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Nitrogen and carbon concentrations, and stable isotope ratios in Mediterranean shrubs growing in the proximity of a CO_2 spring

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Abstract

Seasonal changes in foliage nitrogen (N) and carbon (C) concentrations and $\delta^{15}N$ and $\delta^{13}C$ ratios were monitored during a year in *Erica arborea*, *Myrtus communis* and *Juniperus communis* co-occurring at a natural CO_2 spring (elevated [CO_2], about 700 μmol mol^{-1}) and at a nearby control site (ambient [CO_2], 360 μmol mol^{-1}) in a Mediterranean environment. Leaf N concentration was lower in elevated [CO_2] than in ambient [CO_2] for *M. communis*, higher for *J. communis*, and dependent on the season for *E. arborea*. Leaf C concentration was negatively affected by atmospheric CO_2 enrichment, regardless of the species. C/N ratio varied concomitantly to N. Leaves in elevated [CO_2] showed lower $\delta^{13}C$, and therefore likely lower water use efficiencies than leaves at the control site, regardless of the species, suggesting substantial photosynthetic acclimation under long-term CO_2-enriched atmosphere. Leaves of *E. arborea* showed lower values of $\delta^{15}N$ under elevated [CO_2], but this was not the case of *M. communis* and *J. communis* foliage. The use of the resources and leaf chemical composition are affected by elevated [CO_2], but such an effect varies during the year, and is species-dependent. The seasonal dependency and species specificity suggest that plants are able to exploit different available water and N resources within Mediterranean sites.

Additional key words: elevated CO_2, *Erica arborea*, global change, *Juniperus communis*, Mediterranean ecosystems, *Myrtus communis*, water stress.

Introduction

The present increase of atmospheric CO_2 concentration ([CO_2]) is predicted to continue throughout the century due to human activities. Plant communities of Mediterranean-type ecosystems are expected to be particularly sensitive to this increase because they will also have to face more severe drought conditions due to the predicted greenhouse gas driven increase in mean global temperature at the surface of the earth and to a concurrent decrease in precipitation at Mediterranean latitudes forecast by General Circulation Models (*e.g.* Kattenburg *et al.* 1996).

Increased [CO_2] has been observed to affect chemical composition of plant material (*e.g.* Lincoln 1993). In particular, the within plant allocation of carbon (C) and nitrogen (N) may be modified by the greater C availability (Ceulemans and Mousseau 1994), inducing increased C/N ratio (McGuire *et al.* 1995, Peñuelas *et al.* 1997) and C-based secondary compounds of foliage (Peñuelas and Estiarte 1997), thus possibly affecting plant susceptibility to herbivores (Lincoln 1993) and litter decomposition rate (Cotrufo *et al.* 1999). However, the latter effect on decomposition is not clear because it seems that the amount of N retained in naturally senesced litter produced under elevated [CO_2] may vary too little to affect subsequent decomposition (Norby and Cotrufo 1998). In semi-arid environments, such as Mediterranean-type ecosystems, the forecast drier conditions for the next decades would presumably bring about an additional effect on nutrient cycling (Cotrufo *et al.* 1999). Possible changes in the N source and the relative activity of

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different N cycle processes (losses and supplies) in the surrounding system, as a response to elevated [CO_2], could be assessed by plant $\delta^{15}N$ value (Peñuelas and Estiarte 1997, 1998).

Partial stomatal closure is a common, although not universal, response to elevated [CO_2] (Eamus 1991). This decreased stomatal conductance allows plants to use water more efficiently by enhancing the ratio of leaf net photosynthetic rate to evapotranspiration, termed instantaneous water-use efficiency. This increased water-use efficiency, for which leaf $\delta^{13}C$ is a widely used substitution in C_3 species (Farquhar *et al.* 1989), could be especially advantageous to Mediterranean plant growth and survival when water supply is limiting.

