the near future, if human induced greenhouses gas emissions and the associated temperature rise are further increasing, the tendency could as well reverse. Under this scenario, the cost for maintaining facilitation interaction, in our case mycorrhizal symbiosis, could be too costly for both host and fungus, and the intensity of this relationship would be reduced, thereby affecting negatively the regeneration and persistence of *Quercus ilex* forests.

5.4 Vulnerability of regeneration in Mediterranean forests

Mediterranean forests are projected to suffer largest impacts by climate change (Schroter, 2005). Nevertheless, the results of this PhD revealed that the regeneration of *Q. ilex* seedlings under a closed-dense canopy Mediterranean forest in the South of France would be assured under increasing droughts. A recent study assessing the effect of climate change on different biomes, revealed the high sensitivity to both land-use changes and climate change of Mediterranean biomes. In detail, it reports reductions of species richness in secondary vegetation, pastures and areas of harvested agriculture, but no impact on species richness of primary vegetation with land-use and climate changes (Newbold et al., 2020).

In order to understand and predict the *Q. ilex* regeneration vulnerability to climate change across the Mediterranean region, we have to understand (i) the ecological succession of Mediterranean forests and (ii) the history of past land-uses that determine the actual composition of Mediterranean forests and its probability to persist. In the Mediterranean region the natural pathway of ecological succession in forest recovery is a gradual colonization by pioneer shrubs *Cistus* species followed by colonization by *Arbutus* and *Erica* species, and finally natural colonization by oak that is known to be facilitated by nursery shrub plants or by pioneer *Pinus* species providing shade and fungal inoculum (Richard et al., 2009). A study analysing *Q. ilex* survival after first and second-year of summer drought under different habitats of Mediterranean successional stages: open (pioneer), shrubland (mid-successional) and woodland (late-successional) with different irrigations levels revealed significantly higher seedling survival in woodlands than in open areas and increased survival under wetter conditions (Mendoza et al., 2009) (Figure 5.3).

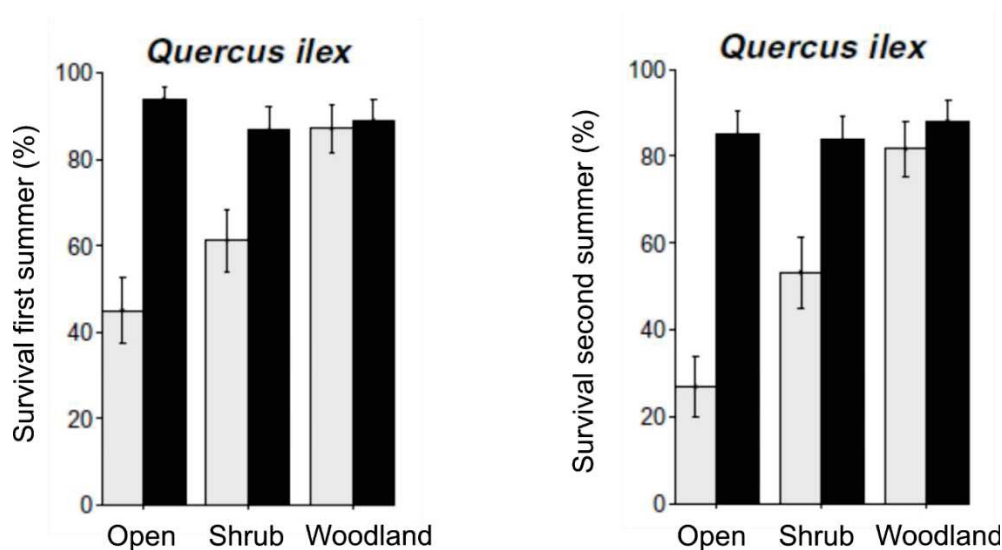


Figure 5.3. First and second summer seedling survival in different habitats and irrigation levels (black irrigation treatment and grey control). Second summer survival was calculated as the number of seedlings surviving the second summer divided by the number of emerged seedlings. Figure extracted from Mendoza et al. (2010).

These results suggest that regeneration in open areas or transition zones between forests and shrublands is more endangered by incoming changes than in closed-dense canopy forests. Indeed, species living in transition zones or ecotones are near the edge of their tolerances, and therefore are more vulnerable to global changes (Gea-Izquierdo et al., 2014; Noble, 1993) than within stands closed canopy vegetation. In terms of establishment and

survival, open areas and shrublands possess microhabitats with more light and less shelter for drought (than closed canopy forests microhabitats), which are microhabitats less favourable for holm oak recruitment and for ectomycorrhizal colonization (Gómez-Aparicio et al., 2005; Richard et al., 2009). Indeed, ectomycorrhizal colonization is higher under shelter trees (under shadow) than in open areas (Bingham and Simard, 2012), additionally, the number of fungal species sharing between late-stage plants (trees) is higher than between early stage plants (shrubs) (Taudiere et al., 2015), which enhances seedling establishment under closed canopy late-stage forests (Dickie et al., 2002).

