



UNIVERSITÀ DEGLI STUDI DI TRIESTE

XXXII CICLO DEL DOTTORATO DI RICERCA IN AMBIENTE E VITA

Effects of biodiversity on soil C dynamics, tree response to extreme events and water-use efficiency in forest ecosystems

Settore scientifico-disciplinare: AGR-05

DOTTORANDA

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ANNO ACCADEMICO 2018/2019

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GENERAL SUMMARY

Forests provide fundamental services to the humanity, contribute to climate regulation, water provision and represent one of the most important biodiversity reservoir on the Earth. Climate change due to anthropogenic greenhouse gases emission is altering forest ecosystem functioning and services through significant changes in the frequency and intensity of extreme events (i.e. drought, floods, fires, heatwaves).

This thesis focuses on the role of tree species richness and functional diversity in supporting carbon sequestration and in mitigating the possible negative effects of extreme events, by increasing stand resistance and/or resilience. We also considered the long-term effects of atmospheric CO₂ increase on growth and water-use efficiency in old-growth forests in the Balkans. To do this, we combined soil C stocks assessments and dendrochronological measurements with stable (C) isotope analysis and with the calculation of functional diversity indexes.

We found a positive effect of tree species richness and functional diversity in enhancing soil C sequestration after land use change from croplands to tree plantations. On the contrary, we detected only a partial positive effect of tree richness on growth during extreme drought events. Finally, we found that the old-growth forests at the studied sites are still actively fixing CO₂ and their efficiency in water use is increasing. However, our data support the idea that these positive trends might reach a maximum or even undergo a decline in the next future.

1. INTRODUCTION

Atmospheric composition and, in particular, CO₂ concentration was relatively stable over the last 10,000 years, around 260-280 ppm (Köhler *et al.* 2017). After the second industrial revolution, CO₂ concentration has been raising up to the actual 408.53 ppm (data from Mauna Loa Observatory, October 2019, <https://www.esrl.noaa.gov/gmd/ccgg/trends/>). Moreover, according to the Intergovernmental Panel on Climate Change (IPCC), the anthropogenic greenhouse gases (GHGs) emissions have reached the highest levels in the last 800.000 years (49 ± 4.5 GtCO_{2eq} yr⁻¹ in 2010; Figure 1.1).

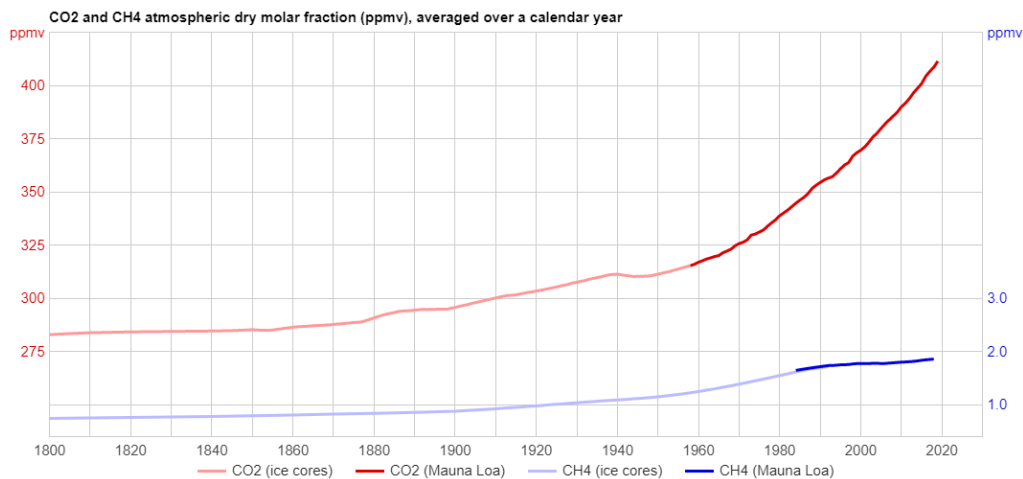


Figure 1.1 - CO₂ and methane (CH₄) concentration (ppm) trends since 1800. Source: sealevel.info.

GHGs can modify the radiative climate forcing (i.e. the rate of energy change per unit area of the globe; Rockström *et al.* 2009), thus altering the energy balance of our planet with direct effects on the mean annual temperature ($+0.87\pm 0.12$ °C in period 2006-2015; IPCC 2018), on the distribution of total precipitation and on the frequency and intensity of extreme events worldwide (Herring *et al.* 2015; Meehl *et al.* 2000; Parmesan *et al.* 2000; Rebetez *et al.* 2006). In particular, extreme droughts, heat waves, floods, forest fires, hurricanes have become more frequent and more violent, resulting in strong social impacts and economic losses. All these changes are altering natural ecosystems, their functioning and the services they can provide to the humanity.

Forests cover about 30% of the biosphere (FAO 2018), harbouring about 60% of all terrestrial plant species (Huang *et al.* 2018) and representing an important sink for atmospheric CO₂ (Canadell and Raupach 2008; McMahon *et al.* 2010). They are experimenting, and are becoming more vulnerable, to extreme climatic events such as severe drought periods, heat waves and wildfires, which can cause changes in species composition, tree mortality rate, growth, ecosystem functioning, acclimation and adaptation processes (Anderegg *et al.* 2012; Lindner *et al.* 2014; Seidl *et al.* 2014). As a result, climate change is modifying the services forests can provide to the human society. Such services are usually grouped in four main categories: provisioning (e.g. wood production), regulating (e.g. carbon (C) sequestration, microclimate), supporting (e.g. biogeochemical cycling, water retention and redistribution) and cultural (e.g. recreational, human health) benefits (Grossiord *et al.* 2014c; Millar and Stephenson 2015; Thompson *et al.* 2011).

Biodiversity in forests have a fundamental role in regulating ecosystem functioning and services. Indeed, several studies carried out both in natural ecosystems and in controlled experiments have found positive effects of biodiversity on productivity (Liang *et al.* 2016; Ouyang *et al.* 2019), on biogeochemical cycles (Li *et al.* 2019), on soil C sequestration enhancement (Lange *et al.* 2015), on resistance and resilience to climatic extremes (Isbell *et al.* 2015) and on both microbial and pedofauna communities (Chen *et al.* 2019; Dinnage *et al.* 2012; Ebeling *et al.* 2014). However, most of these studies are often affected by the lack of one or more of the following basic requirements (Baeten *et al.* 2013; Pretzsch *et al.* 2017): orthogonality, i.e. the detection and quantification of diversity effects against disturbing factors; representativeness, i.e. the proportion of variation in the studied population captured by the design; comprehensiveness, i.e. the spectrum of ecosystem functions and services considered in the study.

Dendrochronology is one of the main tool used to address the impacts of climate change on forests as it allows a retrospective evaluation of tree growth patterns related to climatic variability and extreme events. Each tree-ring is the result of plant-intrinsic factors (i.e. photosynthetic rate, water potential regulation, hormonal regulation) and plant-external factors (climate, pathogens, stress

factors) that can modulate quantity (i.e. width) and quality of annual wood (De Micco *et al.* 2010; Deslauriers and Morin 2005; Lupi *et al.* 2010; Figure 1.2). For example, hot and dry vegetative seasons can lead plants to important hydraulic deficits coupled to drops in photosynthetic rate. As a result, annual tree-rings will be thinner than those formed during favourable seasons (Gao *et al.* 2018).

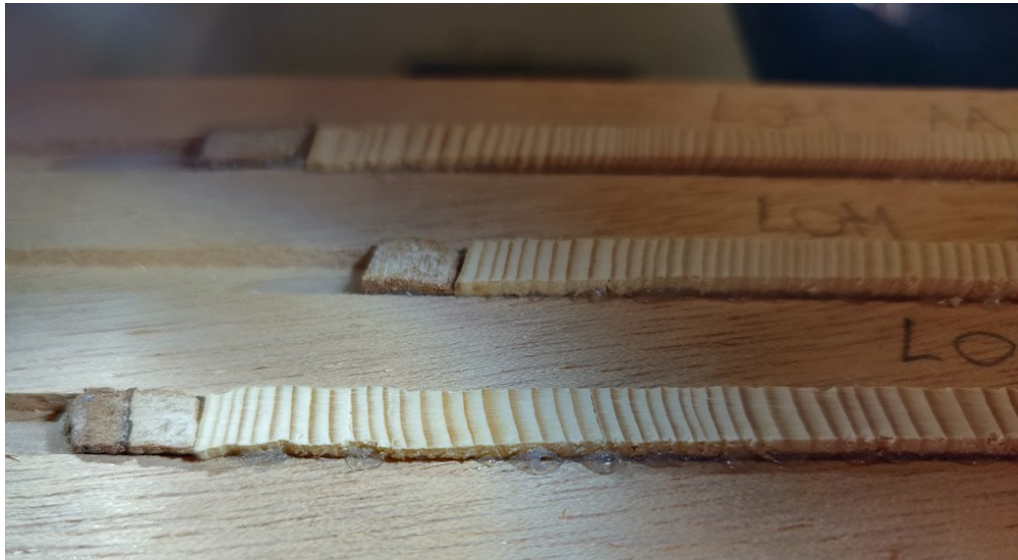


Figure 1.2 - Example of tree-ring cores with relative ring-width variability in different years.

Dendrochronology is often coupled with stable isotope analysis to get a deeper insight on the impact of climate change on tree growth and physiology. As far as C is concerned, there are about 15 different isotopes, but only two of them are stable: ^{12}C (98.93% of total carbon) and ^{13}C (1.07%). They can be found in different proportions in organic and inorganic compounds and this ratio can be measured using the $\delta^{13}\text{C}$ (‰) notation, namely the ratio of the proportions of heavier and lighter isotope of a sample to a standard. The standard represents a specific material with an isotopic signature ($\delta^{13}\text{C}$) of 0‰. For C isotopes, the standard is the marine limestone fossil Pee Dee Belemnite (PBD). Stable C isotope signature variation in organic and inorganic matter depends on fractionation processes, that is, the change from source to product in $^{13}\text{C}/^{12}\text{C}$ ratio after temporal, physical or chemical processes (Fry 2006). Tree response to climatic variability can be reflected in changes in the above mentioned fractionation processes and thus in the isotopic signature of the tree rings. For example, during hot and dry days, trees reduce stomatal conductance in order to avoid excessive water loss through transpiration. The consequence is a lower discrimination to the heavier C isotope (i.e. ^{13}C) by

RUBISCO enzyme and an increase in $\delta^{13}\text{C}$ in the produced organic matter. Thus, isotopic analyses represent a useful tool in natural ecosystems to disentangle complex dynamics.

2. THESIS OUTLINE

After this general introduction, the thesis is made up of three chapters representing three different research papers (one already published and two ready for submission).

The first paper (Chapter 3) assesses the impact of tree species richness and functional diversity on soil C dynamics in six deciduous plantations in Friuli-Venezia Giulia plain (Italy) by using stable C isotope analysis.

The second paper (Chapter 4) investigates the effects of species richness on tree response to extreme drought periods. The study was carried out in the same deciduous plantations of the first paper, coupling dendrochronological analyses with isotopic analyses.

The third paper (Chapter 5) analyses the long-term effects of the increase in atmospheric CO₂ concentration on tree growth and water-use efficiency in three old-growth forests in the Balkans using dendrochronology and isotopic analysis.

Finally, the overall conclusions are reported in Chapter 6.

3. TREE DERIVED SOIL CARBON IS ENHANCED BY TREE SPECIES RICHNESS AND FUNCTIONAL DIVERSITY¹

Summary

We assessed the impact of tree species richness (SR) and functional diversity (FD) on soil carbon (C) accumulation in plantations. We selected six mixed plantations established in 1999 on continuous maize field soils. Plantations differed only in the number of species whereas climatic conditions, stand age, tree density and soil type were the same. In each plantation, four random plots of 400 m² each were identified. In each plot and in the nearby maize fields, we collected soil cores to quantify organic C content, soil bulk density and $\delta^{13}\text{C}$. We then calculated the proportion of new and old C by using a mass balance approach. Total soil C stock significantly increased 19 years after tree planting (+12%). Most of this increase was detected at 0-15 cm and, on average, almost half of the soil C derived from the trees. Both SR and FD had a positive and significant effect on the proportion of C derived from the current forest vegetation. Plantations with higher biodiversity, either in terms of SR or FD, enhance the proportion of C derived from trees.

¹ Palandrani C, Alberti G (2020) Tree derived soil carbon is enhanced by tree species richness and functional diversity. *Plant and Soil* 446: 457–469.

3.1 Introduction

The human alteration of the global environment has caused, and is still causing, widespread changes in the global distribution of species and habitats, with possible large impacts on biodiversity levels (Cardinale *et al.* 2012; Schmid *et al.* 2009). A long history of ecological experimentation and theories supports the idea that ecosystem functions (EFs) and services (ESs) are strongly related to biodiversity (Balvanera *et al.* 2006; Cardinale *et al.* 2002; Hooper *et al.* 2005; Isbell *et al.* 2011; Loreau *et al.* 2001; Mace *et al.* 2012; Nadrowski *et al.* 2010). In particular, several studies have shown that increasing species richness positively affects productivity (Liang *et al.* 2016; Brassard *et al.* 2011; Ouyang *et al.* 2019), biogeochemical cycles and soil carbon (C) sequestration (Lange *et al.* 2015; Li *et al.* 2019), resilience and resistance to climatic extremes (Isbell *et al.* 2015; Pretzsch *et al.* 2013), microbial biomass (Chen *et al.* 2019) and pedofauna diversity (Dinnage *et al.* 2012; Ebeling *et al.* 2014; Haddad *et al.* 2001). However, most of the studies performed on forests are mainly represented by large-scale forest inventories (Gamfeldt *et al.* 2013; Vilà *et al.* 2013), long-term forest plots (Pretzsch *et al.* 2015) or worldwide meta-analysis (Chen *et al.* 2019; Paquette and Messier 2011; Piotta 2008; Zhang *et al.* 2012). Even though their main results highlight a positive effects of diversity on all the above-mentioned EFs and ESs, they are often affected by the lack of one or more of the following basic requirements (Baeten *et al.* 2013; Pretzsch *et al.* 2017): orthogonality, i.e. the detection and quantification of diversity effects against disturbing factors; representativeness, i.e. the proportion of variation in the studied population captured by the design; comprehensiveness, i.e. the spectrum of EFs and ESs considered in a study. Indeed, most of these studies are usually affected by climatic and environmental heterogeneity, low number of considered tree species, incomparable species identity and/or different stand ages. *Ad hoc* experiments, considering a wider spectra of tree species richness levels within defined climatic conditions and soil characteristics, have been only recently established (Huang *et al.* 2018; Scherer-Lorenzen *et al.* 2007), but most of them have been mainly considering aboveground processes (i.e. primary productivity), while little is still known

about the impact of tree diversity on below-ground processes and soil C sequestration in forest ecosystems (Li *et al.* 2019).

