Changes in leaf $\delta^{13}C$ and $\delta^{15}N$ for three Mediterranean tree species in relation to soil water availability

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**Abstract**

A rain exclusion experiment simulating drought conditions expected in Mediterranean areas for the following decades (15% decrease in soil moisture) was conducted in a Mediterranean holm oak forest to study the response of leaf $\delta^{13}C$, $\delta^{15}N$, and N concentrations to the predicted climatic changes for the coming decades. Plant material was sampled in 2000, 2003, 2004, and 2005 in eight plots: four of them were control plots and the other four plots received the rain exclusion treatment. Although there was a negative relationship between $\delta^{13}C$ and soil moisture, for each species and year, the rain exclusion treatment did not have any significant effect on $\delta^{13}C$, and therefore on the intrinsic water use efficiency (iWUE) of the three dominant species: Phillyrea latifolia, Arbutus unedo, and Quercus ilex. On the other hand, rain exclusion clearly increased the $\delta^{15}N$ values in the three species studied, probably indicating higher N losses at the soil level leading to a $^{15}N$ enrichment of the available N. It suggested that rain exclusion exerted a greater effect on the nitrogen biogeochemical cycle than on the carbon assimilation process. $\delta^{15}N$ values were inversely correlated with summer soil moisture in Q. ilex and A. unedo, but no relationship was observed in P. latifolia. This latter species showed the lowest iWUE values, but it was the only species with no decrease in annual basal increment in response to the rain exclusion treatment, and it also had the highest resistance to the hot and dry conditions projected for the Mediterranean basin in the coming decades. The different strategies to resist rain exclusion conditions of these species could induce changes in their competitive ability and future distribution. The losses of N from the ecosystem may further limit plant growth and ecosystem functioning.

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1. **Introduction**

Stable isotope discrimination of several elements has been widely studied in plant physiology (Griffiths, 1991). Plant photosynthesis discriminates against the stable $^{13}C$ isotope (O’Leary, 1988, 1993; Farquhar et al., 1989) when atmospheric CO$_2$ passes through stomata and during CO$_2$ carboxylation in RuBisCo. $^{13}C$ discrimination decreases with a decrease in intercellular CO$_2$ concentration due to stomatal closure, and consequently with water use efficiency (WUE). In this way, higher $\delta^{13}C$ is observed in drier sites and during drier years (Ehleringer, 1993; Lajtha and Marshall, 1994; Damesin et al., 1997; Peñuelas et al., 2000). $^{15}N$ discrimination also occurs in some ecological processes such as the transfer of N from...
mycorrhizal fungi to plants, or the N losses from the ecosystem which enrich the remaining N in $^{15}\text{N}$, such as gaseous N losses and the leaching of NO$_3^-$ when nitrification is incomplete. After the rain event which ends a rain exclusion period, there are losses of nitrogen that tend to discriminate against the heavier $^{15}\text{N}$ isotope, favouring larger proportional loss of $^{14}\text{N}$ and increasing $^{15}\text{N}$ of the remaining N in the ecosystem (Peñuelas and Aznó-Bieto, 1992; Handley and Raven, 1992; Högb erg and Johannisson, 1993; Peñuelas and Estiarte, 1997; Hobbie et al., 2005; Craine et al., in press).