On the other hand, the actual composition of Mediterranean forests is the result of past land-uses that nowadays determine the trajectory of the vegetation in the context of climate change (Figure 5.4). In the Mediterranean basin, there is a gradient from North to South with different periods of the industrialization that turned out on different land-uses (Acácio et al., 2007). The original Mediterranean forests colonized by holm and cork oaks were degraded by clearing, livestock grazing and agriculture activities (Terradas, 1999). In the 1960's, economic changes in the society (industrialization, migrations to cities, international markets) produced the abandonment of crops and pastures that were further colonized by shrubs species following the natural succession (Acácio et al., 2007). In the north of the Mediterranean basin (South France, Catalonia) the industrialization occurred earlier than in the South, and forest clearing and coppicing for agriculture or wood energy supply was progressively abandoned. Coppicing for wood production resulted in actual very dense *Q. ilex* forests with high number of sprouts per tree, and thus in microhabitats that seem favourable for holm oak recruitment under the expected drier conditions (as we showed in this PhD).

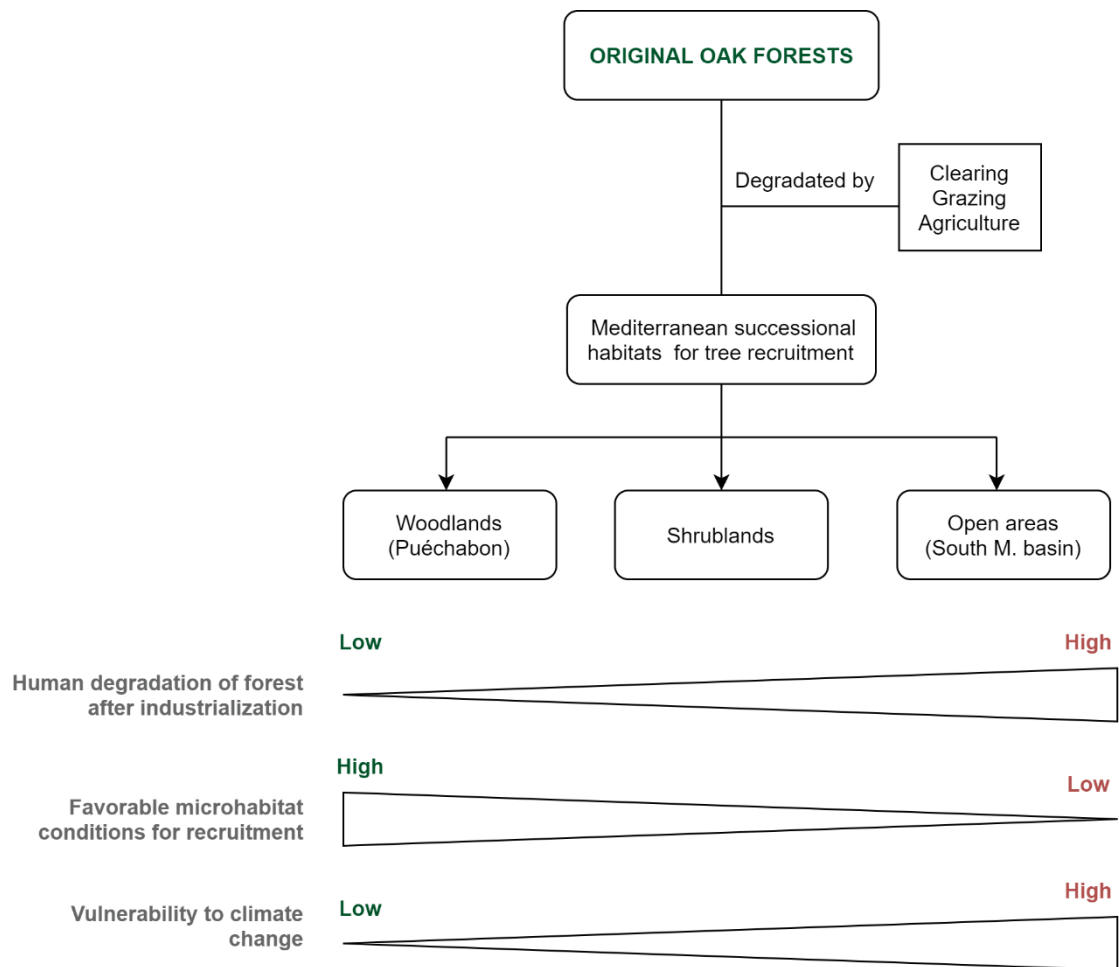


Figure 5.4. Schematic representation of the holm oak regeneration vulnerability to climate change according to its past land-uses. The different past land determines the vulnerability of holm oak forest regeneration to climate change.