Soil can store far more C than plants tissues and represents an important sink for atmospheric CO₂ (Jobbágy and Jackson 2000). Soil C stock results from a dynamic equilibrium between C inputs (i.e. aboveground litter, dead roots and root exudates) and outputs (i.e. microbial decomposition and leaching). Therefore, this balance may be affected by changes in soil physical and chemical properties, species composition, plant growth through litter production, persistence of recalcitrant compounds or assemblage of microbial community (Jastrow *et al.* 2007). As far as this last aspect is concerned, soil microbial biomass and activity show a significant response to changes in species richness and functional trait diversity (Alberti *et al.* 2017; Chen *et al.* 2019; Handa *et al.* 2014; Thakur *et al.* 2015; de Vries *et al.* 2012). In fact, different tree species mixtures influence litter decomposition directly through species-specific litter traits (i.e. nutrient content, physical traits) (Deveau *et al.* 2018; Santonja *et al.* 2017) and, indirectly, through distinct modifications of the local micro-environment (Joly *et al.* 2017). Thus, changes in litter decomposition may translate into significant changes in soil C stocks along a tree species richness or functional diversity gradient. However, detecting such responses is challenging, as C pools only change slowly (Lange *et al.* 2015; Smith 2004), soil heterogeneity is usually large (Schrumpf *et al.* 2011), and the processes involved are complex (Cotrufo *et al.* 2015; Manzoni *et al.* 2012). In this context, C stable isotopes may represent a useful tool to detect the expected little changes in soil C stocks along species richness gradients (Ehleringer *et al.* 2000), especially if mixed deciduous plantations (C3 plants) have been established on soil with a strong C4 signal (i.e. maize cultivated soil). In fact, as ¹³C signal from C3 plants is significantly different from those in soil with C4 crops (ca. -28‰ and ca. -20‰, respectively), the relative contribution of new vs. old soil organic C (SOC) can be better quantified using the mass balance of stable isotope contents instead of looking at the overall soil C stock change (Del Galdo *et al.* 2003). In Friuli Venezia-Giulia plain (North Eastern Italy), mixed deciduous plantations with different mixture levels have been established on previously maize cultivated soil with a strong C4 isotopic C

signature in the last 20 years and offer a unique opportunity to study the impact of species richness on belowground processes and C sequestration. In addition, continuous maize fields were maintained in the surroundings (Del Galdo *et al.* 2003). Among these plantations, we selected stands differing only in the number of species (3, 4, 6, 7, 8 and 9) whereas climatic conditions, stand age (19 years), tree density and soil characteristics were exactly the same as well as we selected nearby maize fields as controls. The aim of our study was to quantify the impact of species richness and functional diversity on soil C accumulation after land use change by combining traditional assessment of soil C stocks and stable isotopes. We hypothesized that tree mixtures with higher biodiversity, either in terms of tree species richness or functional diversity, show an increase in soil C sequestration about 20 years after land use change.

3.2 Materials and methods

3.2.1 Study area

Six nearby deciduous plantations established 20 years ago (1999) in Friuli Venezia-Giulia plain (Italy; 46°5'50''N, 13°1'7''E, 121 a.s.l.) on former arable lands (i.e. maize) were selected. The stands differed only for their surface area and number of tree species (3, 4, 6, 7, 8 and 9) whereas initial tree density ha⁻¹ were the same. None irrigation, fertilization and pruning have been done since planting. Climatic conditions as well as soil characteristics were the same at all sites: mean annual temperature was 13.3°C, mean annual precipitation was 1500 mm and soil was an alluvial mesic Udifluent (sand 58%, loam 28%, clay 14%).

The continuous maize cultivation in the surrounding croplands preserved the typical isotopic signature of C4 plants, here used as background value (Del Galdo *et al.* 2003).

3.2.2 Sampling strategy

In each plantation four random plots of 400 m² each were identified. In each plot, tree species and abundance were assessed and tree diameter at 1.30 m (d.b.h.) and total height were measured. In

summer 2017 and summer 2018, three healthy and fully-developed leaves from three randomly chosen individuals for each species in each plot were sampled, sealed and stored together in cool-bags in order to prevent weight loss, until measurements in laboratory.

Five soil samples up to 15 cm were collected in each plot and in the respective nearby maize field with a manual soil auger (MSA samples). Moreover, one soil core per plot and four soil cores in the nearby maize field up to 60 cm depth were sampled using a petrol driven pneumatic auger (PPA samples; Eijkelkamp, the Netherlands) to quantify organic carbon (C) content, soil bulk density and isotopic signature of SOC at different depths (0-15; 15-30; 30-45; 45-60 cm).

3.2.3 Leaf analysis

Once in lab, leaves without petiole were fresh-weighted, dried for 48 hours at 60°C, dry-weighted, ball-milled and stored in plastic vials.

Before drying, leaves were scanned and leaf area was measured by analysing each picture with ImageJ software (ImageJ 1.51j8, Wayne Rasband, National Institutes of Health, USA, <http://imagej.nih.gov/ij>, Java 1.8.0_112). Specific leaf area (SLA; cm² g⁻¹) was calculated as ratio between leaf surface and leaf dry weight. Such a variable is well-related with tree growth rate and, thus, with tree biomass contribution to soil C dynamics (Poorter and Remkes 1990).

Leaf thickness (TH; mm) was computed as (Vile *et al.* 2005):

$$TH \approx \frac{1}{SLA \times LDMC}$$

where LDMC is the leaf dry matter content. TH is another functional trait related with tree growth (Poorter 1990), productivity (White and Montes 2005) and ecological performance (Diaz *et al.* 2004). Finally, C and nitrogen (N) content, C to N ratio, C and N isotopic signature ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively) of each dry sample were measured using a CHNS Elemental Analyser (Vario

Microcube, © Elementar) coupled to a stable isotope ratio mass spectrometer (IRMS; Isoprime 100, © Elementar).

3.2.4 Soil sample analysis

In the lab, MSA samples were air-dried, sieved at 2 mm, ball-milled and stored in plastic vials for chemical analysis. As said, soil cores (PPA samples) were divided in four different horizons, air-dried and sieved at 2 mm. Then, soil bulk density (kg m^{-3}) was calculated for each layer as the ratio between the weight of sieved soil and sample total volume. Subsamples of sieved soil were taken, ball-milled and stored in plastic vials for the further chemical analysis.

Before analysis, all soil samples were treated with HCl to eliminate carbonates (Nieuwenhuize *et al.* 1994). Then, C and N content, C to N ratio and $\delta^{13}\text{C}$ were measured as previously described for leaf samples.

3.2.5 Data analysis

Total leaf biomass (t ha^{-1}) was computed from leaf area ($\text{m}^2 \text{ ha}^{-1}$) derived using allometric relationships from literature (Table 3.1) and leaf mass per area (LMA [g cm^{-2}] = SLA^{-1}) measured on the sampled leaves.

Species richness was expressed as the total number of tree species sampled in each single plot. Functional diversity can be mathematically expressed using several approaches and indices (Laliberté and Legendre 2010; Mason *et al.* 2005; Petchey and Gaston 2002, 2006; Villéger *et al.* 2008). In our study, we computed functional dispersion index (FDis), i.e. the mean distance in a multidimensional trait space of individual species to the centroid of all species, weighted by their relative abundances (Laliberté and Legendre 2010). Species distance was weighted by the basal area of each species within the mixture thus to take into account the different sizes that tree species reached after 19 years of growth. FDis was computed using the R-package FD (Laliberté *et al.* 2014).

Species	a	b	Reference
<i>Acer campestre</i>	0.446	1.799	(Burton <i>et al.</i> 1991)
<i>Acer pseudoplatanus</i>	0.446	1.799	(Burton <i>et al.</i> 1991)
<i>Carpinus betulus</i>	2.655	1.091	(Shahrokhzadeh <i>et al.</i> 2015)
<i>Celtis australis</i>	0.210	1.863	Generic equation for broad-leaved species; (Forrester <i>et al.</i> 2017)
<i>Fraxinus excelsior</i>	0.148	2.392	(Forrester <i>et al.</i> 2017)
<i>Fraxinus ornus</i>	0.210	1.863	Generic equation for broad-leaved species; (Forrester <i>et al.</i> 2017)
<i>Juglans nigra</i>	1.900	1.422	(Zellers <i>et al.</i> 2012)
<i>Juglans regia</i>	1.900	1.422	(Zellers <i>et al.</i> 2012)
<i>Morus alba</i>	0.210	1.863	Generic equation for broad-leaved species; (Forrester <i>et al.</i> 2017)
<i>Ostrya carpinifolia</i>	2.655	1.091	Same equation used for <i>Carpinus betulus</i> ; (Shahrokhzadeh <i>et al.</i> 2015)
<i>Prunus avium</i>	0.089	1.420	(Forrester <i>et al.</i> 2017)
<i>Quercus robur</i>	0.168	2.138	(Forrester <i>et al.</i> 2017)
<i>Tilia cordata</i>	0.210	1.863	Generic equation for broad-leaved species; (Forrester <i>et al.</i> 2017)
<i>Ulmus minor</i>	0.210	1.863	Generic equation for broad-leaved species; (Forrester <i>et al.</i> 2017)

Table 3.1 - Coefficients used to estimate leaf area (LA; m²) according to the model $LA = a \times D^b$, where D is the diameter at breast height (cm).

Soil carbon stock (C_{soil} ; MgC ha⁻¹) was calculated as:

$$C_{soil} = \frac{d \times 10000 \times \rho \times C_m}{1000}$$

where d is the horizon depth, ρ is the soil bulk density of fine soil particles (<2 mm) (kg m⁻³) and C_m is the mean C content (%).

With respect to the adjacent maize field, the $\delta^{13}C$ values of the soil organic matter (SOM) were used to calculate the proportion of new C (f_{new} , i.e. the C derived from the current forest vegetation) and of old C ($f_{old}=1-f_{new}$, i.e. the C of the organic matter previous to afforestation), by using the mass balance equation (Del Galdo *et al.* 2003):

$$f_{\text{new}} = \frac{\delta_{\text{new}} - \delta_{\text{old}}}{\delta_{\text{veg}} - \delta_{\text{old}}}$$

where δ_{new} is the $\delta^{13}\text{C}$ of SOM of the afforested soil, δ_{old} is the $\delta^{13}\text{C}$ of the cropped soil and δ_{veg} is the mean $\delta^{13}\text{C}$ of tree leaves weighted for species mean basal area. Thus, knowing the f values for the old and new C, concentrations and amounts of old and new C were obtained.

3.2.6 Statistical analysis

All statistical analysis were performed using RStudio software (Version 1.0.136 – © 2009-2016 RStudio, Inc.).

Mean aboveground characteristics among the plantations, leaf functional traits across species, overall soil C stocks between land uses, $\delta^{13}\text{C}$ values between leaves and soil were compared using one-way ANOVA. Soil C stocks at different soil depth in plantations and maize fields were compared using a two-way ANOVA. All data were eventually log-transformed before performing the statistical analysis to meet the requirements for parametric statistical tests using `powerTransform` and `bcPower` functions in `car` package. Post-hoc Tukey test was performed when a significant difference was detected.

Linear models were used to describe correlations of both soil C stocks samples and f_{new} with species richness gradient or FDis. Shapiro-Wilk normality test was applied on models' residuals in order to test their normal distribution.

3.3 Results

3.3.1 Stand characteristics

Fraxinus excelsior, *Juglans sp.p.* and *Prunus avium* were the three common species across the selected plantations (Table 3.2).

Even though the planting density was the same (between 1905 and 2020 plants ha⁻¹), a significant difference in the actual tree density was detected at the time of field survey, 19 years after planting ($p < 0.001$; Table 3.3). On average, the selected plantations had a mean basal area (G) of 18.00 ± 1.39 m² ha⁻¹ (n=6) a mean tree diameter (d_{avg}) of 12.42 ± 0.37 cm (n=6) and a mean height (H) of 10.37 ± 0.17 m (n=6) (Table 3.3). Both tree basal area ($p = 0.019$) and leaf biomass ($p = 0.003$) significantly decreased with increasing tree species richness (Figure 3.1).

TREE SPECIES	PLANTATION					
	1	2	3	4	5	6
<i>Acer campestre</i>			X	X	X	X
<i>Acer pseudoplatanus</i>		X			X	X
<i>Carpinus betulus</i>			X	X		X
<i>Celtis australis</i>					X	X
<i>Fraxinus excelsior</i>	X	X	X	X		X
<i>Fraxinus ornus</i>			X	X		
<i>Juglans nigra</i>	X					X
<i>Juglans regia</i>			X	X	X	X
<i>Morus alba</i>				X		
<i>Ostrya carpinifolia</i>					X	
<i>Prunus avium</i>	X	X	X	X	X	X
<i>Quercus robur</i>					X	
<i>Tilia cordata</i>					X	
<i>Ulmus minor</i>		X				X
TREE SPECIES RICHNESS	3	4	6	7	8	9

Table 3.2 - Surveyed tree species by plantation and total species richness.

ID	AREA (ha)	SPECIES RICHNESS	FUNCTIONAL DISPERSION (FDis)	PLANNED STAND DENSITY (N ha⁻¹)	ACTUAL STAND DENSITY (N ha⁻¹)	d_{avg} (cm)	G (m² ha⁻¹)	H_{avg} (m)	LAI (m² m⁻²)
1	1.19	3	6.84±0.70 (a)	1905	1494±25 (ab)	12.75±0.21 (ab)	21.02±0.52 (ab)	13.28±0.08 (d)	6.20±0.42 (b)
2	1.00	4	6.61±0.49 (a)	1905	1479±56 (ab)	13.75±0.19 (a)	23.09±0.83 (a)	10.31±0.05 (bc)	4.28±0.18 (ab)
3	0.52	6	7.22±0.87 (a)	2020	1190±81 (a)	12.42±0.36 (ab)	15.61±1.20 (ab)	9.75±0.11 (ab)	4.63±0.36 (ab)
4	0.52	7	10.98±1.39 (ab)	1905	1252±53 (a)	13.06±0.20 (ab)	17.98±0.76 (ab)	9.08±0.06 (a)	5.15±0.35 (ab)
5	1.59	8	9.69±1.37 (ab)	1905	1736±59 (b)	11.26±1.21 (ab)	15.79±4.14 (ab)	10.93±0.54 (c)	3.48±0.91 (a)
6	0.58	9	15.92±3.05 (b)	1905	1321±72 (a)	10.70±0.33 (b)	14.52±0.83 (b)	8.89±0.15 (a)	4.50±0.49 (ab)

Table 3.3 - Mean characteristics of the selected plantations (ID). D_{avg} = mean tree diameter (cm); G = tree basal area (m² ha⁻¹), H_{avg} = mean tree height (m); LAI = leaf area index (m² m⁻²). Mean ± standard error (n=4). Different letters in parenthesis indicate a significant difference among plantations ($p < 0.05$).

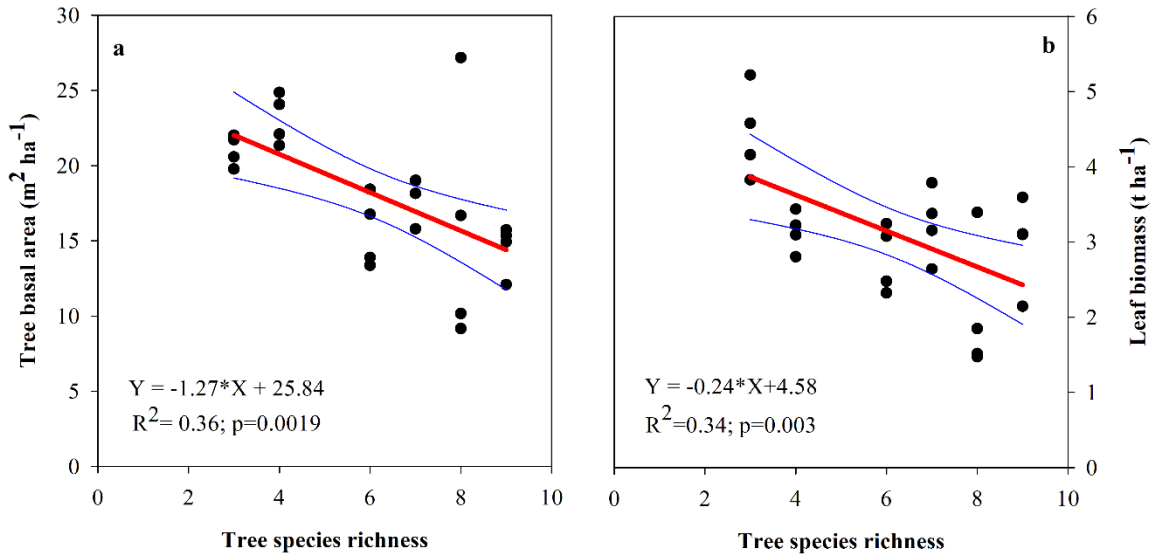


Figure 3.1 - Changes in tree basal area (a) and leaf biomass (a) with increasing tree species richness. Each point represent a sampling plot ($n=24$). Red line represent the regression line, while blue lines represent the 95th confidence interval.