Mediterranean environments are often characterized by dry summers with low precipitation and high temperatures (Mitrakos, 1980), high irradiance and high water vapour pressure deficit (Di Castri and Mooney, 1973; Terradas and Savé, 1992; Pereira and Chaves, 1995). This rain exclusion period is frequently considered a key factor in Mediterranean environments. Many studies have described low photosynthetic rates during summer rain exclusion due to a greater stomatal control of transpiration water loss (Tenthunen et al., 1981, 1990; Lange et al., 1982; Lösch et al., 1982; Harley et al., 1987; Gratani, 1993, 1995; Faria et al., 1998; Filléa et al., 1998; Peñuelas et al., 1998; Gratani and Bombelli, 1999; Karavatas and Manetas, 1999; Llusià and Peñuelas, 2000). The relatively greater decrease in stomatal conductance than in photosynthetic rates produce an increase in WUE (Peñuelas and Aznó-Bieto, 1992; Peñuelas et al., 2000). Rain exclusion also changes N use (Peñuelas and Estiarte, 1997; Peñuelas et al., 2000) since a decrease in water availability induces significant changes in the biogeochemical cycles of the ecosystem that end up reducing nutrient availability (Sardans and Peñuelas, 2004, 2005), and soil nitrogen content (Sardans et al., in press).

An increase in air temperatures is predicted for the Mediterranean basin during the coming decades, and a decline in rainfall is also often projected for this area (IPCC, 2007). Higher evapotranspiration and lower water availability for vegetation are also projected by GCM and ecophysiological models (Sabaté et al., 2002; Peñuelas et al., 2005; IPCC, 2007). Our aim was to study the effect of these projected changes in water availability on $^{13}\text{C}$ and $^{15}\text{N}$ considered as indicators of changes in WUE (Farquhar et al., 1989) and N availability and use (Peñuelas and Estiarte, 1997). Thereby we can estimate future water and N use alterations induced by climatic change in the dominant species of a Mediterranean holm oak forest, Quercus ilex, Arbutus unedo and Phillyrea latifolia. Holm oak (Quercus ilex L.) is a drought tolerant tree widely distributed in the Mediterranean basin. Phillyrea latifolia L. is a small tree associated with holm oak forests and more tolerant to drought and high temperatures than Q. ilex (Peñuelas et al., 1998; Ogaya and Peñuelas, 2003a, b). Arbutus unedo L. is another small tree, typical of holm oak forests, that in this area has shown greater drought sensitivity than P. latifolia (Ogaya and Peñuelas, 2007a). Under a moderate rain exclusion, a decrease in net photosynthetic rates and in the formation of new leaves in Q. ilex, and a decrease in flower and fruit production in A. unedo have been observed. These effects contributed to a decrease in stem growth and biomass accumulation in Q. ilex and A. unedo, but not in P. latifolia, species without any significant effect produced by the moderate rain exclusion (Ogaya and Peñuelas, 2006, 2007a, b). In this study we aimed to test our hypothesis that under drier climatic conditions, drought-resistant species, such as P. latifolia, could be less affected by water and N availability than some other species, such as Q. ilex, the dominant species of this forest. We also expected an increase in $^{15}\text{N}$ values as a result of ecosystem N losses induced by rain exclusion treatment.

2. Materials and methods

2.1. Study site

The present study was carried out in Prades holm oak forest in southern Catalonia (NE Spain) (41° 21’ N, 1° 2’ E, 950 m) on a south-facing slope (25% slope). The soil is a Dystric Cambisol over Paleozoic schist, and its depth ranges from 35 to 90 cm. This holm oak forest has a very dense multi-stem crown (16,616 stems ha$^{-1}$) and it is dominated by Q. ilex (8633 stems ha$^{-1}$), P. latifolia (3600 stems ha$^{-1}$) and A. unedo (2200 stems ha$^{-1}$), with abundant presence of other evergreen species well adapted to dry conditions (Erica arborea L., Juniperus oxycedrus L., Cistus albidus L.) and occasional individuals of deciduous species (Sorbus torminalis (L.) Crantz, and Acer monspessulanum L.).

2.2. Experimental design

In the study site, eight 15 × 10 m plots were delimited at the same altitude along the mountain face. Four of them (randomly selected) received the treatment consisting of partial rain exclusion by suspending PVC strips at a height of 0.5–0.8 m above the soil (covering 30% of the soil surface), and the excavation of a 0.8 m deep ditch at the upper part of the plots to intercept runoff water supply. The rain exclusion treatment started in January 1999. Water intercepted by strips and ditches was conducted outside the plots, below their bottom edge. The other four plots did not receive any treatment and were considered control plots.