In contrast, in the south of the Mediterranean basin (South of Spain, North of Africa) the industrialization occurred later in time, which turned out in an over-exploitation of forests resources that transformed the dominant holm oak forests into agroforestry systems and transitions zones between forests and shrublands (Joffre et al., 1999; Pulido et al., 2001) which are microhabitat less favourable to holm oak recruitment. Nowadays regeneration of holm oak facing climate change is more endangered in the South than in the North parts of the basin because the tree layer is less present and the climatic conditions for regeneration are less favourable due to the differences in annual precipitations across the latitudinal gradient. We can predict two potential scenarios for the South. If human activities remain low with conservation policies that controls land-uses and regeneration policies that counteract climate change effects, the persistence of oak forests, even with the threats of changes of climate, could be assured. However, if human activities continues combined with the increasing aridity

induced by climate change with associated wildfires, the recovery of oak forests will be impeded and may be transformed definitely into shrublands (Acácio et al., 2007).

I conclude that the positive effects of long-term increased drought on holm oak regeneration observed in my PhD cannot be extrapolated to other Mediterranean forest from the south of the basin, where *Q. ilex* is less present or is in mid-stages of ecological succession, and where the different past human activities and current disturbance regimes may not have maintained enough canopy cover for holm oak regeneration. To compensate the past land uses and to increase resilience under climate change in the southern parts of the basin, land-use and forest regeneration policies will be necessary for maintaining forest cover and enhance oak recruitment. Furthermore, if the climate scenarios of the most pessimist models becomes real, the regeneration of holm oak could be also compromised in the north of the basin since the facilitation and acclimation responses will be too costly for forest species leading to tree mortality and consequent transformation of forests into transition zones and shrublands with less oak regeneration success. As a last detail, we have to be cautious about the “positive” effects of survival found in this PhD because we evaluated survival in eleven-month old seedlings which is too short to conclude about the final survival, since seedlings depends mainly on acorn reserves in their first year, and more on environmental conditions on the following years, (Mendoza et al., 2009).

5.5 Perspectives

The first perspective of the work carried in my PhD would be to investigate the vulnerability of regeneration across the Mediterranean basin. To do so I would replicate the same germination experiment that I did in my PhD in the rainfall exclusion of Puéchabon (Chapter 2 and 3) across different rainfall exclusion experiments in the southern parts of the basin like in the experimental site of Prades (Catalonia) and in Los Alcornocales (Andalusia). It would be interesting to analyse the emergence and survival of *Q. ilex* seedlings as well as the ectomycorrhizal communities associated to roots across experimental sites and precipitation treatments to check whether the positive effects of increased drought on seedling survival are maintained along the Mediterranean basin.

A crucial perspective and further investigation of this PhD is the study of the potential heritability of different methylated regions (DMRs) found in mother trees to really evaluate

the role of epigenetics in the transgenerational adaptation to drought. For that I propose to analyse whether DMRs found in mothers could be also found in offspring, which could be done with Chop-PCR experiment (Dasgupta and Chaudhuri, 2019) in leaves collected in offspring (see Caveats in Annex). In addition, it would be interesting to evaluate the functionality of these DMRs in order to analyse different gene expressions among seedlings from different maternal origins, and under different drought treatments. If the identified differently methylated regions (DMRs) are stable, they could be considered as important loci (epialleles) for local adaptation to drought in *Quercus* species and be used in breeding programs of regeneration plans (Bräutigam et al., 2013). Finally, if one day *Q. ilex* genome is fully sequenced, it would be interesting to re-analyse the mapping of bisulfite reads and the identification of DMRs. This will allow us to complete the *Q. ilex* methylome and increase our understanding of how long-lived non-model species will respond to incoming climate changes contributing to the new emerging field of ecological epigenetics (Richards et al., 2017).

Recent research has demonstrated the effect of epigenetics in regulating the mechanisms that control the interactions between plants and microbes, and their influence on the survival and adaptation of plant responses to infections and symbiosis (Alonso et al., 2019; Zogli and Libault, 2017). Therefore, given the importance of ectomycorrhizal symbiosis for the survival of seedlings found in my results, I propose to study the potential role of DNA methylation on the expression of genes involved in the establishment of the symbiotic relationships and the defence against pathogens in *Q. ilex* seedlings through RNA-seq experiments. These effects could be first studied in mother trees and then in seedlings to evaluate the potential intergenerational transmission.