3.3.2 Leaf traits and functional diversity

Mean leaf traits per species are summarized in Table 3.4. All functional traits were significantly different among tree species with the exception of $\delta^{15}\text{N}$ that differed significantly only between *C. betulus* and *F. excelsion* vs. *J. nigra*. *U. minor* differed from the majority of all the other species for all traits, with the exception of $\delta^{15}\text{N}$, followed by *J. nigra* and *M. alba*. On the contrary, *Q. robur* did not show any difference when compared to all the other species for all traits. *T. cordata* had the highest specific leaf area ($255.29 \pm 8.09 \text{ cm}^2 \text{ g}^{-1}$) and the highest N content ($5.62 \pm 0.67\%$), *U. minor* has the highest leaf thickness ($0.19 \pm 0.01 \text{ mm}$), *O. carpinifolia* had the highest leaf C content ($48.75 \pm 0.27\%$), while *A. campestre* had the lowest $\delta^{13}\text{C}$ ($-30.04 \pm 0.14\%$).

The computed FDis vary from 6.61 ± 0.49 to 15.92 ± 3.05 in 4-species and in 9-species plantations, respectively (Table 3.3).

SPECIES	N	SLA	TH	N	C	C/N	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$
AC	15	189.64±6.02 (cde)	0.11±0.00 (abc)	2.67±0.26 (bcd)	46.96±0.31 (bc)	19.05±1.07 (bc)	-30.04±0.14 (bc)	-4.85±1.03 (ab)
AP	10	211.09±12.54 (def)	0.13±0.01 (bcd)	3.09±0.33 (cd)	46.89±0.33 (cd)	16.2±1.11 (bcd)	-28.97±0.24 (cd)	-6.32±0.48 (ab)
CA	6	115.9±6.87 (cbde)	0.17±0.01 (cd)	1.93±0.08 (bcd)	44.22±1.06 (bcd)	23.2±1.08 (bcd)	-29.65±0.48 (bcd)	-9.6±2.42 (ab)
CB	12	200.8±11.69 (ef)	0.11±0.00 (cd)	2.31±0.11 (cd)	47.74±0.17 (cd)	21.12±0.93 (cd)	-28.59±0.26 (cd)	-2.08±0.46 (a)
FE	20	135.58±3.45 (ab)	0.19±0 (bc)	2.17±0.06 (ab)	44.66±0.22 (ab)	20.9±0.54 (ab)	-28.48±0.13 (b)	-4.97±0.63 (a)
FO	8	165.59±4.57 (bcde)	0.14±0.01 (abcd)	2.18±0.07 (bc)	46.32±0.58 (bc)	21.44±0.78 (abcd)	-29.16±0.2 (bc)	-3.53±0.46 (ab)
JN	8	189.17±15.41 (f)	0.14±0.01 (d)	2.27±0.08 (d)	44.81±0.34 (d)	19.92±0.68 (d)	-29.93±0.4 (d)	-5.52±0.96 (b)
JR	16	176.92±9.66 (bcd)	0.17±0.01 (bcd)	2.63±0.18 (bc)	46.1±0.34 (bc)	18.61±1.02 (abc)	-29.41±0.2 (bc)	-5.31±0.93 (ab)
MA	4	215.07±15.74 (bce)	0.14±0.01 (abcd)	2.76±0.09 (bcd)	44.84±1.62 (abcd)	16.29±0.26 (abcd)	-28.06±0.34 (abcd)	-2.96±0.49 (ab)
OC	4	218.17±21.75 (bce)	0.1±0.01 (abc)	4.1±0.39 (bcd)	48.75±0.27 (abcd)	12.29±1.34 (ab)	-29.61±0.31 (abc)	-7.45±1.52 (ab)
PA	24	187.9±7.95 (bc)	0.13±0.00 (a)	2.51±0.18 (ab)	46±0.23 (ab)	19.67±0.87 (ab)	-28.51±0.15 (ad)	-6.16±0.69 (ab)
QR	2	130.23±2.21 (abcdef)	0.16±0.01 (abcd)	3.46±0.23 (bcd)	48.54±0.01 (bcd)	14.14±0.88 (abcd)	-26.77±0.15 (abcd)	-7.81±0.34 (ab)
TC	4	255.29±8.09 (def)	0.1±0.00 (abcd)	5.62±0.67 (cd)	47.27±0.3 (bcd)	8.89±1.25 (ab)	-27.72±0.34 (bcd)	-5.91±0.96 (ab)
UM	8	131.11±6.31 (a)	0.19±0.01 (ab)	2.24±0.1 (a)	45.52±0.3 (a)	20.64±0.95 (a)	-28.28±0.18 (a)	-8.07±1.01 (ab)

Table 3.4 - Mean values of leaf functional traits per species. AC = *Acer campestre*; AP = *Acer pseudoplatanus*; CA = *Celtis australis*; CB = *Carpinus betulus*; FE = *Fraxinus excelsior*; FO = *Fraxinus ornus*; JN = *Juglans nigra*; JR = *Juglans regia*; MA = *Morus alba*; OC = *Ostrya carpinifolia*; PA = *Prunus avium*; QR = *Quercus robur*; TC = *Tilia cordata*; UM = *Ulmus minor*. N are the plots where the species have been found. SLA = Specific Leaf Area ($\text{cm}^2 \text{g}^{-1}$); TH = leaf thickness (mm); N = leaf nitrogen content (%); C = leaf carbon content (%); C/N = leaf carbon to nitrogen ratio; $\delta^{13}\text{C}$ = stable carbon isotope ratio (‰); $\delta^{15}\text{N}$ = stable nitrogen isotope ratio (‰). Mean ± standard error. Different letters in parenthesis indicate a significant difference among plantations ($p < 0.05$).

3.3.3 Variation in soil C among plantations

A clear decrease in total soil C was detected with depth in both the plantations and the adjacent maize fields (plantations: $p=0.005$; maize: $p=0.08$; Figure 3.2a). Nineteen years after the land use change, the overall soil C stock (0-60 cm; PPA samples) was 12% higher in the forest stands (96.99 ± 6.10 MgC ha⁻¹) than in the adjacent maize crops (85.68 ± 8.26 MgC ha⁻¹) and corresponded to an annual total C sequestration of $+0.60$ MgC ha⁻¹ year⁻¹. This increase in soil C was mainly due to the significant changes detected between 0 and 15 cm ($+32\%$; p -value <0.001 ; Figure 3.2a). Such a difference at this depth was also confirmed by the intensive soil survey (SSA samples; $p<0.001$), even though any clear pattern with species richness level was evident ($p=0.98$, Figure 3.2b).

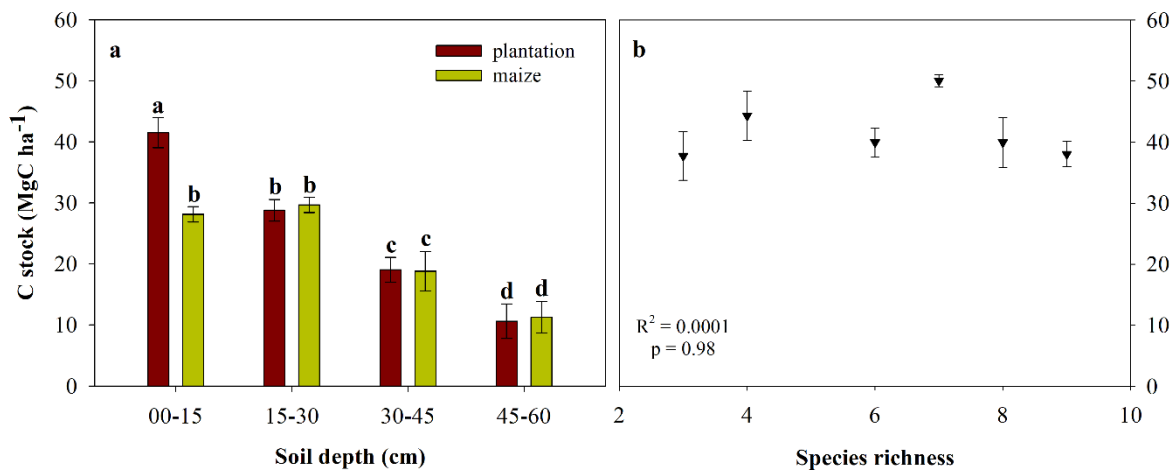


Figure 3.2 - Total soil C stocks with soil depth as derived by PPA samples (a) and soil C stocks at 0-15 cm at different levels of species richness (b) as derived by MSA samples in plantations. Vertical bars indicate standard error ($n=6$ and $n=4$ for panel a and b, respectively). Different letters in panel a indicate a significant difference ($p<0.05$). Adjacent maize C stock is reported as reference in panel a.

A significant difference in isotopic signature ($\delta^{13}\text{C}$) among tree leaves ($-28.95\pm 0.18\%$; $n=6$), soil organic matter in the plantations ($-24.74\pm 0.18\%$; $n=6$) and soil organic matter in the adjacent maize fields ($-20.14\pm 0.61\%$; $n=6$) was detected ($p<0.001$; Figure 3.3), thus allowing the calculation of f_{new} according to the equation proposed by Del Galdo *et al.* (2003).

On average, f_{new} was equal to 0.49 ± 0.02 and, assuming a mean total soil C stock at 0-15 cm of 41.7 ± 1.9 MgC ha⁻¹ across all plantations ($n=6$; Figure 3.2a), we estimated an overall contribution of trees to soil C storage at this depth of 20.4 ± 1.2 MgC ha⁻¹.

Both species richness (Figure 3.4a) and FDis (Figure 3.4b) had a significant and positive effect on f_{new} following the land use change (species richness: $R^2=0.23$; $p=0.017$; FDis: $R^2=0.18$; $p=0.039$).

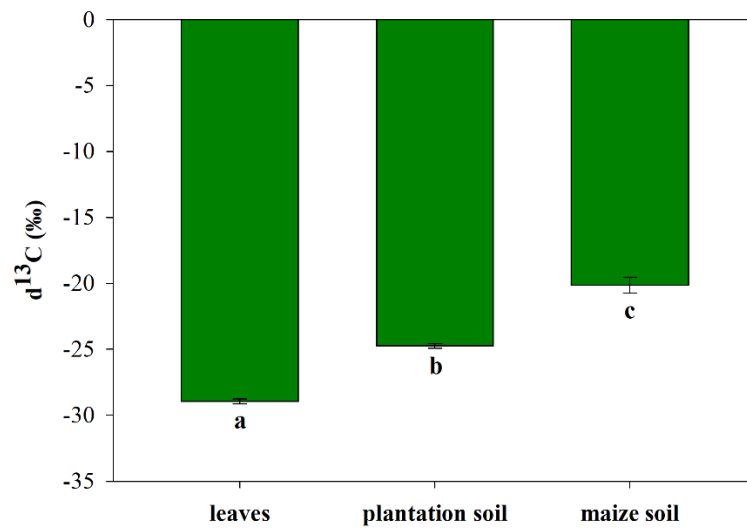


Figure 3.3 - Mean $\delta^{13}C$ of leaves, soil organic matter in plantation and in adjacent maize fields. Vertical bars indicate standard error ($n=6$). Different letters indicate a significant difference ($p<0.05$).

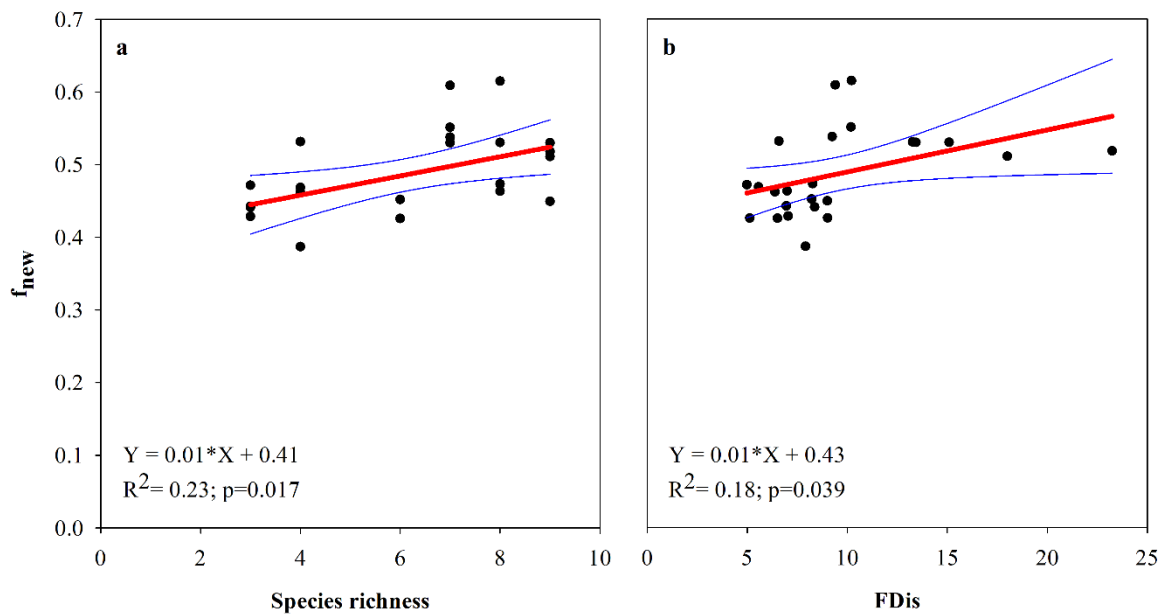


Figure 3.4 - Proportion of new soil organic C (f_{new}) derived from the current forest vegetation with increasing species richness (A) and with increasing functional dispersion (FDis; B). Points represent the sampling plots ($n=24$). Red line represents the regression line, while blue lines represent the 95th confidence interval.

3.4 Discussion

Nineteen years after planting we measured an overall increase in soil C stock (+12%; 0-60 cm; Figure 3.2a). Most of this increase was detected in the upper soil layer (0-15 cm), being this layer the first to receive the organic C entering the soil. On the contrary, any significant decrease in total soil C with depth was detected in the control maize fields, probably because of tillage practices. Similarly to our results, Guo and Gifford (2002), in their meta-analysis on soil C stock and land use change, reported an overall mean increase in soil C stock of 18% 15-18 years after planting and Popleau and Don (2013) found about a 30% increase at 0-30 cm depth in soil C stocks after about 40 years in six European sites after cropland afforestation. Our results are also similar to those of Del Galdo *et al.* (2003) obtained in similar plantations. On the contrary, Nave *et al.* (2013) and Vesterdal *et al.* (2002) found that afforestation of agricultural land does not lead in soil C accumulation in less than 30-35 years. They highlighted an increase in soil C stocks in shallow soil layers, offset by a huge decrease of soil C stocks in deeper layers after afforestation mainly due to low C accumulation in the younger stands and still higher decomposition rates of organic matter by soil microbial communities. However, forest ecosystems have far more complex C dynamics both above- and belowground and they require long-term experimentations to be disentangled and understood (Leuschner *et al.* 2009). Using stable isotopes ($\delta^{13}\text{C}$), we estimate that, on average, almost half of the total soil C at 0-15 cm in the selected plantations derived from the trees. Similarly, Del Galdo *et al.* (2003) reported that tree-derived C contributed 43% and 31% to the total soil C storage at 0-10 and 10-30 cm depths, respectively. Comparing the old C stocks (calculated by difference between total and new C stocks) and soil C stocks from maize fields (can be considered approximatively the same value of pre-planting situation), we found that plantations lost approximately 23% of soil C derived from previous maize cultivation. Such loss can be attributed to the complex dynamic in both shallow and deep soil layers. For example, Li *et al.* (2019) performed SOC analysis on biodiversity-ecosystem functioning experiment in China, established on previous natural forest. They found, from 2010 to 2015 a significant decrease of SOC in the topsoil (0-10 cm), probably due to soil disturbance during site

preparation, and a significant increase of SOC in deeper layers (10-30 cm) attributed to downward transport processes of organic matter (Gleixner *et al.* 2009) or slower SOC turnover and consequent SOC accumulation (Jobbágy and Jackson 2000). On the other hand, Fontaine *et al.* (2007) showed how stable organic compounds in deep soil layers can be easily degraded when interacting with more recent and less stable soil organic matter, enhancing the process called “priming effect”. This process can alter the proportion of old C stocked in soil and the new one. Moreover, in shallow layers, plants can stimulate and modulate microbial activity via recalcitrant compounds release, root exudates and root turnover (De Deyn *et al.* 2008). Indeed, many studies highlighted the positive relationship between species richness or diversity and fine root productivity, thus, influencing belowground carbon dynamics (Brassard *et al.* 2013; Lei *et al.* 2012; Ma and Chen 2017).