Uncertainties on the effects of elevated [CO_2] on foliage chemical composition and water-use efficiency arise because predictions have mainly been extrapolated from studies of short-term exposure of plants in manipulated environments (greenhouses and different kind of chambers), but physiological processes might adjust or acclimate to long-term increase in [CO_2] (Saxe *et al.* 1998). Natural CO_2 springs are an option to expensive FACE (free-air CO_2 enrichment) experiments and offer an opportunity for studying long-term responses of entire plant communities to enriched [CO_2], being unique environments of intrinsic ecological interest (Hättenschwiler *et al.* 1997, Körner and Miglietta 1994, Miglietta *et al.* 1993). *Quercus* species at the CO_2 spring of Bossoleto (Italy) have shown altered C physiology under elevated [CO_2], the response being species specific. Higher isoprene emissions, concentrations of phenolic compounds and total non-structural carbohydrates, and C exchange rates have been found in plants (*Quercus* species) grown at elevated [CO_2] (Johnson *et al.* 1997, Tognetti *et al.* 1998, Stylinski and Oechel, personal communication). However, much less information is available for the shrub layer (Jones *et al.* 1995, Barták *et al.* 1999) which constitutes the great bulk of the vegetation in Mediterranean-type ecosystems (Di Castri 1981).

Erica arborea, *Myrtus communis* and *Juniperus communis* are widely distributed in Mediterranean-type ecosystems, and form together with other shrubs species the dominant biomass in large areas of macchia vegetation. It is therefore highly relevant to investigate the effects of environmental changes which may alter the C and N interactions in these Mediterranean shrubs and which might in turn influence ecological interactions with other organisms and the functioning of macchia-dominated ecosystems. This study investigates the effect of prolonged elevation of [CO_2] on C and N concentrations in leaves of the three species. We also determined whether variations in N acquisition, water use efficiency, and acclimation, assessed by leaf N and C stable isotope composition, of individuals grown in the proximity of a natural CO_2 spring differ from those of comparable shrubs of the same species grown nearby but exposed to ambient [CO_2]. We tested *1*) whether changes in C and N concentrations, and isotopic signatures occur at elevated [CO_2] or there is a long-term acclimation over a period of generations, and *2*) whether they are general or particular for different shrub species. We hypothesized that these changes can be related to site histories (soil N dynamics and plant development as influenced by elevated [CO_2]), reflecting differences in atmospheric [CO_2] between the CO_2 spring and the control site.

Materials and methods

Three common and widespread Mediterranean macchia shrub species, *Erica arborea* L., *Myrtus communis* L. and *Juniperus communis* L., growing at a natural CO_2 spring near Lajatico (Pisa, Italy) called "I Borboi" (43°26'N, 10°42'E) were measured. The enriched area extends over 7 000 m^2. The area is covered by a coppiced stand dominated by *Quercus ilex* L., but in which *Quercus pubescens* Willd., and *Arbutus unedo* L. (and scattered individuals of *Quercus cerris* L. and *Fraxinus ornus* L.) and the shrub level (including *Smilax aspera* L., *Cytisus scoparius* L., *Cistus salviifolius* L., *Genista* sp., *Ligustrum vulgare* L., *Pistacia lentiscus* L. and *Phyllirea latifolia* L.) constitute the great bulk of the flora. The CO_2 spring is located on the north-facing slope (20 %) of a hill near the bottom of a small valley about 200 m a. s. l. (see Raiesi Gahrooee *et al.* 1999, Tognetti *et al.* 2000*a*). Almost pure CO_2 emissions occur from a series of vents located along a narrow seasonal creek, where [CO_2] tends to decrease upslope. The vents emit small amount of H_2S, never exceeding 0.04 µmol mol^{-1}. The whole canopy of the studied plants around the CO_2 spring is exposed to daytime [CO_2] of about 700 µmol mol^{-1} throughout the year with short-term variations between 500 and 1000 µmol mol^{-1}, depending on wind speed and convective turbulence (Hättenschwiler *et al.* 1997).

The shrubs growing in close association in the proximity of the CO_2 spring were measured. Additional measurements were made at a control site chosen along the same creek about 150 m upstream. Thus roots of selected plants experienced similar soil environment and branches similar aerial environment, except for [CO_2]; see Tognetti *et al.* (2000*a*) for a [CO_2] map of the area. The area has non-calcareous, brown loamy clayey soils, developed from calcareous marl (pH 6 - 7) (Raiesi Gahrooee 1998). Total soil N and C/N ratio in the forest floor and mineral horizon (0 - 10 cm) are comparable at both sites (Raiesi Gahrooee *et al.* 1999). The climate is typical Mediterranean, with cool winters, relatively wet fall and spring, and hot, dry summers; details on environmental conditions during the experiment can be found in Tognetti *et al.* (2000b). At both sites, shrubs of similar exposure were selected. Measurements were made on sunny leaves in the upper or outer canopy (individuals were not shaded by dominant species). Individuals within a species were of similar size.