Last but not least, I propose the investigation of soil pathogens abundance in the soil of the experimental site of Puéchabon in both drought and control plots. In chapter 1 we assumed that wetter conditions in spring could increase the development of soil pathogens and therefore partially influence the higher mortality rates found under wetter conditions. However, we do not have evidence for this, and I propose that it should be investigated in order to understand the role of the balance between fungal mutualists and pathogens in determining the survival of *Q. ilex* seedlings under increased drought.

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ANNEX

7.1 Résumé de la thèse en français

INTRODUCTION

Les projections du changement climatique pour l'Europe suggèrent une augmentation de la température et des changements du régime des précipitations avec une augmentation de la fréquence des événements extrêmes tels que les sécheresses et les inondations, en particulier dans la région méditerranéenne (IPCC, 2014). Le changement climatique devrait donc entraîner des changements dans la distribution, la composition et la diversité des espèces forestières, avec des conséquences sur le fonctionnement et les services rendus par les écosystèmes forestiers (Lindner et al., 2010).

L'augmentation de température, combinée à une aridification, diminue la croissance des arbres et accélère leur mortalité dans les forêts du monde entier (Allen et al., 2010; Choat et al. 2012; Anderegg et al. 2016). Le changement climatique en cours va non seulement augmenter la mortalité des arbres mais devrait également avoir un impact sur l'établissement et la performance des semis, et donc menacer la régénération naturelle et la composition future des forêts (Lloret et al., 2004). En réponse à une sécheresse accrue, les arbres peuvent soit (i) ajuster de façon plastique leur développement et leur physiologie pour devenir plus résistants au stress (plasticité phénotypique), (ii) s'adapter aux nouvelles conditions (adaptation génétique), ou (iii) migrer vers d'autres régions où ils sont mieux adaptés, entraînant des extinctions locales. Parmi ces trois options, la plasticité phénotypique semble la plus rapide et la plus prometteuse pour la survie des arbres (Nicotra et al., 2010). Les modifications épigénétiques (changements dans l'expression des gènes mais pas dans la séquence d'ADN) induites par des stress environnementaux sont un mécanisme susceptible d'influencer la variation du phénotype et les réponses d'acclimatation à court terme (Bossdorf et al., 2007). Les variations phénotypiques épigénétiques peuvent être transmissibles d'une génération à l'autre, être adaptatives et influencer la microévolution et l'adaptation. Comme les changements épigénétiques peuvent se produire beaucoup plus rapidement que les mutations génétiques, ils peuvent jouer un rôle clé dans les réponses adaptatives aux changements rapides du climat.

En outre, les nouvelles conditions climatiques modifient également les cycles biochimiques du sol par des modifications de l'activité microbienne. L'activité microbienne est essentielle pour la nutrition des plantes, car elle est responsable de la décomposition de la

matière organique et de la transformation des nutriments en composés chimiques assimilables par les plantes. Du point de vue des communautés fongiques, des nombreuses études ont montré des changements dans la composition mais pas dans la richesse des espèces en réponse à l'augmentation du CO₂ (Fransson et al., 2007), ou des diminutions de la diversité et des changements dans la composition des communautés en réponse à l'augmentation de la sécheresse et au réchauffement (Herzog et al., 2013; Shi et al., 2002).

Il est essentiel de comprendre comment les forêts réagiront aux conditions climatiques futures prévues pour mieux anticiper les conséquences du changement climatique et élaborer des stratégies de gestion forestière appropriées. Dans cette thèse, je souhaite contribuer à ce domaine en étudiant comment des sécheresses plus intenses affecteront la régénération et la survie du chêne vert (*Quercus ilex*) ainsi que les communautés fongiques associées dans les forêts méditerranéennes.

Facteurs affectant le recrutement des arbres forestiers

Le succès du recrutement des arbres dépend fortement (i) de la quantité de réserves (sucres, protéines, hormones...) allouée aux graines par les arbres mères et qui influence positivement les taux de germination et de survie des semis (Khan and Shankar, 2001; Quero et al., 2007), (ii) des conditions du micro-habitat dans lesquelles les semis germent, et (iii) de leurs interactions biotiques avec les espèces de leur environnement.

Au sein d'une forêt, les micro-habitats diffèrent par l'hétérogénéité de la couverture de la canopée qui crée une gamme de conditions allant de trouées lumineuses à des abris très ombragés. Ces micro-habitats peuvent varier considérablement en termes de disponibilité de la lumière et d'humidité du sol. Les semis qui émergent sous la canopée des arbres bénéficient d'une température et d'une demande évaporatoire réduite par rapport aux semis sous les trouées de la canopée, ce qui peut réduire le déficit hydrique local et le stress des semis.