An automatic meteorological station installed between the plots monitored temperature, photosynthetic active radiation, air humidity, and precipitation. Soil moisture was measured each month throughout the experiment by time domain reflectometry (Tektronix 1502C, Beaverton, OR, USA) connecting the time domain reflectometer to the ends of three stainless steel cylindrical rods, 25 cm long, fully driven into the soil (Zegelin et al., 1989). Four sites per plot were randomly selected to install the steel cylindrical rods for soil moisture measurements.

2.3. Isotope and elemental analyses

In each plot, five Q. ilex and P. latifolia trees were sampled in August 2000, 2003, 2004, and 2005, and five A. unedo trees were sampled only in August 2005. All leaves collected were fully developed; leaf flushing occurred during May in the three species studied (Ogaya and Peñuelas, 2004). Only current-year leaves were collected for analyses, and the same stem per tree was sampled during the overall studied period. Leaf nitrogen concentrations (% dry weight) and foliar $^{15}\text{N}$ and $^{13}\text{C}$ analyses were carried out in an elemental analyser EA1108 (Carlo Erba, Milano, Italy) coupled to a Delta C isotope ratio mass
spectrometer with a CONFLO II interface (Thermo Finnigan MAT, Bremen, Germany). The calibrations were performed using interspersed international isotopic standards of carbon and nitrogen (IAEA, Vienna, Austria). The elemental analysis calibration was performed using atropine (ThermoQuest Italia, Milan, Italy) as the standard.

Values were expressed relative to PDB standard for δ\(^{13}\)C and relative to atmospheric nitrogen for δ\(^{15}\)N, as (\(\delta_{\text{air}}\)), according to the following equation:

\[
\delta Z = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000
\]  

where Z is the heavy isotope of either nitrogen or carbon, and R is the ratio of heavier to lighter isotope for the sample and standard (\(^{13}\)C/\(^{12}\)C or \(^{15}\)N/\(^{14}\)N). The accuracy of the measurements was ±0.15\(^{\circ}\) for δ\(^{13}\)C and ±0.3\(^{\circ}\) for δ\(^{15}\)N.

Intrinsic water use efficiency (iWUE) was calculated according to the following equations (McCarroll and Loader, 2004):

\[
iWUE = A/g = c_a[1 -(c_i/c_a)] \times (0.625)
\]  

\[
c_i = c_a \left( \frac{\delta^{13}C_{\text{plant}} - \delta^{13}C_{\text{air}} + a}{b - a} \right)
\]

where \(a\) is the discrimination against \(^{13}\)CO\(_2\) during diffusion through the stomata (±4.4\(^{\circ}\)), \(b\) is the net discrimination due to carboxylation (±27\(^{\circ}\)), and \(c_i\) and \(c_a\) are intercellular and ambient CO\(_2\) concentrations (Farquhar et al., 1982).\(A\) is the rate of CO\(_2\) assimilation and \(g\) is the stomatal conductance. Data of \(\delta^{13}C_{\text{air}}\), \(c_i\) and \(c_a\) were obtained from McCarroll and Loader (2004) which used the high precision records of atmospheric δ\(^{13}\)C from Antarctic ice cores (Francey et al., 1999), and the atmospheric CO\(_2\) concentration (ppm) from Robertson et al. (2001).

2.4. Basal area measurements

All living stems of all the species with a diameter of more than 2 cm at a height of 50 cm were tagged and their circumference measured at a height of 50 cm with a metric tape. A line was painted on the exact point of the stem where circumference had been measured the first time in January 1999. Every January from 1999 to 2005, the circumferences of the stems were measured again to calculate the basal area increment.