Every two months during one year, shoots were collected from six individuals of each species (*E. arborea*, *M. communis* and *J. communis*) at each site (CO_2 spring and control), early in the morning. Mean leaf longevity of the three shrubs is 2 - 3 years, bud break and leaf abscission took place in April - May. To avoid possible isotope differences due to phenological effects, we sampled plants and leaves in the same stage of development at both sites. The shoots were transported to the laboratory where they were oven-dried at 60 °C to constant mass (d.m.). Leaves were then ground to a fine powder in a mill. Carbon and N leaf concentrations were analysed with an elemental analyser (*NA 1500, Carlo Erba, Milano*, Italy), using the standard configuration for those determinations (Peñuelas *et al.* 1999); 10 - 12 current-year leaves of the same stage of development were bulked for each plant at each sampling date.

The $\delta^{15}N$ and $\delta^{13}C$ ratios were measured on subsamples (about 1 mg) of ground leaves by a *Delta C* isotope ratio mass spectrometer (*Finnigan Mat*, Bremen, Germany) operated in continuous flow mode, after combustion of the samples in an elemental analyser (*NA 1108, Series 1, Carlo Erba*). A system check of analysis was achieved with interspersed working standards of acetanilide and atropine (*Carlo Erba*) for elemental analyses, and standards of sucrose, graphite, polythylene fosil, ammonium sulphate and potassium nitrate (*IAEA*, Vienna, Austria) for isotopic analyses (Peñuelas *et al.* 1999). The $\delta^{13}C$ value for source atmospheric CO_2 during the study averaged -8.3 ‰ and -8.9 ‰ at the control and CO_2 spring site, respectively (M. Saurer, personal communication); these average values are very similar and relate to the same area and time over which the average [CO_2] was assessed, also resembling data from long-term geological surveying. The accuracy of the measurement was ± 0.2 ‰ for $\delta^{15}N$ and ± 0.1 ‰ for $\delta^{13}C$.

Within each species, two-way analysis of variance (*ANOVA*) with sampling date and site (with different [CO_2]) as the main effects were conducted for all cases after testing for normality and homogeneity of variances. A repeated-measure analysis was not considered appropriate because we did not sample the same plants over the sampling time period. Differences amongst the three species were also tested with *ANOVA*. Statistical analysis was conducted by using *Statistica 5.5*.

Results

In *M. communis* the N concentration was significantly lower in leaves of CO_2 spring plants (Table 1), while in *J. communis* it was lower ($P < 0.05$) in leaves of control plants. In *E. arborea* it was consistently lower in leaves of CO_2 spring plants in mid-winter only. Leaf N concentration did not display consistent seasonal variation throughout the year (Fig. 1). In general CO_2 spring plants showed less seasonal variations than control plants. Differences amongst species were significant ($P < 0.001$), with *J. communis* (control plants) showing generally lower values (N %) than *E. arborea* and *M. communis.*

Markedly lower C concentrations were generally found in leaves of CO_2 spring plants (Table 1), regardless of the species, but the leaf C concentration seasonal course was not clear (Fig. 2). Differences amongst species were strong ($P < 0.0001$), with *E. arborea* showing constantly higher values (C %) than the other two species.

In *M. communis* the C/N ratio was overall significantly higher in leaves of CO_2 spring plants (Table 1), while in *J. communis* it was particularly higher in leaves of control plants during late winter and early spring (Fig. 3); leaves of *E. arborea* showed higher C/N ratio in CO_2 spring than control plants only in late winter. *M. communis* displayed increasing values of C/N ratio from the fall to the next growing season. Again, CO_2 spring plants had overall less seasonal variations than control plants. Differences in C/N ratio amongst species were highly significant ($P < 0.0001$).

Table 1. Summary of analysis of variance (*ANOVA*) results showing the effects of [CO_2] (site), measuring month (month) and interactions amongst effects (s × m) on leaf chemical composition of three Mediterranean shrubs; differences between species are reported in the text. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, n.s. $P > 0.05$ (not significant).