We did not observe any significant trend in total soil C stocks at 0-15 cm depth along our tree species richness gradient (Figure 3.2b). This can be related to the large soil heterogeneity under tree cover, which would have required a more intensive sampling (Schrumpf *et al.* 2011, Smith 2004), and to the fact that the relative change along the species gradient is too small when compared to the overall soil C stock to be detected using soil C concentrations and soil bulk densities. However, using stable isotopes, we showed that both tree species richness and functional diversity had a positive and significant effect on the proportion of C derived from the current forest vegetation (f_{new} ; Figure 3.4a and Figure 3.4b), thus indicating that higher biodiversity can locally enhance soil C sequestration. Such results are in agreement with Gamfeldt *et al.* (2013), who showed an increase of 11% of soil C storage passing from 1 to 5 species in Swedish forests plots.

As far as mechanisms are concerned, plant diversity can act on microbial community and, consequently, on soil C accumulation through litter input into the soil (quantity and quality) and root dynamics. Several field studies in forest ecosystems have reported positive effects of tree species diversity on productivity (e.g. Chen *et al.* 2018; Huang *et al.* 2018; Liang *et al.* 2016; Paquette and Messier 2011; Zhang *et al.* 2012). Thus, higher tree productivity would also mean more litter production in broad-leaf stands (both above- and below-ground) and, thus, more C entering into the

soil compartment. However, there are also several other study showing none or even negative relationships between tree productivity and species richness (e.g. Chen and Klinka 2003; Jacob *et al.* 2010; Vilà *et al.* 2003). In our study, we did not directly measure tree productivity, but, as it is positively linked to tree basal area (Bohn and Huth 2017), we use this last variable to infer the overall effect of tree species richness on tree productivity. According to our results, we found a significant decrease in total tree basal area with increasing the number of tree species (Figure 3.1a). This unexpected decrease can be related to the lower actual stand density than at planting (Table 3.3), mainly due to natural mortality following a high competition for resources. In fact we can expect higher competition as the number of species increases as the richest plantation (#6) had two couples of congeneric species (*Acer sp.* and *Juglans sp.*) and three couples of the same family (*Acer sp.* of Aceraceae, *Juglans sp.* of Juglandaceae and *Celtis australis* and *Ulmus minor* of Ulmaceae). Such a decrease in tree basal area also translated into a significant decrease in the leaf biomass (i.e. leaf productivity; Figure 3.1b) and, thus, in annual litter production.

On the contrary, we detected significant differences in the measured leaf functional traits (Table 3.4) among the considered tree species, reflecting different qualities of the litter mixtures. Such differences may thus explain the measured increase in tree-derived soil C with increasing species richness and tree functional diversity. In fact, Dawud *et al.* (2017) highlighted that across the major European forest types soil C stocks are driven by tree species functional groups, while Ruiz-Jaen and Potvin (2011) in the tropics underlined a strong positive effect of species richness and functional diversity on soil C storage in plantations and in natural forest, respectively. It has also been shown that leaf N content, SLA and chlorophyll content per unit leaf area had positive effects on decomposition, explaining together 65–69% of the variation (Bakker *et al.* 2011). Litter with a high N concentration is easily mineralised, thus producing more microbial residues, which are then bound to the mineral soil matrix, leading to an increase of stable SOC content (Cotrufo *et al.* 2015; Faucon *et al.* 2017). In our case, species with higher leaf N content (>3%), higher SLA and lower C:N ratio were only present in the plantations with the highest tree species richness and functional diversity (Table 3.4), thus

contributing to higher tree-derived soil C values. Moreover, as N concentrations in leaves and absorptive roots are positively correlated (Wang *et al.* 2017), we can hypothesized that soil organic matter should be more stable where species with higher leaves N concentration are present (Angst *et al.* 2019). Our measured leaf trait values are coherent with those reported in literature. SLA is one of the most measured and easy to be measured trait (Cornelissen *et al.* 2003; Pérez-Harguindeguy *et al.* 2013). Our SLA data ($168.15 \pm 8.16 \text{ cm}^2 \text{ g}^{-1}$, on average) are in agreement with those derived from specific leaf mass in (Poorter *et al.* 2009). Looking at TH, N and C content and C to N ratio mean values, they laid in the range of data obtained from TRY-Plant Trait Database (Kattge *et al.* 2011). Similarly, our mean leaf $\delta^{13}\text{C}$ values are in the range reported by Del Galdo *et al.* (2003). Craine *et al.* (2009) reported a global mean value of leaf $\delta^{15}\text{N}$ of 0.9‰ with 95% of data ranging between -7.8‰ and 8.7‰, confirming our data. However, leaf $\delta^{15}\text{N}$ can be locally very variable due to several factors, that is, the signature of N deposition, the amount of N gained from symbiotic fixation or from mycorrhiza, the fractionation processes in soil and the signature of N lost from the ecosystem (Craine *et al.* 2015).

3.5 Conclusions

We found that both species richness and functional diversity have a positive effect on the proportion of tree derived C (0-15 cm) in forest plantations on ex-agricultural fields even though mean tree basal area and tree leaf biomass along the tree diversity gradient decreased. Therefore, litter quality can be more important than its quantity (i.e. annual production): higher leaf N content, coupled with higher SLA and lower C/N values can lead to higher mineralisation rates and higher soil C accumulation. Thus, more diverse forest stands can enhance and better modulate microbial community and, thus, soil C dynamics through more diverse litters.

4. INFLUENCE OF TREE SPECIES RICHNESS ON TREE GROWTH AND INTRINSIC WATER-USE EFFICIENCY AFTER EXTREME EVENTS IN TREE PLANTATIONS IN NORTH-EASTERN ITALY²

Summary

We investigated the effects of species richness on tree response to extreme drought periods. The study was carried out in the same deciduous plantations of the first paper, coupling dendrochronological analyses with isotopic analyses. We compared the differences in calculated normalised tree growth and intrinsic water-use efficiency in both a dry and a mild year along the species richness gradient. Tree species diversity had a positive effect on the response to drought, but only at lower levels of tree species richness, when facilitation and/or complementarity mechanisms prevailed. Instead, negative responses were typical at higher levels of species richness, when competition was the dominant process within the stand.

² Palandrani C, Battipaglia G, Alberti G (submitted) Influence of tree species richness on tree growth and intrinsic water-use efficiency after extreme events in tree plantations in North-eastern Italy. *European Journal of Forest Research*.

4.1 Introduction

In the last decades, anthropogenic impacts are threatening natural ecosystems worldwide and are exacerbating extreme climate events, which are increasing both in intensity and in frequency (IPCC 2014; Trenberth *et al.* 2014; Williams *et al.* 2013). Consequently, forests are becoming more vulnerable to these events (i.e. windstorms, drought events, heatwaves or wildfires), which are causing increasingly loss of biodiversity and loss in several ecosystem functions (EFs) and services (ESs) (Allen *et al.* 2010; Scholze *et al.* 2006). Indeed, forests can adapt to this new disturbance regimes or stresses by adjusting tree physiological mechanisms, shifting in latitudinal and altitudinal distributions or changing species composition, but such changes are usually slow through space and time (Beckage *et al.* 2008; Christmas *et al.* 2016; Jump and Peñuelas 2005; Kremer *et al.* 2012; Parmesan 2006). Thus, it has become important to understand the extreme events' effects on forests in order to identify and adopt the best management and mitigation policies and practices in the short/medium term (Mori *et al.* 2017).

In this perspective, studies about the role of species diversity in enhancing ecosystem resistance (i.e. the ability to withstand harsh events) and/or resilience (i.e. the ability to regain the pre-disturbance growth rates) (Merlin *et al.* 2015) to climatic extremes are spreading out (Baeten *et al.* 2013; Bruelheide *et al.* 2014; Verheyen *et al.* 2015) as biodiversity can sustain EFs and ESs through species complementarity, facilitation processes and/or niches partitioning, with a net increase in overall ecosystem performances (Cardinale *et al.* 2012; Hector *et al.* 2011; Morin *et al.* 2011). Several studies have highlighted that higher species richness can increase the resistance and/or the resilience to disturbances and stresses, especially to pests, pathogens and other diseases (Jactel and Brockerhoff 2007; Zhu *et al.* 2000). However, the effects of tree species diversity on the response to climatic extreme events (i.e. drought and heatwaves) are still debated and the results are often contrasting (Yin and Bauerle 2017). For example, during drought events, plant's physiological activity and growth can be seriously compromised: hydraulic failure and/or depletion of carbon (C) pools (i.e. C starvation), interacting with pests and other biotic attacks, are considered the main factors leading in reduced

growth or higher tree mortality (McDowell 2011). In some cases, different tree species, with different functional traits (e.g. including genetic diversity and physiological characteristics), both above- and belowground, have been seen to positively influence forest resistance to drought events with a better resource acquisition and/or use (Lebourgeois *et al.* 2013; Pretzsch *et al.* 2013). In others, species sharing the same ecological niches strongly compete for the same resources, thus leading in a reduced resistance or resilience to extreme events (Grossiord *et al.* 2014a; Grossiord *et al.* 2014b).

Recent studies have used dendrochronological analyses to assess the impact of well- documented dry years on forests and targeted tree species (Lloret *et al.* 2011; Merlin *et al.* 2015; Vitali *et al.* 2017) as well as stable C isotope to compare dry and wet years and to better underline the effects of drought on tree physiological processes (Grossiord *et al.* 2014a). In fact, every tree-ring is the result of plant-intrinsic factors (i.e. photosynthetic rate, water potential regulation, hormonal regulation) and plant-external factors (i.e. climate, pathogens, stress factors), which can modulate the quantity (i.e. width) and the quality (i.e. isotopic signature) of annual wood production (Battipaglia *et al.* 2009; De Micco *et al.* 2010; Deslauriers and Morin 2005; Lupi *et al.* 2010). Hot and dry vegetative seasons can lead plants to important hydraulic deficits coupled to drops in photosynthetic rate and, as a result, annual tree-rings will be thinner than those formed during favourable seasons (Gao *et al.* 2018). On the other hand, the analysis of stable C isotopes in tree-rings gives additional information about the occurrence of drought events and on the amount of carbon assimilated as biomass per unit of water used by trees (i.e. intrinsic water-use efficiency; iWUE) (Farquhar *et al.* 1982). During drought events, plants can close stomata to avoid unnecessary water loss. Consequently, stomatal conductance to CO₂ decreases and rubisco fixes more ¹³C than usual. The results are rings enriched in heavier isotope, which translates in less negative isotopic signatures (Francey and Farquhar 1982). Few studies (Li 1999; Walker *et al.* 2015) have specifically investigated, so far, the role of tree species richness on the tree growth's response to drought by combining both dendrochronological indexes and C isotope in forests or in controlled experiments.

In Friuli-Venezia Giulia plain (North Eastern Italy), several deciduous plantations were established at the end of last century to sustain regional wood production, but also to increase C storage and/or biodiversity. Such plantations might be composed by target tree species only (i.e. commercial ones) or by target species consociated with accessory tree species. The same climate and soil conditions make these plantations a useful tree species richness gradient experiment in homogenous environmental conditions to assess the role of tree diversity in mitigating extreme event impacts on forest ecosystems. The aim of our study was to understand the role of the overall tree species richness in determining growth and iWUE patterns in the target tree species (i.e. *Fraxinus excelsior*, *Juglans sp.p.* and *Prunus avium*) during drought events, by coupling dendrochronology and C isotope analysis. We hypothesized that the higher is the number of consociated species (i.e. tree species' diversity), the higher is the positive effect of tree growth and iWUE, in response to drought.

4.2 Materials and methods

4.2.1 Study area

Six deciduous plantations were identified in Friuli-Venezia Giulia plain (Italy; 46°5'50''N, 13°1'7''E, 121 a.s.l.). Climatic conditions and soil characteristics were the same across all sites: mean annual temperature was 13.3°C and mean annual precipitation was 1500 mm from 1995-2017 data recorded at Fagagna meteorological station (46°06'51''N; 13°04'50''E; www.osmer.fvg.it); soil was alluvial mesic Udifluent (sand 58%, loam 28%, clay 14%). All plantations were established in 1999 and stand planting density was between 1905 and 2020 plant ha⁻¹. No fertilization, irrigation (with the exception of emergency irrigation during the first 4-5 years) and pruning have been performed at all sites. Plantations differed only for their total surface area and for tree species richness (3, 4, 6, 7, 8, and 9). A detailed description of each selected stand (i.e. species composition and dendrometric characteristics) is reported in Chapter 3, tables 3.2 and 3.3.

4.2.2 Sampling strategy

The study focused on the three commercial tree species (target species), which were present at all sites (i.e. *Fraxinus excelsior*, *Juglans sp.p.* and *Prunus avium*). In each plantation, 15 dominant trees for each target species (45 trees per site) were selected, avoiding individuals at the border of the stand. Two perpendicular wood cores at 30 cm height from the ground for each selected plant were taken using an increment borer, stored in cardboard and naturally dried. Once in the lab, samples were glued on woody supports and sanded with paper with 60, 120, 240, 320 and 600 grit to make tree rings more visible.

4.2.3 Dendrochronological analysis

Tree-ring width was estimated for each core using Dendrotab 2003 (© Walesch Electronic GmbH). Chronologies were visually and statistically cross-dated using TSAP (© TSAPWin Scientific, version 4.81, 2002-2018, Frank Rinn / RINNTECH) and COFECHA (Grissino-Mayer 2001; Holmes 1983) softwares, respectively. Cross-dated chronologies were then normalized using the Hegershoff correction (Fang *et al.* 2010; Warren 1980; Warren and MacWilliam 1981) with the function *detrend* in dplR package in RStudio (© Rstudio, version 1.2.1335, ©2009-2019 Rstudio, inc.) in order to remove noises associated with age trends, which may be significant especially in young plants and during the first years of growth.

4.2.4 Climate index

The driest and the reference year (i.e. a year representing average climate conditions at the experimental sites, neither too warm and dry nor too cold and wet) since tree planting were identified by calculating the De Martonne Index (DMI; Maliva and Missimer 2012; Vitali *et al.* 2017):

$$DMI = P/(T + 10)$$

where P is the total rainfall (mm) and T is the mean temperature ($^{\circ}\text{C}$) at the experiment sites, calculated yearly on the vegetative season (from April to October) from 1995-2017 data from Fagagna meteorological station, the nearest station to our selected sites (www.osmer.fvg.it). We considered the years below the 10th percentile of DMI distribution as the driest and the years between the 45th and the 55th percentile as the references (i.e. years representing mean climate conditions at the experimental sites). Using such an approach, 2006 and 2014 were identified as the driest and the reference year, respectively (Table 4.1). Even though 2003 resulted to be the driest year ever, we decided to not consider it as the selected plantation were 4 years-old, canopies were not completely closed and emergency irrigations were still performed according to local records.

YEAR	T_{avg} (°C)	P (mm)	DMI
1995	17.7	984.4	35.59
1996	16.9	1219.2	45.29
1997	17.4	656.2	23.99
1998	17.7	1406.4	50.74
1999	18.1	935	33.30
2000	18.3	913	32.22
2001	17.9	768.4	27.54
2002	17.6	1169	42.36
2003	19.3	458.4	15.66
2004	17.3	882.8	32.38
2005	17.6	1194.6	43.30
2006	19.0	714.8	24.67
2007	18.7	809.4	28.21
2008	18.0	988.6	35.29
2009	19.2	805.2	27.60
2010	18.1	982.3	34.91
2011	19.7	807.6	27.16
2012	19.5	1082	36.73
2013	18.4	1032.8	36.31
2014	18.2	970.6	34.45
2015	18.6	1009	35.32
2016	18.3	775.9	27.39
2017	18.2	696.4	24.68
MEAN	18.2	924.4	/

Table 1 - Yearly climatic parameters considered during the vegetative season (from April to October) for the De Martonne Index (DMI): T_{avg}=yearly mean temperature (°C); P=yearly cumulated precipitations (mm); DMI=De Martonne Index. DMI 10th percentile=24.67; DMI 45th percentile=32.36; DMI 55th percentile=34.50. Data from Fagagna meteorological station (www.osmer.fvg.it).