2.5. Statistical analyses

Repeated measures ANOVAs were performed with δ\(^{13}\)C, δ\(^{15}\)N, %N, and IWUE as dependent variables, year as grouping factor, and species and treatment as independent factors. For A. unedo data other ANOVAs with the same variables were performed with data from 2005, the only year when A. unedo was sampled. Analyses of covariance (ANCOVAs) were conducted with δ\(^{13}\)C, δ\(^{15}\)N, %N, and IWUE as dependent variables, species as independent factors, and spring and summer soil moisture in each plot as covariates. For each species, correlations were conducted between δ\(^{13}\)C, %N, and δ\(^{15}\)N. Other ANCOVAs were performed with δ\(^{13}\)C, δ\(^{15}\)N, and %N as dependent variables, species and treatment as independent factors, and mean temperature and total rainfall from April to August for each year as covariates. Finally, other ANCOVAs were performed for each species, with annual basal area increment as the dependent variable, treatment as an independent factor, and annual rainfall and mean annual temperature as covariates. Percentages of annual basal area increment (\(p\)) were transformed to arcsin \(p^{1/2}\) to meet the normality assumptions of the ANCOVA. All analyses were performed with the Statview software package (Abacus Concepts Inc., Cary, NC, USA).

3. Results

Meteorological data for the period studied was typical for a Mediterranean climate: hot and dry summers, relatively cold winters, and rainfall periods concentrated in spring and autumn (Fig. 1). Mean annual temperature varied from 11.7°C in 2004 to 12.6°C in 2003, and total annual rainfall varied from 403 mm in 2005 to 927 mm in 2003 (Fig. 1). Rain exclusion treatment decreased soil moisture by ca. 15% during the...
overall period studied, but larger differences (ca. 20%) were observed during rainfall periods, usually in spring and autumn, and lower differences (ca. 10%) were observed in drier seasons, usually in summer and sometimes in winter (Fig. 1).

Q. ilex experienced higher $\delta^{13}C$, $\delta^{15}N$, and %N values than P. latifolia ($P < 0.01$), whereas A. unedo showed higher $\delta^{13}C$ values than the two other species ($P < 0.01$). However $\delta^{15}N$ and %N values in A. unedo fell in between those of Q. ilex and P. latifolia (Fig. 2). Rain exclusion treatment increased $\delta^{15}N$ in the three species ($P < 0.05$), and did not change $\delta^{13}C$ and %N in any of them (Fig. 2). $\delta^{13}C$ values of each repetition plot were negatively correlated with soil moisture in P. latifolia and A. unedo ($P < 0.05$), and $\delta^{15}N$ values of each repetition
plot were negatively correlated with soil moisture in Q. ilex and A. unedo ($P < 0.01$), but no correlation was observed between $\delta^{13}C$ and soil moisture in Q. ilex, nor between $\delta^{15}N$ and soil moisture in P. latifolia. Furthermore, no correlation was observed between %N and soil moisture for any of the three species studied (Fig. 3). There were no significant correlations between $\delta^{13}C$, $\delta^{15}N$, and %N values except in P. latifolia, where $\delta^{13}C$ values were correlated with %N values ($P < 0.05$), and in Q. ilex, where $\delta^{15}N$ values were correlated with $\delta^{13}C$ values

![Graph 1](image1.png)

![Graph 2](image2.png)

![Graph 3](image3.png)

Fig. 2 – Annual time course of leaf $\delta^{13}C$, $\delta^{15}N$, and %N in Q. ilex, P. latifolia, and A. unedo trees in summer 2000, 2003, 2004, and 2005. Vertical bars indicate standard errors of the mean ($n = 4$ plot averages of 5 individuals each). Statistical differences between control and rain exclusion plots depicted in the panel were tested by an ANOVA.