		N [%]	C [%]	C/N	$\delta^{15}N$	$\delta^{13}C$
E. arborea	site	n.s.	**	n.s.	**	****
	month	n.s.	*	n.s.	***	n.s.
	s × m	n.s.	n.s.	n.s.	n.s.	n.s.
M. communis	site	****	**	*	n.s.	****
	month	*	n.s.	n.s.	n.s.	****
	s × m	n.s.	n.s.	n.s.	n.s.	*
J. communis	site	*	*	*	n.s.	****
	month	n.s.	*	n.s.	n.s.	***
	s × m	n.s.	n.s.	n.s.	n.s.	n.s.

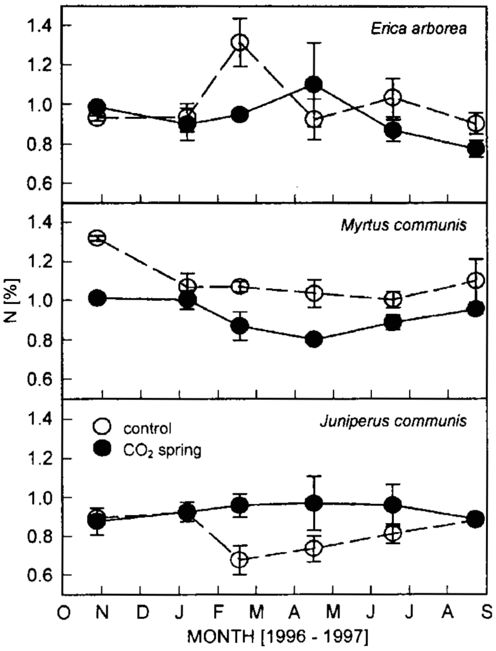

Fig. 1. Seasonal course of N concentration (% dry mass) measured in leaves of *E. arborea, M. communis* and *J. communis* plants at the CO_2 spring site and at the control site during 1996 - 1997. *Vertical bars* indicate SE of the mean (n = 6 individuals). Species and sites are referred to by symbols in the legend.

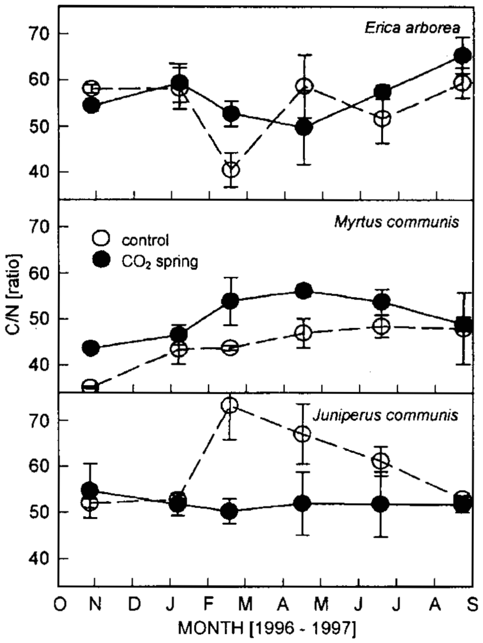

Fig. 3. Seasonal course of C/N ratio measured in leaves of *E. arborea, M. communis* and *J. communis* plants at the CO_2 spring site and at the control site during 1996 - 1997. *Vertical bars* indicate SE (n = 6). Species and sites are referred to by symbols in the legend.

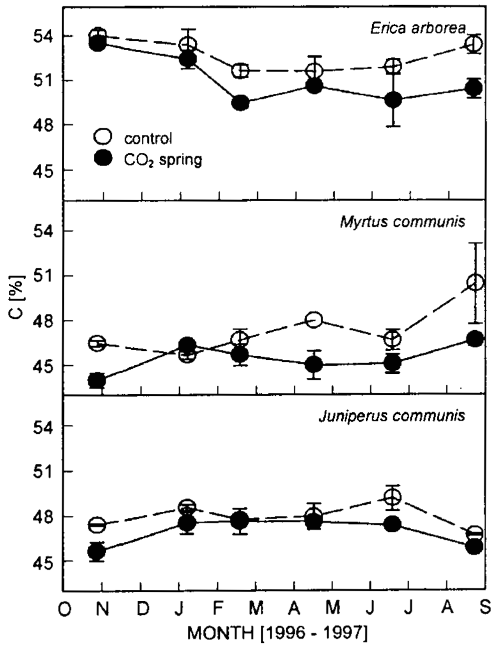

Fig. 2. Seasonal course of C concentration (% dry mass) measured in leaves of *E. arborea, M. communis* and *J. communis* plants at the CO_2 spring site and at the control site during 1996 - 1997. *Vertical bars* indicate SE (n = 6). Species and sites are referred to by symbols in the legend.