4.2.5 Isotopes analysis and iWUE

Three wood cores for each species in each plot were randomly selected. Rings corresponding to 2006 and 2014 were accurately cut and stored in different plastic tubes. To avoid contamination, samples were washed with pure ethanol and dried at 40°C overnight. Then, samples were milled and weighted in tin capsules and $\delta^{13}\text{C}$ was assessed using a CHNS Elemental Analyser (Vario Microcube, © Elementar) coupled to a stable isotope ratio mass spectrometer (IRMS; Isoprime 100, © Elementar).

We calculated $iWUE$ ($\mu\text{molCO}_2 \text{ molH}_2\text{O}^{-1}$), defined as the ratio between photosynthesis and leaf transpiration, using $\delta^{13}\text{C}$ values of the single tree ring according to the model of Farquhar *et al.* (1982):

$$iWUE = \frac{A}{g_{H_2O}} = c_a * \frac{(b - \Delta^{13}C)}{1.6 * (b - a)}$$

knowing that C isotopic discrimination is equal to (Farquhar *et al.* 1982):

$$\Delta^{13}C = \frac{\delta^{13}C_a - \delta^{13}C_p}{1 - \delta^{13}C_p}$$

where C_a (ppm) is mean annual atmospheric CO_2 concentration, a is the fractionation during CO_2 diffusion ($a = 4.4\text{‰}$), b is the fractionation during carboxylation ($b = 27\text{‰}$), $\delta^{13}C_a$ is the isotopic mean annual isotopic composition of atmosphere, and $\delta^{13}C_p$ is the isotopic composition of tree-ring samples. C_a and $\delta^{13}C_a$ were downloaded from Mauna Loa Observatory database (<https://www.esrl.noaa.gov/gmd/>).

4.2.6 Data analysis

We considered as control (ctrl) the plantation without any consociate species (plantation n. 1; Table 3.2), while we expressed tree species diversity in the other plantations as the total number of consociated species. Then, we compared normalized tree-ring width and $iWUE$ of the target species between the selected years (2006 and 2014), between species richness and years x species richness using a two-way ANOVA. All data were eventually transformed before doing the statistical analysis to meet the requirements for parametric statistical tests using `powerTransform` and `bcPower` functions in `car` package. Post-hoc Tukey test was done when a significant difference was detected. All statistical analysis were applied using RStudio software (Rstudio, version 1.2.1335, ©2009-2019 Rstudio, inc.).

4.3 Results

The annual growth rates of the three target tree species were similar, on average, across all the considered plantations both considering raw and normalized tree-ring width (Figures 4.1a and 4.1b): all target species showed peaks in tree-ring width in 2002 and in 2004 followed by a constant decrease during the last 8-9 years. Similarly, they showed a significant drop in the growth rate in 2003, 2006 and 2015, while synchronous increases were recorded in 2007, 2014 and 2016.

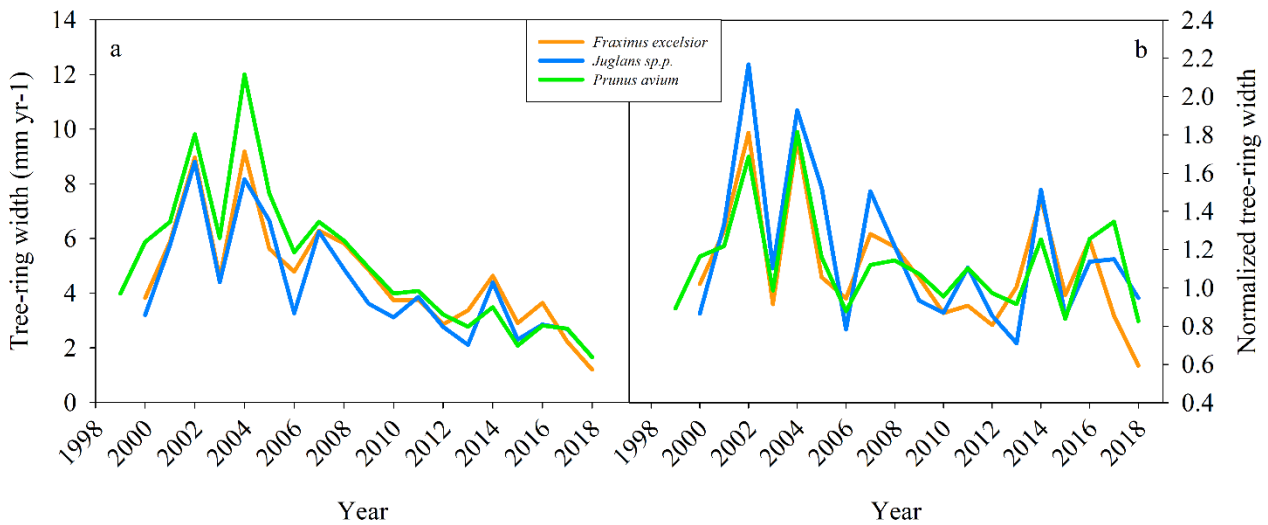


Figure 4.1 - Average (mm yr⁻¹; a) and normalized tree-ring width (b) of the three commercial species (*Fraxinus excelsior*, *Juglans sp.p.* and *Prunus avium*) across all the studied plantations .

When normalized ring width is considered across all plantations, *Fraxinus excelsior*, *Juglans sp.p.* and *Prunus avium* grew 36%, 47%, 30% less in the driest (2006) and in the reference year (2014), respectively ($p < 0.001$; Figure 4.2). Such a difference in ring width between those two years is also confirmed when each single plantation (i.e. increasing the number of associated species) is considered ($p < 0.05$; Figure 4.3). However, while in the driest year (2006) increasing the number of consociated species had a positive influence on the ring width of the target species (+14% when compared to control; $p = 0.001$), in the reference year (2014) such a difference was not significant (-8%; $p > 0.05$; Figure 4.3). Moreover, in 2006 the major difference (+23%, $p < 0.001$) was detected in the plantation with only one consociated species, while lower differences were found at higher levels (+3% and $p = 0.30$ when consociated species = 6). In 2014 (reference year), significant differences were only found between plantations with five and six consociated species ($p = 0.002$).

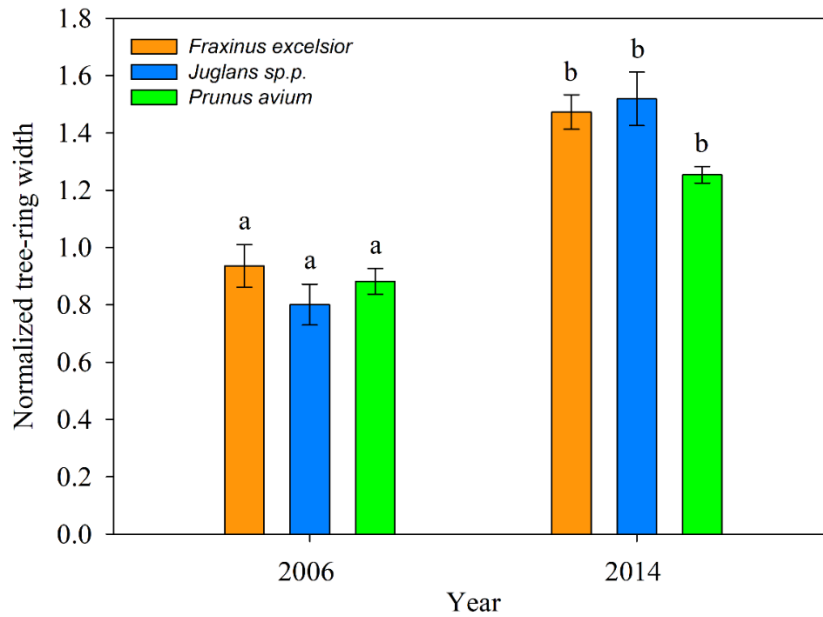


Figure 4.2 - Mean normalized tree-ring width in 2006 and 2014 for the three commercial tree species across all the plantations. Vertical bars indicate standard errors ($n=5$ for *Fraxinus excelsior* and *Juglans sp.p.*; $n=6$ for *Prunus avium*) and different letters indicate a significant difference ($p<0.05$).

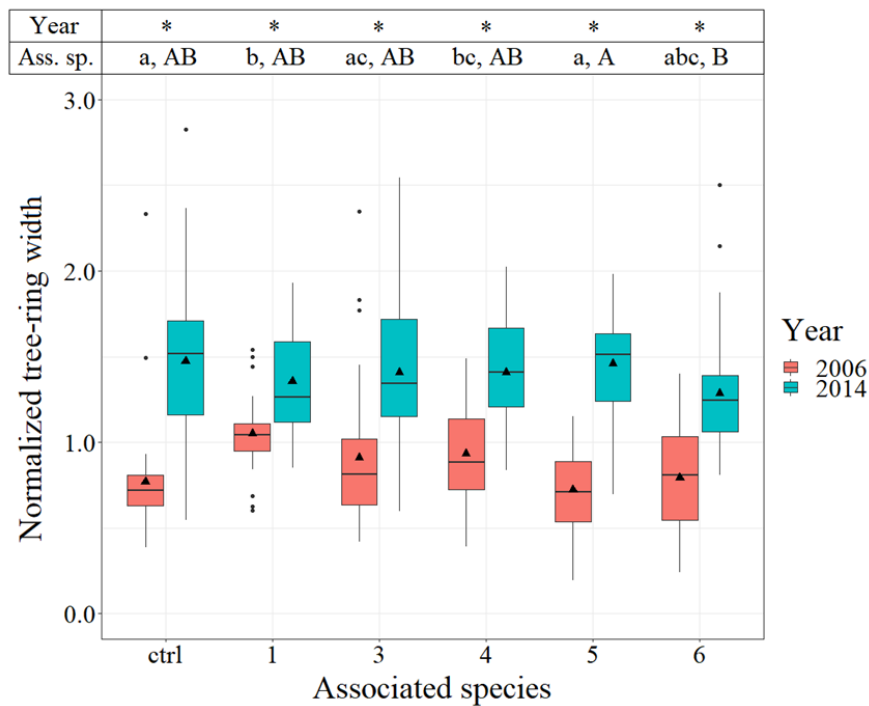


Figure 4.3 - Normalized tree-ring growth with the increase of associated tree species in the different plantations. “ctrl” represents the reference plantation (i.e. the three commercial species, only). Black triangles indicate mean values for each boxplot. Asterisks in “Year” box indicate significant differences between 2006 (dry year) and 2014 (reference year) inside the same plantation ($p<0.05$). Different lowercase letters in “Ass. Sp.” box indicate significant differences among treatments in 2006 ($p<0.05$), while different capital letters in the same box indicate significant differences among plantations in 2014 ($p<0.05$).

As far as C isotopic signature is concerned, $\delta^{13}\text{C}$ values ranged from $-26.45\pm 0.65\text{‰}$ and $-26.54\pm 0.84\text{‰}$ in 2006 and 2014, respectively, reflecting typical ranges of C3 plants. We measured a significant lower mean iWUE across all plantations (-6% ; $p<0.001$; Figure 4.4) in the driest year (2006) than in the reference year (2014). Increasing the number of consociated species (Figure 4.5) caused a significant difference in iWUE in driest year, only ($p=0.02$). In particular, major and significant differences with control were detected when five consociated tree species were present ($+11\%$; $p=0.02$), while minor and non-significant differences were found when six consociated species were present ($+4\%$; $p=0.84$).

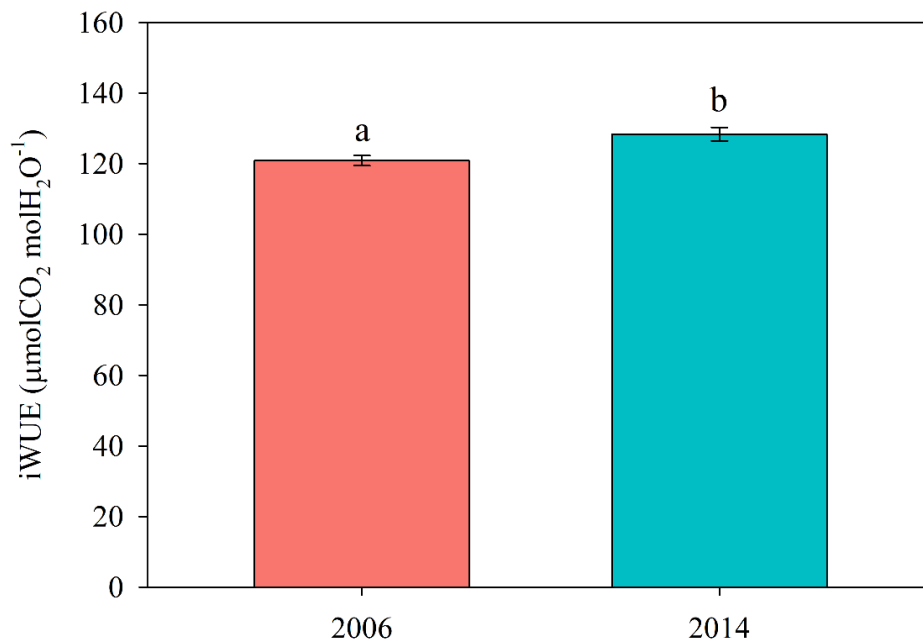


Figure 4.4 - Mean intrinsic water-use efficiency (iWUE; $\mu\text{mol CO}_2 \text{ mol H}_2\text{O}^{-1}$) in 2006 and 2014 across all plantations. Vertical bars indicate standard errors ($n=48$) and different letters indicate a significant difference ($p<0.05$).

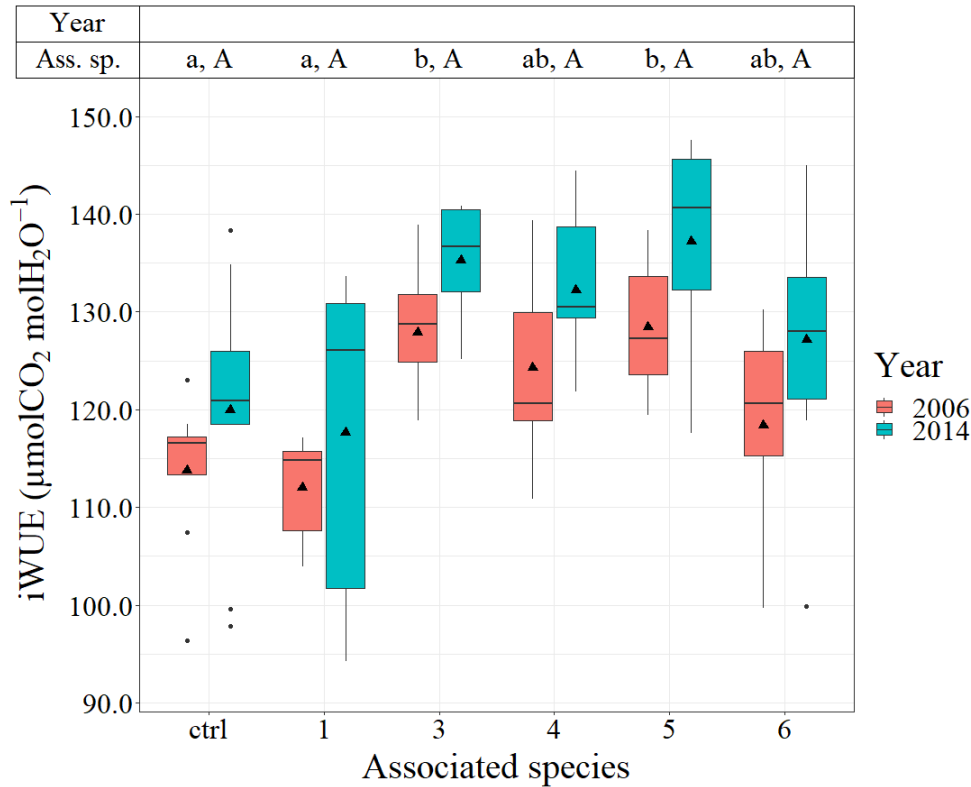


Figure 4.5 - Intrinsic water-use efficiency ($iWUE \mu\text{mol CO}_2 \text{ mol H}_2\text{O}^{-1}$) with increasing the number of associated species. “ctrl” represents the reference plantation (i.e. the three commercial species, only). Black triangles indicate mean values for each boxplot. Asterisks in “Year” box indicate significant differences between 2006 (dry year) and 2014 (reference year) inside the same plantation ($p < 0.05$). Different lowercase letters in “Ass. Sp.” box indicate significant differences among treatments in 2006 ($p < 0.05$), while different capital letters in the same box indicate significant differences among plantations in 2014 ($p < 0.05$).