Les interactions biotiques peuvent (i) influencer positivement les performances des semis, comme dans le cas de la symbiose mycorhizienne et de la facilitation plante-plante, ou (ii) influencer négativement les performances des semis, comme l'herbivorie ou l'infection par un pathogène. Ci-après, je me concentrerai sur les interactions biotiques positives de la symbiose mycorhizienne, en raison de son rôle clé dans l'établissement des arbres.

Les mycorhizes sont des associations symbiotiques entre les champignons et les racines des plantes où les sucres tels que le glucose et le saccharose produits par la photosynthèse de

la plante sont fournis au champignon, et où le champignon fournit en retour à la plante hôte une capacité accrue d'absorption d'eau et de nutriments dans le sol, ce qui augmente potentiellement la résistance de l'hôte aux stress (sécheresse, salinité, agents pathogènes). Les mycorhizes jouent ainsi un rôle clé en augmentant la résistance à la sécheresse des plantes (Lehto and Zwiazek, 2011). Dans cette thèse, je me concentre sur les ectomycorhizes (ECM) car elles s'associent aux espèces ligneuses, comme le modèle de mon doctorat, le chêne vert.

L'établissement, la performance et la survie des semis sont favorisés par la colonisation ectomycorhizienne (Bingham and Simard, 2012; Rincón et al., 2007), en particulier par l'intégration dans un réseau commun de mycélium ectomycorhizien (Selosse et al., 2006 ; Nara et al., 2006). En effet, la plupart des plantes mycorhizées sont interconnectées par un réseau ectomycorhizien commun entre les plantes hôtes grâce à leurs mycéliums extramatriciel.

Agerer (2001) a décrit quatre stratégies d'exploration des ectomycorhizes en fonction des différences d'anatomie et de morphologie du mycélium extramatriciel qui rayonne dans le sol : exploration par contact, à courte distance, à moyenne distance et à longue distance. Un mycélium extramatriciel plus long est capable d'absorber les nutriments et l'eau à des distances plus grandes de la racine, augmentant ainsi la résistance à la sécheresse des hôtes. En effet, dans des conditions plus sèches les morphotypes avec un mycélium plus long sont favorisés par rapport aux ECM de contact (Bakker et al., 2006)

OBJECTIFS ET HYPOTHESES

L'objectif principal de cette thèse est d'étudier les effets de l'augmentation de l'intensité des sécheresses sur l'établissement, la performance et la survie des semis de chêne vert (*Quercus ilex* L.) dans un contexte d'aridité croissante causée par le changement climatique en cours. En détail, les objectifs de ma thèse sont d'étudier :

- (i) L'effet du micro-habitat où se développent les semis sur l'établissement, la survie et la performance des semis dans un contexte de sécheresse accrue.
- (ii) Les effets de l'aridification sur les communautés fongiques du sol susceptibles d'influencer la survie des semis en s'associant aux racines.
- (iii) Les conséquences physiologiques et épigénétiques de l'acclimatation des arbres mères à la sécheresse, en faisant l'hypothèse que les réponses épigénétiques peuvent être transférées aux plantules et améliorer leur survie.

Cette thèse est structurée en trois chapitres :

Chapitre 1 - Les effets du micro-habitat et de la colonisation ectomycorhizienne sur l'établissement et la survie des semis en contexte de sécheresse accrue. Dans ce chapitre, je présente les résultats d'une étude expérimentale de terrain visant à étudier le rôle des effets maternels (masse des glands), de la disponibilité de la lumière, de l'humidité du sol et des types d'exploration des ectomycorhizes (ECM) sur la germination des glands, le recrutement et la croissance des semis.

Chapitre 2 - Effets d'une augmentation à long-terme de la sécheresse sur la composition de la communauté ectomycorhizienne dans le sol et associée aux des racines des semis. Dans ce chapitre, je me concentre sur la quantification de l'impact d'une sécheresse accrue sur la composition de la communauté fongique du sol, ainsi que sur l'existence de liens potentiels entre la diversité fongique, et la survie et la croissance des semis, avec un accent particulier sur la guilda ectomycorhizienne.

Chapitre 3 - Effets à long terme de la sécheresse sur les réponses physiologiques et épigénétiques (méthylation de l'ADN) des arbres mères porteurs de graines, avec une transmission intergénérationnelle potentielle. Dans ce chapitre, je présente les résultats d'une étude de terrain qui a évalué les réponses physiologiques à la sécheresse ainsi que les changements de méthylation de l'ADN chez plusieurs individus adultes de *Q. ilex* soumis à des régimes de précipitations différents, dans le but de tester les liens potentiels entre les réponses physiologiques et les régions différemment méthylées (DMR) du génome.