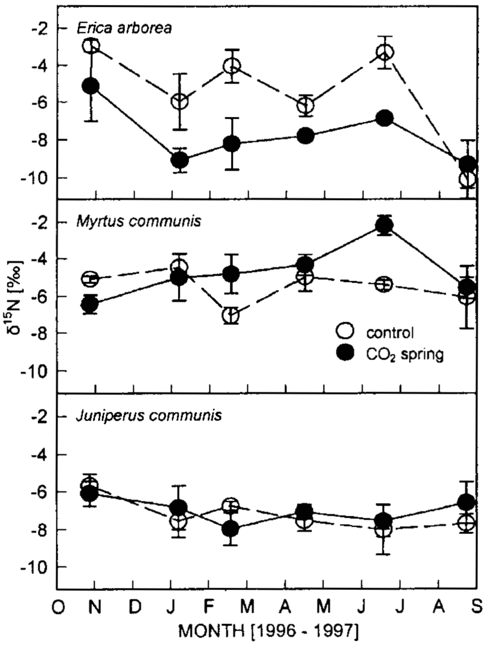

Fig. 4. Seasonal course of $\delta^{15}N$ measured in leaves of *E. arborea, M. communis* and *J. communis* plants at the CO_2 spring site and at the control site during 1996 - 1997. *Vertical bars* indicate SE (n = 6). Species and sites are referred to by symbols in the legend.

Strong site differences in leaf $\delta^{15}N$ values (‰) were evident only in *E. arborea* (Table 1), with control plants showing generally higher values than CO_2 spring plants (except for late summer and early fall). Patterns of leaf $\delta^{15}N$ in *E. arborea* showed a consistent minimum in late summer (Fig. 4), while the other two shrubs did not display significant seasonal changes (except for a maximum in early summer in *M. communis* CO_2 spring plants). Differences in $\delta^{15}N$ values amongst species were highly significant ($P < 0.0001$).

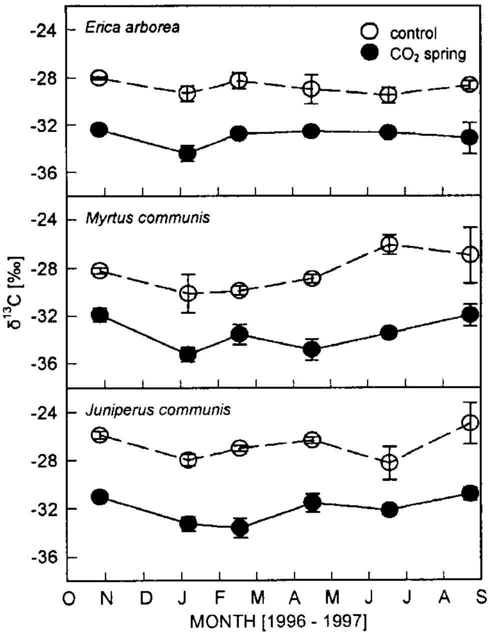

Fig. 5. Seasonal course of $\delta^{13}C$ measured in leaves of *M. communis*, *E. arborea* and *J. communis* plants at the CO_2 spring site and at the control site during 1996 - 1997. *Vertical bars* indicate SE (n = 6). Species and sites are referred to by symbols in the legend.

The C stable isotope analyses yielded significantly lower values of $\delta^{13}C$ in leaves of plants growing at the CO_2 spring site, regardless of the species (Table 1). Seasonal patterns were approximately constant for *E. arborea* (Fig. 5). Relatively larger variations were observed in *M. communis* and *J. communis*. There was significant variation ($P < 0.0001$) in $\delta^{13}C$ amongst the leaves of *E. arborea*, *M. communis* and *J. communis*. *J. communis* had relatively higher $\delta^{13}C$ than *M. communis* and *E. arborea*.