4.4 Discussion

Tree species respond differently to drought events depending on the intensity and the frequency of these lasts, on their occurrence during the vegetative season (i.e. spring or summer droughts) (Merlin *et al.* 2015), on tree age, site characteristics and forest management (Sohn *et al.* 2016).

In our study, we observed a strong age-related trend in tree ring width for each of the considered commercial species (Figure 4.1a): major widths were recorded between 2001 and 2005, followed by a subsequent and constant decline. Such a growth pattern reflects the typical growth behaviour in young trees (Fang *et al.* 2010), thus confirming our choice to use a normalizing function to eliminate these age-related trends (Figure 4.1b). Each of the target tree species showed a significant drop in tree

ring width values during 2003, which was recorded as one of the driest and hottest years in Europe since the beginning of XX century (De Bono *et al.* 2004; Rebetz *et al.* 2006; Schär and Jendritzky 2004). Other growth declines were also observed in 2006 and in 2015, which have been reported as exceptionally hot and dry years at regional scale (www.osmer.fvg.it), as confirmed also by our DMI index analysis (Table 4.1). On the contrary, increases in the ring width values were measured in 2004 and 2014, years with vegetative seasons characterised by mild temperatures and relatively abundant precipitations (www.osmer.fvg.it).

Looking at normalized tree ring widths, the three target species responded differently during the selected dry (2006) or the reference year (2014), with a 37% decrease in the first compared to the latter (Figure 4.2). Such a difference was consistently maintained throughout the tree species richness gradient, even though with different intensities (from 48% in the reference to 23% in the plantation with one consociated tree species; Figure 4.3). Moreover, comparing the control plantation to all the other stands, the difference in the normalized ring width values was more evident in the driest year than in the reference year (Figure 4.3). Our results support the idea that plantations with less than five consociated tree species may enhance tree growth in the target commercial tree species through niche complementarity and/or facilitation processes (Pacala and Tilman 1994; Petchey 2003; Schoener 1974), while higher numbers of consociated species (≥ 5) may decrease growth during the harshest seasons because of competition for resources (Lang *et al.* 2010; Wagner and Radosevich 1998). Such a behaviour has been already observed in literature, even though the majority of the studies have been performed on forest species as fir, pine, beech and spruce. Metz *et al.* (2016) found that European beech performed better (higher tree growth) when associated with pine compared to pure beech stands as tree ecophysiology, canopy structure and sunlight tolerance different between these two species. Nevertheless, at higher tree-species richness levels, a stronger competition for water was recorded and a reduced tree-ring growth in beech was measured (Metz *et al.* 2016). Moreover, Pretzsch and Dieler (2012) highlighted facilitative effects of oak on beech when conditions are less favourable, for example during drought periods. Del Rio *et al.* (2014) report that when environmental conditions are

favourable, a strong competition between beech and other species and, consequently, a decrease in tree growth, is detected while when conditions are less favourable (i.e. drought year) the opposite occurs (the so called “stress-gradient hypothesis”, as defined by Bertness and Callaway 1994). These results were also confirmed by Grossiord *et al.* (2014a) and, in a wider study across Europe, by Jucker *et al.* (2016). Thus, competitive and facilitative mechanisms can interact simultaneously inside the same community, depending on the local climatic conditions, producing a wide spectrum of positive or negative effects on tree growth (Callaway 1998; Callaway and Walker 1997). Moreover, the absence of both complementarity and competition or an equilibrium between these two mechanisms when environmental conditions are not limiting growth might bring to no effect of tree species richness on tree growth (Pretzsch *et al.* 2013), as we found at our experimental sites in 2014. We also measured a significant decrease in iWUE between 2006 and 2014 (Figure 4.4). The highest values were found in the plantation with five consociated species, whereas the lowest values were measured in the plantations with four and six consociated species. However, such differences were significant in 2006 only (Figure 4.5), confirming again that tree species richness has a positive effect on tree growth only when environmental conditions are harsher. Similar patterns in iWUE with species diversity have been also observed in boreal forests by Grossiord *et al.* (2014a) and by Gebauer *et al.* (2012).

4.5 Conclusions

Our results partially confirmed our working hypothesis that the higher is the number of consociated species (i.e. tree species’ diversity), the less the target commercial tree species are impacted by a drought event. In fact, a positive species richness effect on tree growth and iWUE was found only when less than five consociated species are present and only when environmental conditions are harsher (2006). For higher tree-species richness levels, competition for resources prevails thus leading to a decrease in radial tree growth and iWUE. Similarly, our data support the ‘stress gradient hypothesis’: the absence of both complementarity and competition or an equilibrium between these

two mechanisms when environmental conditions are not limiting growth (2014) bring to no effect of tree species richness on tree growth.

5. EVIDENCE FOR A LONG-TERM INCREASE IN TREE GROWTH AND INTRINSIC WATER-USE EFFICIENCY IN THREE OLD-GROWTH FORESTS IN BOSNIA-HERZEGOVINA AND MONTENEGRO³

Summary

We analysed the long-term patterns of tree growth and intrinsic water use efficiency (iWUE) in three old-growth forests in the Balkans (Bosnia-Herzegovina and Montenegro) using dendrochronology and isotopic analysis. Tree cores have been sampled from dominant silver fir (*Abies alba* Mill.) trees in each forest. Tree-ring widths were measured and basal area increments (BAI) was assessed for each sampled tree and, from the six longest chronologies, five decades were chosen for cellulose extraction, isotopic analysis ($\delta^{13}\text{C}$) and iWUE determination.

We observed a continuous increase in iWUE from 1800 to 2010 in all the old-growth forests, implying a continuous increase in the ratio between assimilation rates and stomatal conductance. Our results, coupled with the positive increment in BAI over all the studied period, support the idea that the global rise in atmospheric CO_2 has positively influenced tree growth. However, a stabilization of BAI was detected in the last few decades suggesting that a maximum, or even a decline, in the growth rate will be reached in the near future. This could translate in an overall decrease in the net C sequestration in these old-growth forests, thus limiting their role in terrestrial C sink capacity.

³ Palandrani C, Motta R, Cherubini P, Čurović M, Dukić V, Tonon G, Alberti G (submitted) Evidence for a long-term increase in tree growth and intrinsic water-use efficiency in three old-growth forests in Bosnia-Herzegovina and Montenegro. *Plant, Cell and Environment*.

5.1 Introduction

Human activities have caused approximately 1.0°C of global warming above values recorded in pre-industrial times, with important changes in the intensity and the frequency of some climate and weather extremes (IPCC 2018). Warming caused by anthropogenic greenhouse gases emissions (GHGs) will persist for centuries to millennia (Matthews and Caldeira 2008; Solomon *et al.* 2009) and will continue to cause further long-term changes either in the climate system or in the biosphere. In particular, atmospheric CO₂ concentration has been constantly rising in the last centuries because of human activities, reaching in the 2000s the highest levels over the last 160.000 years (IPCC 2014). In forest ecosystems, climate change is altering productivity, species distribution, tree physiology and pest disease severity and frequency, with widespread tree mortality phenomena (Allen *et al.* 2010; Anderegg *et al.* 2012; Lindner *et al.* 2014; Seidl *et al.* 2014, 2018). However, increase in CO₂ atmospheric concentration, coupled with the increase in nitrogen (N) deposition, can stimulate tree growth and can change forest dynamics (Ciais *et al.* 2008; Magnani *et al.* 2007; Lewis *et al.* 2009; Pretzsch *et al.* 2014). Indeed, elevated CO₂ concentrations can stimulate tree growth due to enhanced photosynthesis (Ainsworth and Long 2005): higher atmospheric CO₂ levels (C_a) usually lead to stomata closure, thus decreasing transpiration rates and increasing assimilation rates (Tognetti *et al.* 1999), with an improvement in the intrinsic water-use efficiency (iWUE), defined as the carbon (C) gained per unit water vapour lost at leaf level (Saurer *et al.* 2004). Consequently, higher iWUE values, coupled with faster tree growth measured by tree-ring width converted into basal area increment (BAI), have been previously reported (Feng 1999; Liu *et al.* 2007; Saurer *et al.* 2014). However, this relationship has been not always confirmed (Marshall and Monserud 1996; van der Sleen *et al.* 2015; Waterhouse *et al.* 2004). In fact, an increase in iWUE alone may not directly translate into higher BAI as other factors (e.g., high temperature, recurrent drought, nutrient limitation) may negatively influence tree growth (Tognetti *et al.* 2014).

Tree rings offer insight into lifetime growth patterns, allowing climate impacts on trees to be evaluated (Fritts 1976). On the other hand, stable C isotopes represent a useful tool to better

understand changes in tree growth and productivity related to climate conditions through time. The variability of the C isotopic signature ($\delta^{13}\text{C}$) in wood gives information about the plant's sensitivity to different climatic and environmental conditions (McCarroll and Loader 2006). C isotope discrimination ($\Delta^{13}\text{C}$) can be related to CO_2 flux through stomata and to changes in C_a to intercellular CO_2 concentration (C_i) gradient and, by consequence, to iWUE (Farquhar *et al.* 1989; Francey and Farquhar 1982). However, the extent to which rising C_a has affected long-term iWUE, and whether climate could explain deviations from expected C_a -induced growth enhancement, is still poorly understood (Tognetti *et al.* 2014).

Old-growth stands have developed for a long period of time without relevant human impact and/or stand replacing or major natural disturbances and have three main structural characteristics: old and large trees, abundant coarse woody debris in different decay stages and a multilayered vertical structure (Spies 2004). Old-growth have an important role in maintaining animal and plant diversity, are important C reservoirs and are privileged sites to study long-term forest dynamics and climate change impact on forest ecosystems (Motta *et al.* 2011). Several studies have highlighted how old-living trees undergo age-related declines with higher transpiration rates, hydraulic limitations and reduction in photosynthesis (Ryan and Yoder 1997; Ryan *et al.* 2006; Yoder *et al.* 1994), mainly because of higher susceptibility to climatic and environmental stress (Carrer and Urbinati 2004). On the other hand, recent works have suggested that such a decline is not always observed (Luyssaert *et al.* 2008; Yu *et al.* 2008) and that old-growth forests are still accumulating C (Luyssaert *et al.* 2008; Phillips *et al.* 2008; Zhou *et al.* 2006), thus contrasting the traditional "carbon neutrality hypothesis" according to which net ecosystem production (NEP) in these ecosystems is equal to zero (Odum 1969).

In this contest, we studied silver fir (*Abies alba* Mill.), one of the most widespread and long-lived species in southern European forests. Fir woody rings in European old-growth forests represent an important climatic archive to better understand the relationship between growth dynamics, atmospheric CO_2 concentration increase and climate conditions through time. The present study

quantifies silver fir tree growth patterns and iWUE changes during the last two centuries in three mixed old-growth forests across the Balkans. We measured tree-ring width and $\delta^{13}\text{C}$ to calculate iWUE and C_i to check whether tree growth have been increasing during the last 200 years and whether there is any relationship with iWUE. Our hypotheses were: 1) silver fir in all the three old-growth forests is still growing at a remarkable rate and actively sequestering C from the atmosphere in woody tissues and 2) increasing atmospheric CO_2 concentrations have increased iWUE in the last 200 years.

5.2 Materials and methods

5.2.1 Study area

Three study sites were selected in three different old-growth forests, along a 100 km transect from North-west to South-east, in the Balkans, in South-eastern Europe. All of the three sites are in the montane belt and are mixed with silver fir (*Abies alba* Mill.), Norway spruce (*Picea abies* Karst), and European beech (*Fagus sylvatica* L.).

The first site (LOM) is located in the Lom forest reserve (44°27'N, 16°28'E, 1250–1500 m a.s.l.) in the Dinaric Alps in Bosnia and Herzegovina. Climate is continental with maritime airstream influences. Mean annual precipitation is about 1600 mm and mean annual temperature is 7.6°C (Bottero *et al.* 2011).

The second site (PER) is located in the Perućica forest reserve (43°30'N, 18°70'E, 600-2800 m a.s.l.), inside the Sutjeska National Park in the southern Dinaric Mountains, Bosnia and Herzegovina. Climate is a mix of Mediterranean and continental, with mean annual precipitation of 1400 mm and mean annual temperature of 11.3°C (Nagel and Svodoba 2008).

The third site (BIO) is located in the National Park of Biogradska Gora (42°53'N, 19°36'E, 830-2100 m a.s.l.) in the north-eastern part of Bjelasica mountain range, Montenegro (Motta *et al.* 2014).

5.2.2 Tree sampling and dendrochronological analysis

At each site (in 2014, 2016 and 2017 in PER, LOM and BIO sites, respectively), 30 to 40 circular plots were identified, tree species were assessed and tree mean diameter at 1.30 m (d.b.h.) was measured. Then, in the inner part of the forest, one sampling area of about 1 ha per site was identified and tree cores were taken from the dominant individuals of silver fir using an increment borer. Cores were stored in cardboard and, once in laboratory, samples were glued on woody supports and sanded with paper with 60, 120, 240, 320 and 600 grit to better highlight tree rings.

Tree-ring widths were measured for each core using a LINTABTM (Frank Rinn, Heidelberg, Germany) and then were visually cross-dated using TSAP software (© TSAPWin Scientific, version 4.81, 2002-2018, Frank Rinn / RINNTECH). COFECHA software (Grissino-Mayer 2001; Holmes 1983) was used for the statistical cross-date and to check chronologies quality (Castagneri *et al.* 2014).

5.2.3 Isotopes analysis

From all the samples at each site, the six longest chronologies were selected. Then, tree rings for each core were grouped using 10-year intervals, cut and separately milled using an ultra-centrifugal mill with a sieve of 0.5 mm (ZM 100, Retsch Technology, Haan; Germany). In order to have enough replications at each plot, we considered only five consecutive time-intervals along each time-series for the further analysis (2010-2000; 1960-1950; 1910-1900; 1860-1850; 1810-1800). Wood powder was treated with a two-step digestion to extract cellulose according to Green (1963). Briefly, samples were sealed in Teflon bags and at first treated two times with NaOH 5% solution for 2 hours at 60°C. Then, samples were treated with NaClO₂ 7% solution, adjusting the pH to 4-5 with acetic acid, for 10 hours at 60°C. Depending on sample weight and its cellulose content, this phase was repeated for three or four times until samples totally bleached, thus indicating that all fibres but cellulose were completely digested. Dried samples were finally weighted and put in tin capsules for $\delta^{13}\text{C}$ analysis

using a CHNS elemental analyser (Vario Microcube, © Elementar) coupled with a stable isotope ratio mass spectrometer (IRMS; Isoprime 100, © Elementar).

5.2.4 Data analysis

Yearly basal area increment (BAI, cm² yr⁻¹) was assessed using tree-ring width. δ¹³C measurements were used to calculate the C isotopic discrimination (Δ¹³C) (Farquhar *et al.* 1982):

$$\Delta^{13}\text{C} = \frac{\delta^{13}\text{C}_a - \delta^{13}\text{C}_p}{1 - \delta^{13}\text{C}_p}$$

where δ¹³C_a is the isotopic signature of atmosphere and δ¹³C_p is the isotopic signature of the sample. Our δ¹³C_p values represent an average value for 10-year intervals so δ¹³C_a were calculated as the mean value for the same temporal interval of the relative δ¹³C_p measurements using published values in McCarroll and Loader (2006) and those from Mauna Loa Observatory from 2003 (<https://www.esrl.noaa.gov/gmd/>).

Then, we used the fractionation model equation proposed by Farquhar *et al.* (1982) and Francey and Farquhar (1982) to compute intercellular CO₂ concentration (C_i, ppm) knowing Δ¹³C and atmospheric CO₂ concentration (C_a, ppm; data from Mauna Loa Observatory, <https://www.esrl.noaa.gov/gmd/>):

$$\Delta^{13}\text{C} = a + (b - a) * \frac{C_i}{C_a}$$

$$C_i = C_a * \frac{(\delta^{13}\text{C}_p - \delta^{13}\text{C}_a + a)}{(a - b)}$$

where a is isotope discrimination during CO₂ diffusion through stomata (a = 4.4‰), b is isotopes discrimination during carboxylation processes (b = 27‰).