SITE EXPERIMENTAL

Toutes les expériences menées dans le cadre de cette thèse ont été réalisées sur la plate-forme expérimentale CNRS de Puéchabon qui est située à 35 km au nord-ouest de Montpellier. La state supérieure de la forêt est largement dominé par le chêne vert (*Q. ilex*) qui représente plus de 90% de la couverture forestière, et le sous-étage forme une couche arbustive clairsemée avec un pourcentage de couverture inférieur à 25%. Le climat est méditerranéen, les précipitations se produisant principalement en automne et en hiver, avec environ 80 % des pluies entre septembre et avril. Les précipitations annuelles moyennes varient de 550 mm à 1549 mm (moyenne de 901 mm sur les 28 dernières années), et la température annuelle moyenne varie de 11,5°C à 14,1°C (moyenne de 13,1°C sur la même période).

Le site expérimental de Puéchabon comprend une expérience d'exclusion des précipitations qui réduit continuellement l'apport net de précipitations au sol d'environ 29 % par rapport au traitement témoin depuis mars 2003 (Limousin et al., 2008). L'expérience d'exclusion des précipitations est répliquée sur trois blocs (R1, R2, Sc) distants d'environ 100 m les uns des autres, et situés sur un plateau sans écoulement latéral. Dans chaque réplicat, deux parcelles de 100 m² chacune sont soumises à des régimes de précipitation différents : l'une est un traitement témoin recevant les précipitations naturelles et la seconde est soumise à une exclusion partielle des précipitations. L'exclusion des précipitations est réalisée par des gouttières en PVC suspendues sous la canopée et couvrant environ 33 % de la surface au sol. Dans le traitement de contrôle, les mêmes gouttières sont suspendues à l'envers pour égaliser l'effet du microclimat sur le sous-étage dans les deux traitements.

RÉSULTATS

Dans cette thèse nous avons identifié trois voies qui influencent la survie des semis de chêne vert soumis à une sécheresse accrue.

La première voie qui influence la survie des semis correspond aux facteurs environnementaux du micro-habitat, tels que la disponibilité de la lumière et de l'humidité du sol, qui modulent les effets de la sécheresse et influencent la colonisation des racines par différents morphotypes ectomycorrrhiziens (chapitre 1). Plus précisément, la disponibilité de la lumière au printemps, lorsque les sols sont saturés en eau, favorise la levée et la croissance des semis, tandis que l'ombre réduit le risque de dessèchement pendant la sécheresse estivale lorsque l'eau manque et que la lumière augmente l'évaporation.

La deuxième voie qui affecte la survie des semis est la réponse des communautés fongiques à l'augmentation de la sécheresse en termes de diversité et de composition. Mon étude a montré un effet positif de l'augmentation de la sécheresse sur la diversité des communautés fongiques de toutes les guildes. Concernant la guildes fongique ectomycorhizienne associée aux racines, sa réponse à l'aridification a eu un impact positif sur la survie des semis de par (i) l'augmentation de la richesse des espèces d'ectomycorrhizes, (ii) l'augmentation de la colonisation des espèces ectomycorhiziennes à morphotype plus long, et (iii) l'augmentation de l'abondance de deux génotypes de *Cenococcum geophilum* qui ont un impact positif sur la survie des semis de *Q. ilex* (chapitre 1 et chapitre 2).

Enfin, la troisième voie par laquelle la sécheresse peut influencer la survie des semis est l'acclimatation plastique à la sécheresse des arbres mère porteurs de graines. Les arbres exposés à des sécheresses récurrentes peuvent s'acclimater plastiquement aux conditions de sécheresse et transmettre ces réponses à la génération suivante. Dans mon étude, j'ai observé des réponses plastiques à la sécheresse potentiellement régulées par des mécanismes épigénétiques chez les arbres adultes, cependant, la potentielle transmission épigénétique intergénérationnelle n'a pas pu être évaluée. Les arbres porteurs de graines présentent des réponses plastiques à la sécheresse qui confirme la capacité d'acclimatation de cette espèce à la sécheresse. Les réponses consistaient en des modifications physiologiques et morpho-chimiques de leurs feuilles qui leur permettaient d'être plus efficaces dans l'utilisation de l'eau, de l'azote et du carbone. Nous avons également observé une compensation potentielle des activités physiologiques entre l'automne (après la saison de sécheresse) et le printemps (avant le début de la saison de sécheresse). Même si les arbres issus du traitement à sec étaient encore négativement affectés par les effets de la sécheresse précédente durant l'automne, leurs nouvelles feuilles produites au printemps suivant présentaient, des taux d'assimilation du carbone plus élevés et des efficacités d'utilisation de l'azote et du carbone plus importantes par rapport aux feuilles des arbres témoins. Les mécanismes physiologiques qui sous-tendent cette plasticité différente à la sécheresse entre les traitements n'ont pas été analysés directement, mais nous avons observé des corrélations significatives entre les réponses plastiques des arbres à la sécheresse et les différences de méthylation du génome dans les régions différemment méthylées (DMR). Ces corrélations suggèrent un rôle épigénétique potentiel dans la régulation des réponses plastiques et les effets mémoire de la sécheresse chez les arbres.