Fig. 3 – Relationships between leaf $\delta^{13}C$, $\delta^{15}N$, and %N values, and soil moisture values in Q. ilex, P. latifolia, and A. unedo trees (data from summers 2000, 2003, 2004, and 2005). Significant relationships are depicted in the figure. Each point corresponds to a measurement in one plot in one year. Both control and rain exclusion plots are represented. In control plots, soil moisture is, on average, 15% higher than in rain exclusion plots.
Despite the large number of reports describing higher $\delta^{13}C$ values and therefore higher iWUEs when water availability decreases (Farquhar et al., 1989; Peñuelas et al., 2000), we did not find any increase in $\delta^{13}C$ in any of the three species in response to our rain exclusion treatment. This is difficult to explain, especially taking into account that $\delta^{13}C$ and therefore iWUE values were inversely correlated with soil moisture. However, in the same experiment we observed a decrease in the total amount of Q. ilex leaves in rain exclusion plots due to lower production of new leaves (Ogaya and Peñuelas, 2006): Therefore the total transpiration area in Q. ilex trees from rain exclusion plots may be reduced, thus manifesting similar iWUE values as Q. ilex trees from control plots, despite the lower soil moisture in rain exclusion plots.

In contrast, rain exclusion treatment exerted a clear effect on $\delta^{15}N$ values in the three species studied. The increases in $\delta^{15}N$ values under rain exclusion could indicate higher N accumulation during drought, and greater nitrogen losses in the system just after rainfall because the major pathways of nitrogen losses, such as gaseous N losses and leaching of NO$_3^-$ when nitrification is incomplete, are thought to cause $\delta^{15}N$ enrichment of the remaining nitrogen (Shearer and Kohl, 1986; Durka et al., 1994; Schulze et al., 1994). The transfer of N from mycorrhizal fungi to plants also discriminates against $^{15}N$: as the internal N cycle progressively opens and dissolved organic N increasingly mineralizes to ammonium and then nitrifies to nitrate, the reliance of plants on mycorrhizal fungi decreases. This would cause mycorrhizal fungi for N might decline and the retention of $^{15}N$ by mycorrhizal fungi decrease. This could cause mycorrhizal plants to increase in $^{15}N$ (Schimel and Bennett, 2004; Craine et al., in press), but under the rain exclusion treatment, it is difficult to obtain higher fungal activity, given the strong dependence of fungi on water availability. The fungi activity under rain exclusion treatment did not increase, and therefore the increase in $^{15}N$ in treatment plots seems thus a consequence of N losses. In our experimental system, Sardans et al. (in press) observed a decrease in total soil nitrogen content induced by experimental rain exclusion. The $\delta^{15}N$ increase indicates that losses must play an important role in this lower soil nitrogen content. The increases in foliar $\delta^{15}N$ values were not accompanied by decreases in foliar N

!!!Fig. 4–Annual iWUE values of Q. ilex, P. latifolia, and A. unedo trees in control and rain exclusion plots in summer 2000, 2003, 2004, and 2005. Vertical bars indicate standard errors (n = 4 plot averages of 5 individuals each). Control iWUE values were higher than rain exclusion iWUE only in Q. ilex, and when considering the four years (repeated measures ANOVA, P = 0.05).!!!
Changes in δ13C composition are directly assumed to be changes in iWUE due to variations in water availability, whereas changes in δ15N composition are related to a wide variety of processes producing nitrogen losses. The results of this work reveal a greater response in δ15N than in δ13C induced by rain exclusion treatment. In addition, there are some indirect effects of rain exclusion on plant physiology such as a decrease in soil enzymatic activity and nutrient content, and finally, nutrient diffusion by roots is under soil water control (Chapin, 1980).
This experiment shows that a slight decrease in water availability, like that projected for Mediterranean areas in the coming decades (Peñuelas et al., 2005; IPCC, 2007), could induce large N losses in Mediterranean ecosystems. The losses of N as a result of rain exclusion may further limit plant growth and ecosystem functioning. Furthermore, the different species-specific strategies to resist rain exclusion may induce changes in their competitive ability, and finally their future distribution.

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