According to the Fick's law, net photosynthesis (A) is:

$$A = g_{\text{CO}_2} * (C_a - C_i)$$

where g_{CO₂} is leaf conductance to CO₂. Knowing that leaf conductance to water vapour (g_{H₂O}) is equal to 1.6g_{CO₂}, we can calculate iWUE (μmolCO₂ molH₂O⁻¹) (Farquhar *et al.* 1982), expressed as the

ratio between photosynthesis and stomatal conductance to H₂O (A/g_{H2O}) combining all the previous equations:

$$iWUE = \frac{A}{g_{H_2O}} = c_a * \frac{(b - \Delta^{13}C)}{1.6 * (b - a)}$$

5.2.5 Statistical analysis

All statistical analysis were performed using RStudio software (Rstudio, version 1.2.1335, ©2009-2019 Rstudio, inc.). We compare iWUE and basal area increment in the different decades by using a One-way ANOVA, with Tukey's post-hoc test when significant differences were detected. All data were eventually log-transformed before performing the statistical analysis to meet the requirements for parametric statistical tests using `powerTransform` and `bcPower` functions in `car` package. We further correlate C_i to selected 10-year intervals and iWUE to basal area increment through simple linear regressions, using Shapiro-Wilk normality test to check the normal distribution of model's residuals. All data throughout the text and in tables and figures are reported as mean ± standard error.

5.3 Results

The mean stand characteristics at each experimental site are reported in Table 5.1. Total stem density ranged from 412±22 to 489±19 trees ha⁻¹ at BIO and LOM, respectively, but total basal area followed an opposite trend being the highest at LOM and the lowest at BIO (60.1±4.4 and 47.1±1.8 m² ha⁻¹, respectively). European beech was the dominant species in terms of stem density (n ha⁻¹) at all the three sites, but silver fir was always dominant in terms of basal area (m² ha⁻¹). The oldest fir trees were 306, 267 and 273-year-old in LOM, PER and BIO, respectively. Mean age of the sampled trees at LOM was significantly different from that at PER but not from that at BIO (236±52, 157±51 and 191±63 years, respectively; p<0.001).

SITE	STAND DENSITY (n ha ⁻¹)	MEAN DIAMETER (cm)	TOTAL BASAL AREA (m ² ha ⁻¹)	TOTAL SPECIES DENSITY (n ha ⁻¹)			SPECIES BASAL AREA (m ² ha ⁻¹)			NUMBER OF SAMPLED TREES (Fir)	MAX TREE AGE (yrs)	MEAN TREE AGE (yrs)	MIN TREE AGE (yrs)
				<i>Fir</i>	<i>Beech</i>	<i>Spruce</i>	<i>Fir</i>	<i>Beech</i>	<i>Spruce</i>				
LOM	489±19 (a)	35.4±0.8 (a)	47.1±1.8 (a)	122±9 (a)	296±13 (a)	69±9 (a)	22.2±1.6 (a)	14.3±0.7 (a)	10.4±1.5 (a)	28	306	236 ± 52 (a)	129
PER	432±21 (ab)	41.7±1.6 (b)	59.1±4.5 (b)	90±13 (a)	337±20 (a)	6±2 (b)	39.5±4.7 (b)	17.0±1.5 (a)	2.6±1.2 (b)	43	267	157 ± 51 (b)	71
BIO	412±22 (b)	43.2±1.8 (b)	60.1±4.4 (b)	92±11 (a)	298±21 (a)	10±3 (b)	34.1±4.7 (ab)	18.7±2.1 (a)	5.6±2.0 (b)	7	273	191 ± 63 (ab)	114

Table 5.1 - Mean stand characteristics at each experimental site (LOM = Lom; PER = Perucica; BIO = Biogradska Gora). Mean ± standard error.

Fir trees showed a constant and significant increase in tree basal area increment with time ($\text{cm}^2 \text{ tree}^{-1} \text{ yr}^{-1}$; Figure 5.1) at all the three experimental sites (LOM: $R^2=0.62$, $p<0.001$; PER: $R^2=0.47$, $p<0.001$; BIO: $R^2=0.56$, $p<0.001$), especially after the beginning of the 19th century. BIO had generally the highest growth rate even though it showed a slight decline in radial increment in the last two decades, while fir at PER had generally the lowest growth, but did not show any decrease in growth with time. When mean decadal values are considered, such trends are even more clear (Figure 5.2): tree growth was always the lowest during the first two decades (1800-1810 and 1850-1860) than at the end of the period (2000-2010) at all the three forests ($p<0.05$). At BIO, the decade 1900-1910 was also significantly different from the decade 2000-2010.

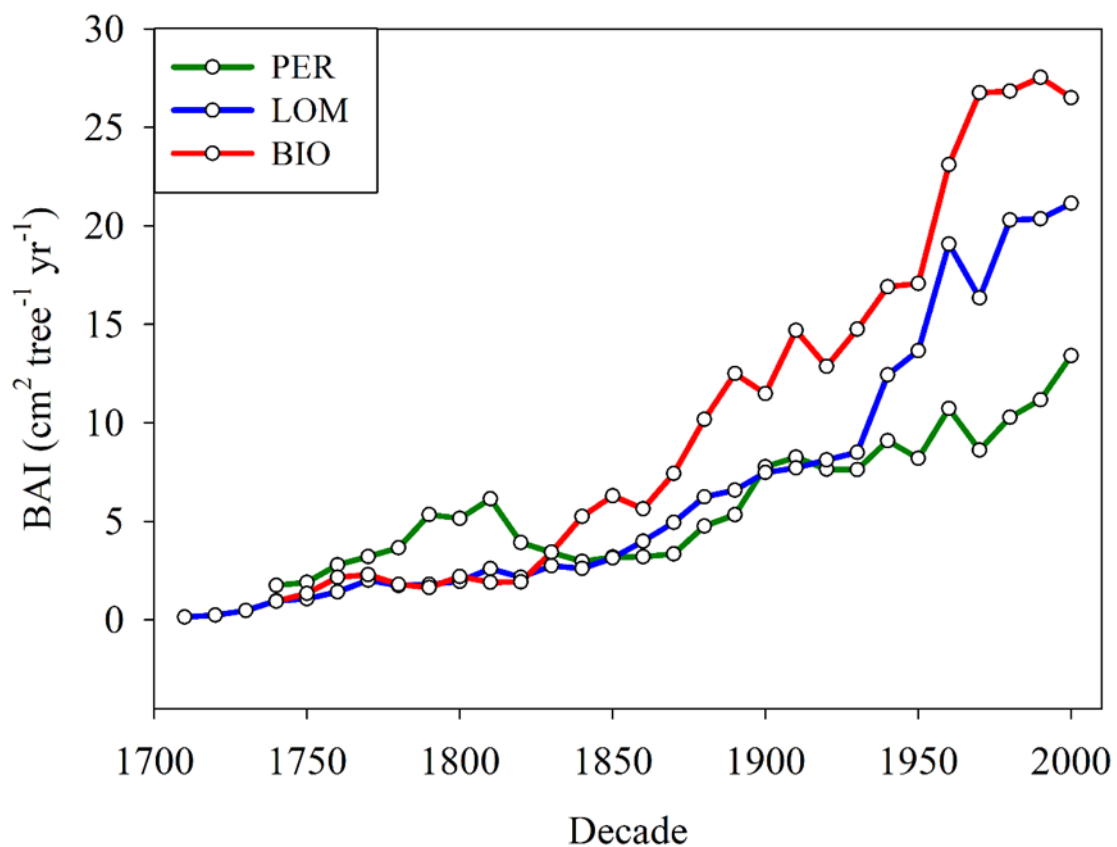


Figure 5.1 - Mean basal area increment (BAI, $\text{cm}^2 \text{ tree}^{-1} \text{ yr}^{-1}$) of the sampled silver fir trees per decade (PER = Perucica; LOM = Lom; BIO = Biogradska Gora).

C_i significantly increased through time at all sites (LOM: $p<0.001$; PER: $p<0.001$; BIO: $p=0.01$; Figure 5.3), with an average increase of +13%, +24% and +18%, in LOM, PER and BIO, respectively,

from 1800 to 2010. We observed also a significant decrease in the ratio C_i/C_a in the last two centuries (LOM: $R^2=0.62$, $p<0.001$; PER: $R^2=0.30$, $p=0.003$; BIO: $R^2=0.30$, $p=0.005$; data not shown). Trees showed an increase in iWUE, with significant higher values at the end of the studied period than at the beginning (Figure 5.4): from 1800-1810 to 2000-2010, iWUE increased, on average, of +45%, +30% and +40% at LOM ($p<0.001$), PER ($p<0.001$) and BIO ($p<0.001$), respectively. Using linear regressions, we found that iWUE and BAI were positively and significantly correlated at all sites (LOM: $R^2=0.93$, $p=0.008$; PER: $R^2=0.91$ $p=0.013$; BIO: $R^2=0.97$, $p=0.002$, Figure 5.5).

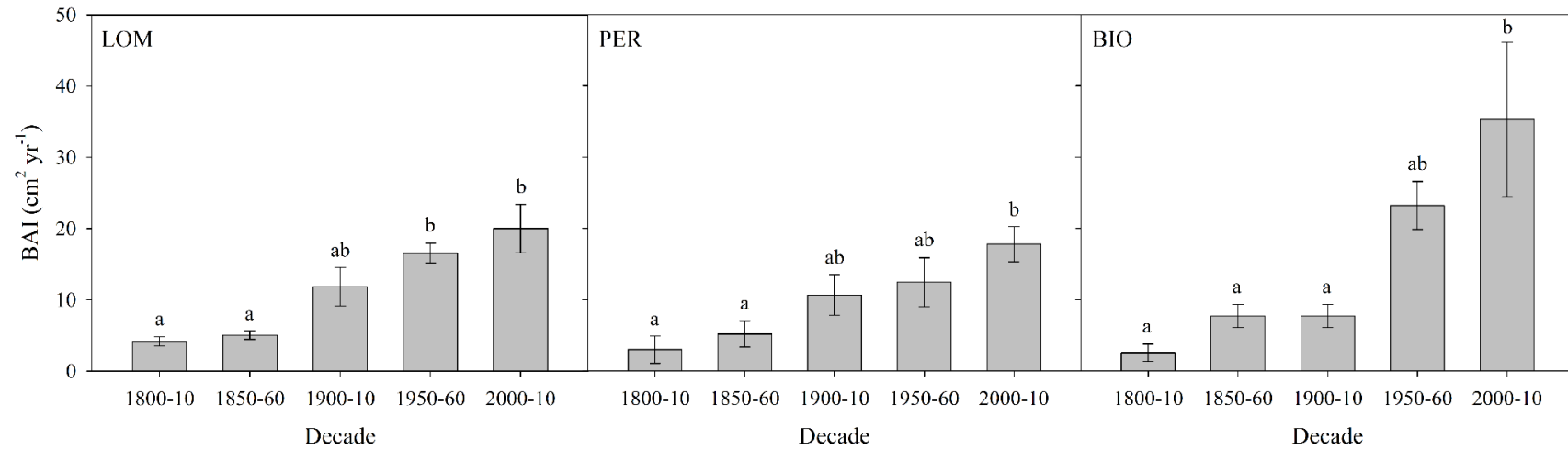


Figure 5.2 - Mean basal area increment (BAI, $\text{cm}^2 \text{tree}^{-1} \text{yr}^{-1}$) comparison between the consecutive selected 10-years intervals at the three experimental sites (PER = Perucica; LOM = Lom; BIO = Biogradska Gora). Mean \pm standard error. Different letters indicate significant differences among periods ($p < 0.05$).

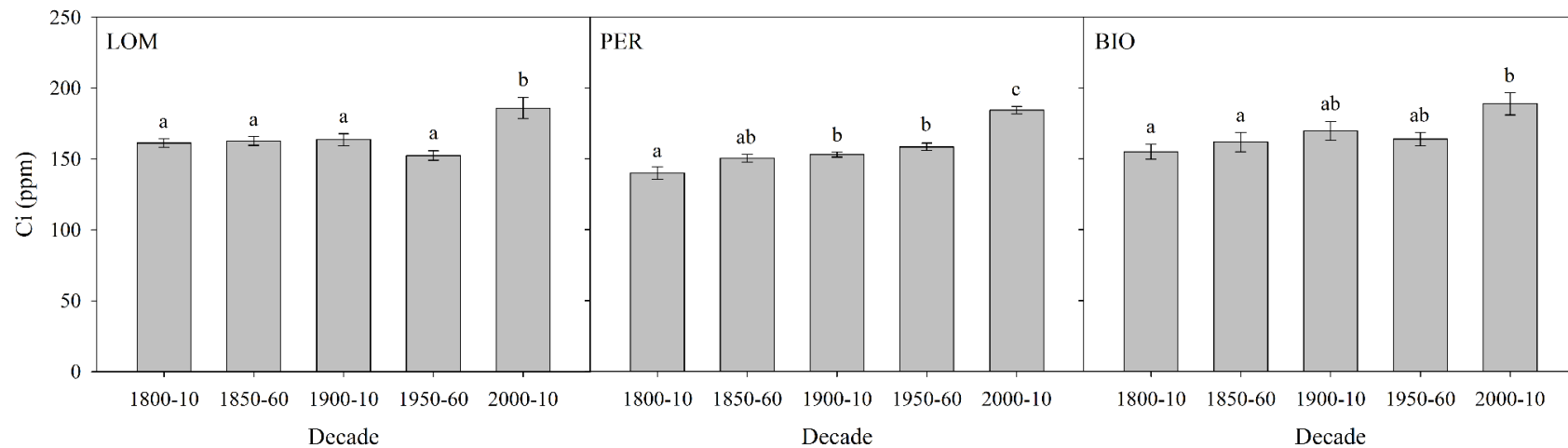


Figure 5.3 - Intercellular CO_2 concentration (C_i , ppm) in the selected 10-years intervals at the three old-growth forests (PER = Perucica; LOM = Lom; BIO = Biogradska Gora). Mean \pm standard error. Different letters indicate significant differences among periods ($p < 0.05$).

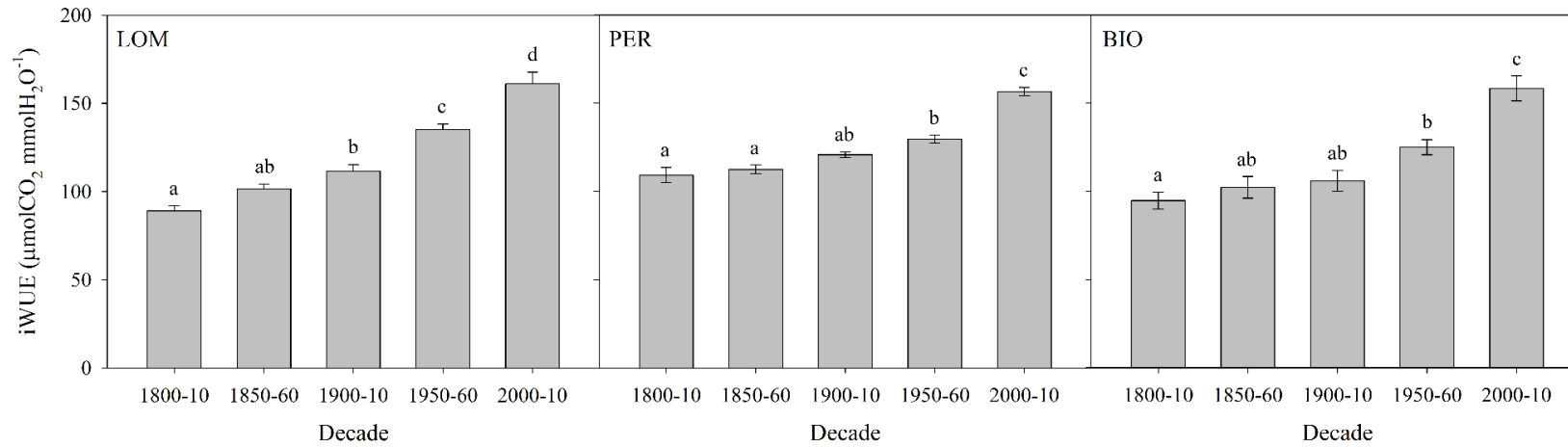


Figure 5.4 - Intrinsic water-use efficiency (*iWUE*, $\mu\text{mol CO}_2 \text{ mol H}_2\text{O}^{-1}$) in the three old-growth forests for the selected 10-years intervals (*PER* = *Perucica*; *LOM* = *Lom*; *BIO* = *Biogradska Gora*). Mean \pm standard error. Different letters indicate significant differences among periods ($p < 0.05$).