DISCUSSION ET CONCLUSIONS

Les effets positifs de l'aridification sur la survie des semis constatés dans notre expérience étaient inattendus. Même si la sécheresse estivale était la principale cause de mortalité des semis, notre traitement d'exclusion partielle de la pluie n'a pas augmenté davantage la mortalité des semis. Ces résultats contrastent avec les résultats précédents où la survie des espèces d'arbres était affectée négativement par l'augmentation expérimentale de la sécheresse (López-Díaz et al. 2019 ; Gómez-Aparicio et al. 2008 ; Hallet et al. 2011). En outre, notre étude a révélé que la diversité fongique était plus élevée dans des conditions de

sécheresse accrue que dans les conditions témoins, ce qui contraste également avec la littérature. Des études antérieures analysant l'effet de la sécheresse sur les communautés fongiques ont constaté des diminutions de la diversité et des changements dans la composition des communautés (Toberman et al. 2008 ; Nickel et al. 2017). D'autres études ont montré que les réductions de l'humidité du sol affectaient la composition mais pas la diversité des communautés fongiques (Schmidt et al. 2017), notamment l'étude de Richard et al. (2011) sur notre site d'étude après cinq années de traitement d'exclusion des précipitations.

Pour réconcilier cette divergence entre les résultats de notre étude et ceux de la littérature, nous faisons l'hypothèse que la longue durée du traitement expérimental pendant quinze ans sur notre site d'étude, a permis aux espèces fongiques de s'adapter à l'aridification et donc d'avoir de meilleures performances que les espèces fongiques du témoin en période de sécheresse. Ce type de réponse n'a pas pu être observé dans les expériences à plus court terme où les espèces n'ont pas eu le temps de s'adapter aux nouvelles conditions expérimentales. La longue durée de traitement avec des sécheresses constamment plus fortes dans notre expérience de réduction des précipitations pourrait avoir exercé des pressions sélectives et filtré les espèces tolérantes et adaptées à la sécheresse, au détriment des autres toujours présentes dans le traitement témoin. Ces espèces fongiques sélectionnées et adaptées à la sécheresse semblent non seulement ne pas être affectées par l'aridification, mais leur diversité a même connu une augmentation dans le traitement sec qui, dans le cas de la guildes ectomycorhizienne, a facilité la survie des semis.

Une autre explication possible pour les taux de survie plus élevés des semis dans des conditions plus sèches, serait l'infection des racines par des agents pathogènes du sol favorisée par des conditions plus humides (Brassier et al. 1992). En effet, les espèces du genre *Phytophthora* sont parmi les organismes causant les dommages et les maladies les plus graves aux racines fines des arbres hôtes, et sont considérées comme le principal facteur de déclin du chêne vert en Espagne et au Portugal (Sánchez et al. 2002 ; Concorbado et al. 2014). Nous pouvons faire l'hypothèse que le développement des *Phytophthora*, qui est favorisé par les conditions humides, pourrait être plus important dans le traitement témoin que dans le traitement sec et donc entraîner une plus grande infestation et mortalité des semis que dans le traitement sec.

Nos résultats dans le cadre de l'hypothèse du gradient de stress

Les effets positifs de l'aridification sur l'établissement des semis de chêne vert peuvent être interprétés dans le cadre de l'hypothèse du gradient de stress (Stress Gradient Hypothesis, en anglais). L'hypothèse du gradient de stress postule que les interactions de facilitation et de compétition varient inversement selon les gradients de stress (Bertness & Callaway 1994). La facilitation est maximale à des niveaux de stress modérés et elle diminue lorsque les niveaux de stress sont extrêmes, tandis que les compétitions augmentent lorsque le stress est à son maximum ou lorsqu'il n'y a pas de stress.

Dans notre étude, la survie des semis a augmenté sous l'effet d'une augmentation à long-terme de la sécheresse en raison d'une plus grande richesse des communautés ectomycorhiziennes, donc lorsque la facilitation des plantes par les champignons est plus élevée. Nous supposons que la réduction de 29 % des précipitations appliquée dans notre expérience est suffisamment importante pour augmenter la facilitation entre les espèces, mais pas trop importante pour exacerber la compétition plus que la facilitation, comme cela pourrait se produire dans des scénarios de changement climatique plus graves. Cette hypothèse pourrait expliquer la plus grande richesse en espèces des communautés fongiques et l'augmentation du taux de survie des semis qui en découle. En ce qui concerne les arbres adultes, nous pouvons émettre l'hypothèse que l'acclimatation à la sécheresse leur a permis de développer des réponses plastiques par lesquelles ils augmentent leur efficacité d'utilisation de l'eau et des nutriments et compensent les effets néfastes d'un stress hydrique plus sévère, ce qui pourrait potentiellement induire une plasticité adaptative se transmettant à leur descendants.

Cependant, si l'aridification devient plus sévère que celle simulée par notre expérience de réduction des précipitations (comme le suggèrent les tendances temporelles de l'indice de stress hydrique à Puéchabon à l'échéance de plusieurs décennies, Lempereur et al. (2017)), nos résultats pourraient éventuellement montrer des tendances opposées. Si le stress hydrique s'approche des limites physiologiquement tolérables par les arbres, la facilitation entre les espèces et donc le maintien de la relation symbiotique pourrait devenir trop coûteuse pour le champignon et la plante. La limitation des ressources exacerberait alors la concurrence entre les espèces fongiques, ce qui aurait un impact négatif sur la diversité fongique et la survie des semis. Dans un tel scénario, on peut imaginer que les communautés fongiques seraient touchées (i) directement par les conditions environnementales extrêmes qui réduisent

l'humidité du sol et les activités enzymatiques microbiennes (Sardans & Peñuelas 2005), et (ii) indirectement par la réduction des relations symbiotiques due à la réduction de l'allocation de carbone par l'arbre hôte (Courty et al., 2010). Dans le cas des arbres hôtes, on peut imaginer qu'un stress hydrique plus sévère ne pourrait pas être compensé par l'acclimatation plastique sous contrôle épigénétique et que les effets néfastes des sécheresses précédentes (cavitation hydraulique, défoliation, mortalité des tissus) réduiraient durablement la performance de l'arbre et son bilan de carbone. Les arbres hôtes pourraient alors réduire leur investissement en carbone dans la symbiose mycorhizienne, ce qui augmenterait encore leur vulnérabilité à la sécheresse (diminuant la force de facilitation).

7.2 Caveats

Along the three years of thesis, my PhD project dealt with unforeseen ecological and methodological limitations that did not allow me to achieve all the initial objectives. This was particularly the case for the experimental work (germination experiment *in situ* as well as in controlled environment at the CNRS Ecotron²) aiming to quantify the potential adaptive effects via intergenerational transmission from mothers to offspring, an aspect that could not be assessed in this study due to several shortcomings. The first reason was that the long and intense summer droughts of 2017 and 2018 reduced significantly the number of viable acorns produced by the trees from the Puechabon experimental site to the point that the collected acorns were not sufficient for an experiment with adequate replication. In consequence, the germination and establishment experiment of chapter 1 was done with acorns from trees outside the Puechabon experimental treatments, and thus, without contrasting drought legacies. This unforeseen circumstance prevented the planned assessment of the role of intergenerational transmission of drought legacies from mothers in the establishment and survival of *Q. ilex* seedlings *in situ*, as well its potential adaptive epigenetic role in seedlings.

In autumn 2018, the very few viable acorns produced were collected within the long-term drought treatments of the Puechabon experimental site and were germinated and transplanted in the Ecotron CNRS facility. The aim of this experiment was to use the Ecotron to impose drought and recovery events to the transplanted seedlings originating from mother trees in different rainfall exclusions treatments. Lack of sufficient acorns and logistic problems at the Ecotron facility forced us to combine our experiment with another project that also included a drought treatment. However, the intensity of drought stress imposed in this experiment was not sufficiently severe for *Q. ilex* seedlings, as indicated by the soil moisture measurements and the seedling physiological measurements. Whilst we detected some changes in physiological measurements (photosynthesis, stomatal conductance ...) in these seedlings before and after the experimental droughts, no significant drought legacy effects were found. Nevertheless, I collected the leaves from these seedlings for DNA methylation analysis. The idea was to investigate whether the DMRs found in mother trees were also

² The CNRS Ecotron facility is a laboratory of the Institute of Ecology and Environment (INEE), where experimental units allow the simulation and measurement of a wide range of environmental scenarios. <https://www.ecotron.cnrs.fr/en/mesocosms/>

present in seedlings. This can be achieved through a Chop-PCR experiment that uses methylation restriction enzymes that cut DNA or not depending on the methylation the DMR. However, although I tried multiple times, I obtained incoherent results on the activity of the enzymes even for the controls, so this analysis could not be completed successfully to test our hypothesis. Collectively, the reasons mentioned above made me decide not to introduce this data in my thesis. This outcome is certainly a consequence of the very interdisciplinary nature of my PhD that included a wide range of different methodologies and technical skills, but with limited time for becoming proficient in all of them.