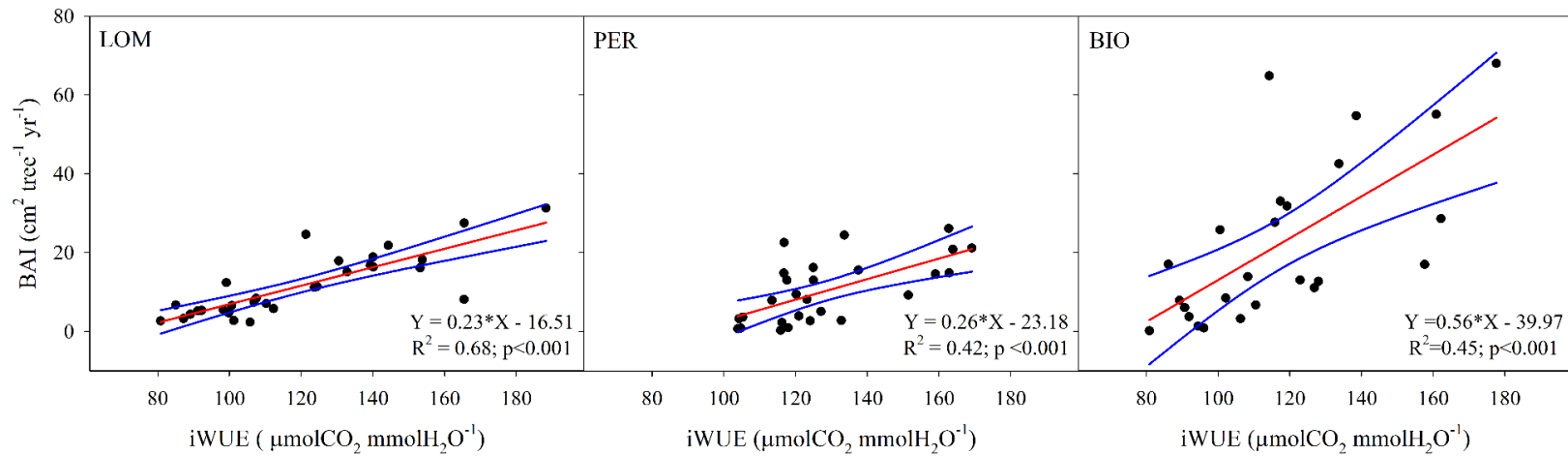


Figure 5.5 - Basal area increment (*BAI*, $\text{cm}^2 \text{ tree}^{-1} \text{ yr}^{-1}$) with increasing intrinsic water-use efficiency (*iWUE* $\mu\text{mol CO}_2 \text{ mol H}_2\text{O}^{-1}$). Each point represents a selected 10-years interval for each sampled tree. Red line represents the regression line and blue lines represent 95th confidence interval.

5.4 Discussion

Over the last two centuries, we found a constant increase in silver fir BAI at all the three old-growth forest sites in the Balkans. Such an increase has been more evident since 1850, especially at BIO, where BAI exceeded $25 \text{ cm}^2 \text{ yr}^{-1}$, followed by LOM and PER. From 1850-1860 to 2000-2010, we measured a mean increase in BAI of +85%, +81% and +71% at LOM, PER and BIO, respectively (Figure 5.2). Similar trends have been already observed for *Abies alba* (Bert *et al.* 1997), *Pinus uncinata* (Granda *et al.* 2017) and *Abies georgei var. smithii* (Huang *et al.* 2017), even though opposite trends have been also reported for similar or other species (Andreau-Hayles *et al.* 2011; Peñuelas *et al.* 2011; Rezaie *et al.* 2018; Saurer *et al.* 2004). In our study, iWUE showed a significant increase of +45%, +30% and +40% in the last two centuries at LOM, PER and BIO, respectively (Figure 5.4). These trends are similar to those measured in a wide range of ecosystems (Frank *et al.* 2015; Peñuelas *et al.* 2011; Silva and Anand 2013), either in temperate (Saurer and Siegwolf 2007; Waterhouse *et al.* 2004) or tropical/sub-tropical forests (Wils *et al.* 2016; Wu *et al.* 2015), as well as at single experimental sites (Battipaglia *et al.* 2013), but none of these studies considered either old-growth forests or period of time longer than 100 years.

The increase in atmospheric CO_2 concentration affects plants photosynthetic rates by increasing carboxylation rate of RUBISCO enzymatic system (Ainsworth and Long 2005; Drake *et al.* 1997; Huang *et al.* 2007), thus positively influencing tree growth rate by improving the production of non-structural C for sink activity (Linares *et al.* 2009; Streit *et al.* 2013). Moreover, increasing C_a induces stomata closure and, by reducing water loss by transpiration, plants may improve iWUE (Farquhar *et al.* 1989; McCarroll and Loader 2004). Variations in both photosynthetic rates and stomatal conductance influence the gradient of C_a to C_i , determining the discrimination intensity against ^{13}C and consequent $\delta^{13}\text{C}$ signature in tree rings (Ehleringer and Cerling 1995). According to Saurer *et al.* (2004): i) C_i may stay constant, C_i/C_a decreases, Δ decreases and iWUE strongly increases; ii) C_i may increase proportionally to C_a so that C_i/C_a is constant, Δ does not change and iWUE still increases; iii) C_i may increase at the same rate of C_a ($C_a - C_i$ is kept constant), C_i/C_a increases, Δ increases, there

is a weak stomatal response and $iWUE$ is not improved. At all our study sites, C_i and $iWUE$ increased with time (Figure 5.3 and Figure 5.4, respectively), while both Δ and C_i/C_a decreased (data not shown). Thus, trees at our study sites behaved according to Saurer *et al.* (2004) hypothesis i), even though a significant increase in C_i was detected during the last considered decade (2000-2010; Figure 5.3). Such a measured increase in C_i indicates that trees are still actively responding to increasing atmospheric CO_2 concentrations (McCarroll and Loader 2004). However, when all decades are considered (Figure 5.1), a stabilization of BAI was detected, especially at BIO, as also observed by Rezaie *et al.* (2018) as well as by Peñuelas *et al.* (2008) in beech forests. Such a decrease in BAI can be related to a complex of interacting factors such as a decrease in nutrient availability (nitrogen and phosphorous), different allocation patterns between above- and below-ground, diversion to secondary metabolites (Tognetti *et al.* 2000).

Peñuelas *et al.* (2011) in their global analysis found no significant BAI increases in response to increases in $iWUE$. On the contrary, we found a strong positive correlation between BAI and $iWUE$ at all our three old-growth forest sites (Figure 5.5), linking positive CO_2 fertilization effect on tree growth and decrease in plant water use.

5.5 Conclusions

In the last two centuries, we observed a continuous increase in $iWUE$ in silver fir at all the three old-growth forest sites in the Balkans. This implies a continuous increase in the ratio between assimilation rates and stomatal conductance. Thus, our results support the idea that the global rise in atmospheric CO_2 and changing climate have positively influenced the gas exchange of these old-growth forests and might have decreased plant water use. Moreover, the observed positive increment in BAI across all the studied period further supports the fertilization effect of the atmospheric CO_2 increase over time. However, a stabilization of BAI was detected in the last few decades, especially at BIO, suggesting that a maximum, or even a decline, in the growth rate will be reached in the near future.

This could translate in an overall decrease in the net C sequestration in these old-growth forests, thus limiting their role in terrestrial C sink capacity.

6. OVERALL CONCLUSIONS

This thesis has focused on the role of tree species richness and functional diversity in supporting soil C sequestration and in mitigating the possible negative effects of extreme events, by increasing stand resistance and/or resilience. In the last part of this work, we have also quantified the long-term effects of atmospheric CO₂ increase on growth and water-use efficiency in some old-growth forests in the Balkans.

As far as the first aspect is concerned (i.e. soil C sequestration), our results showed that both species richness and functional diversity have a positive effect on the proportion of tree derived C in forest plantations, underlying the fact that more diverse stands can enhance and better modulate microbial community and, thus, soil C dynamics through more diverse litters.

Our second study showed that tree species diversity has an overall positive effect on the response to drought, but only at the lower levels of tree species richness, when facilitation and/or complementarity mechanisms prevail. Instead, negative responses are typical at higher levels of species richness, when competition is the dominant process within the stand.

Finally, our last study on the old-growth forests in the Balkans supports the idea that the global rise of atmospheric CO₂ and changing climate have positively influenced the gas exchange in the studied old-growth forests and might have decreased plant water use. In particular, the observed positive increment in BAI across all the studied period further supports the fertilization effect of the atmospheric CO₂ increase over time. However, a stabilization of BAI was detected in the last few decades, suggesting that a maximum, or even a decline, in the growth rate will be reached in the near future. This, could translate in an overall decrease in the net C sequestration in these old-growth forests, thus limiting their role in terrestrial C sink capacity.

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8. ANSWER TO REVIEWERS' COMMENTS

Per prima cosa desidero ringraziare i revisori di questa tesi per i commenti costruttivi e gli spunti per una ulteriore rifinitura di questo scritto. Le risposte ai commenti sono state riportate per i due revisori separatamente e sono stati riportati i singoli commenti con le mie risposte a seguire.

REVISORE: Daniele Ascoli

Cambiare "De Miccio" in "De Micco" in tutto il testo.

Cambiato.

LN456: cambiare "enhanced" in "enhance"

Cambiato.

REVISORE: Tommaso Anfodillo

Introduzione piuttosto generica: invece del grafico della CO₂ in atmosfera (che non è collegata direttamente agli esperimenti effettuati, anche se nel Cap 5 è riportata come causa di aumento della produttività) mi sarei aspettato di vedere citati molti più lavori sulla relazione biodiversità-funzionalità (relazione trattata solo nelle righe 36-42).

Espanderei questa parte.

Questa parte è stata ampliata. Tuttavia, poiché ogni capitolo/articolo presenta una sua introduzione specifica con relativi riferimenti bibliografici, si è ritenuto di non entrare eccessivamente nel dettaglio nell'introduzione generale al fine di non appesantire la tesi o ripetere concetti sviluppati dopo in modo più ampio.

Nell'esperimento del CAP 3 (e anche per il 4) la struttura delle piantagioni è molto diversa non solo per numero di specie ma anche per LAI, altezza e G. Forse sarebbe stato meglio scegliere qualcosa di più omogeneo. Inoltre nelle discussioni si giustifica il maggiore fnew nel suolo con una possibile maggiore produttività delle piantagioni con tante specie ma la biodiversità è inversamente correlata alla G (fig. 3.1). La discussione sulla mortalità maggiore nelle piantagioni con tante specie non è convincente (L. 331-337). Se la mortalità è maggiore allora gli individui rimanenti dovrebbero crescere di più (hanno più risorse ciascuno). Ma l'altezza media è minore nella piantagione con 9 specie. Non saprei aiutare a riguardo ma i processi non sono chiari. L'affermazione che due specie dello stesso genere dovrebbero esprimere una maggiore competizione interspecifica non mi pare plausibile. Forse bisognerebbe portare qualche evidenza sperimentale a riguardo.

Gli impianti sono stati scelti omogenei tra loro per quanto riguarda le condizioni iniziali di impianto (unico dato a disposizione per la scelta). Negli anni i diversi impianti sono andati incontro a fenomeni di mortalità naturale (avendo un sesto di impianto regolare si potevano osservare chiaramente i punti con le piante mancanti), che probabilmente hanno influito sulla stima della biomassa e del LAI. Numerosi lavori (alcuni anche riportati) osservano un aumento della produttività o del carbonio accumulato nel suolo correlato ad un aumento della diversità. Avendo trovato una relazione positiva tra ricchezza specifica e stock di carbonio (utilizzando gli isotopi) ma negativa tra ricchezza specifica e produttività, possiamo ipotizzare che la qualità della lettiera (anche se in quantità minori) ha avuto un ruolo preponderante nel determinare gli stock di carbonio nel suolo.

Ho qualche dubbio (ma non sono dendroecologo) sulle elaborazioni e discussioni delle misure degli anelli legnosi è riportata come "tree growth" (capitolo 4). Prima cosa: la detrendizzazione dell'età non mi pare efficace: è rimasto un trend significativo (forse è "reale" ma da verificare). Seconda cosa: lo spessore dell'anello non puo' essere

rappresentativo della crescita. La crescita annuale non puo' mai diminuire con la dimensione della pianta (infatti vi sono sempre più foglie, come si vede bene nella fig. 5.1 per errore 4.1). Meglio esprimere la crescita come incremento di area basimetrica BAI come nel capitolo 5 (perchè non si segue lo stesso approccio?). Penso che il grafico cambi completamente. Capisco che i confronti sono relativi rispetto all'anno di riferimento ma comunque il termine tree growth è errato. Meglio ring width. Sugerirei di cambiare. Nelle righe 528-532 si parla di detrendizzazione. Forse allora non è stato messo il grafico delle curve detrendizzate?

Ho modificato le figure riportando la dicitura “tree-ring width”. La figura 4.1 rappresentava solamente il trend nelle tre specie dello spessore degli anelli. Adesso la figura 4.1 comprende anche le curve di crescita detrendizzate. Il passaggio di detrendizzazione, legato al confronto fra crescite in anni differenti viene applicato alle crescite degli anelli e non al BAI (si veda Vitali et al. 2017). La crescita annuale può essere inferiore rispetto l'anno precedente, se le condizioni non sono state ottimali, sia che venga espressa come “tree-ring width” che come BAI. Infatti, in figura 5.1 ci sono notevoli oscillazioni tra le decadi e in numerosi lavori eseguiti su foreste vetuste sono stati osservati stati di declino con trend negativi del BAI.

Forse il General summary si poteva espandere un po'. Sono riportati solo gli obiettivi ma non sono minimamente citati né i principali metodi né i risultati fondamentali né qualche conclusione. Penso vada sempre mantenuta la struttura IMRaD.

Ho modificato il General summary seguendo la struttura IMRaD.

Discussione del Cap. 4 (risposta allo stress idrico-diversità): non è facile da piegare perché solo fino a 4 specie la crescita delle singole specie aumenti ed oltre no. Che dire delle foreste triopicali con 50-60 specie diverse? Se fosse così allora tutte le foreste avrebbero al massimo 4 specie. Penso che sia una visione tipica dei forestali delle zone temperate ma che sarebbe

difficilmente condivisa da coloro che lavorano nei tropici. Siccome il tema è molto generale (funzionalità-biodiversità) e quindi si dovrebbe applicare a tutte le foreste queste discussioni non mi convincono tanto.

cap 5. penso ci sia un errore di fondo. In tutte le piante BAI aumenta con dimensione (si veda Sillet et al 2010 nelle piante più grandi al mondo). Quindi non si deve cercare una spiegazione "particolare" (alta CO2) che può essere utilizzata solo per spiegare l'aumento di iWUE .

Il mio studio ha considerato tre specie target e l'effetto dell'aumento della ricchezza specifica sulla loro resistenza ad eventi siccitosi estremi. Non ho quantificato la resistenza complessiva dell'ecosistema e, sicuramente, cambiando specie target l'effetto potrebbe essere diverso. Per quanto riguarda le foreste tropicali, quelle con maggiore ricchezza specifica si trovano in ambienti dove, comunque, l'acqua non è un fattore limitante e quindi i confronti non sarebbero appropriati. Come in una precedente risposta, il BAI non è detto che aumenti con le dimensioni, considerato che si riferisce alla superficie della corona circolare corrispondente all'anello di crescita di un dato anno. Sillet et al., come anche altri lavori, hanno osservato un trend negativo del BAI in piante senescenti, malate o comunque, molto vecchie.