In *E. arborea* leaves, the higher $\delta^{15}N$ values was somewhat accompanied by higher N concentrations (data not shown), though the relationships had low r^2 (0.16 and 0.25 for CO_2 spring and control plants, respectively); such relationships in the other two species showed even lower r^2, except for *M. communis* control plants (0.36). The plot of $\delta^{15}N$ *versus* $\delta^{13}C$ separated the two sites well (data not shown), regardless of the species. In general, at corresponding values of $\delta^{15}N$ the leaves of CO_2 spring plants had more negative $\delta^{13}C$ values. The range of $\delta^{15}N$ values was larger in *E. arborea* than in the other two species. Higher average $\delta^{15}N$ values were found in *M. communis* than in *J. communis*, while the opposite was the case for $\delta^{13}C$ values.

Discussion

Even though total N concentration and C/N ratio do not differ between soil/litter at the CO_2 spring and at the control site (Raiesi Gahrooee *et al.* 1999), significant differences were found in leaf N concentrations. *M. communis* showed lower N concentration and higher C/N ratio in plants grown at the CO_2 spring site as described in most studies of elevated [CO_2] effects (McGuire *et al.* 1995, Peñuelas and Estiarte 1997, Peñuelas *et al.* 1997). However, this decrease in N concentration at elevated [CO_2] was not found in the other two studied species, specially in *J. communis* which showed increased N concentrations, so caution is required in the generalization of such a response among species.

While leaf C concentration was generally lower in CO_2 spring plants, the changes in the C/N ratio followed mainly the pattern of leaf N concentration, and therefore opposite trends between *M. communis* and *J. communis* and seasonal-dependent trends in *E. arborea* were observed. In all shrubs, carboxylation became more restricted relatively to diffusion through stomata, resulting in downward photosynthetic adjustment (Tognetti *et al.* 2000*b*). Plants of semi-arid environments may need to prioritise water saving over C gain. It must be pointed out that differences between sites in leaf N and C concentration (and C/N ratio), if any, may disappear depending on the period of sampling (Peñuelas *et al.* 1997) and, possibly, if allometric relations are considered. Indeed, elevated [CO_2] may cause a shift in the allocation pattern of carbon among leaves, woody stems, fine roots, and rhizomes.

The potential significance of CO_2-induced changes in leaf quality for herbivore feeding and development (Lincoln *et al.* 1993), for litter decomposition (Cotrufo *et al.* 1999) and for C sequestration in soils (Schimel 1995) has been highlighted. In *M. communis* the observed decrease in N concentration may result in herbivore stress under conditions of increased [CO_2] (Lincoln 1993). However, the results of this investigation suggest caution in linking changes in leaf nutrient concentrations and these phenomena under long-term [CO_2] exposure. The water stress experienced by these drought-adapted plants (Tognetti *et al.* 2000*b*) might have been insufficient to produce an evident decline in the acquisition of resources or a change in the C/N ratio, in correspondence with the balance of chemical compounds. Variations in tissue nutrient concentration (and resource use) among plants grown at elevated or ambient [CO_2] may depend on the effect of [CO_2] on plant size (growth and development); other woody plants at the same location did not show differences in size between the CO_2 spring and the control site (Tognetti *et al.* 2000*a*).

All major pathways of ecosystem N loss (nitrification, denitrification, ammonia volatilization and nitrate leaching) are thought to cause a ^{15}N enrichment of the remaining N and a ^{15}N depletion of the lost N (Peñuelas *et al.* 1999). *E. arborea* had lower leaf $\delta^{15}N$ in plants at the CO_2 spring site which may thus indicate higher activities of N-input processes and/or lower activities of N-losing processes in the soil system surrounding the roots, possibly because of higher plant demand under elevated [CO_2]. These processes, however, do not necessarily produce lower soil $\delta^{15}N$ values and the effect of the processes will depend on the $\delta^{15}N$ values of the inputs and of soil-N pool being affected. A preferential C partitioning to below-ground structures under elevated [CO_2] (greater root/shoot ratio) has been sometimes proposed (Ceulemans and Mousseau 1994, Saxe *et al.* 1998). The soil N fractionation activity of mycorrhizal fungi of this species may also have been affected by elevated [CO_2]. Again, the response to elevated [CO_2] was different in the other two studied species; they showed no clear pattern (*J. communis*) or it was season-dependent (*M. communis*). The between species differences in leaf $\delta^{15}N$ values reported here, although not strong, are probably a consequence of specific differences in the exploitation of N resources. *M. communis*, showing less negative averaged leaf $\delta^{15}N$ than the other two species, also had field water relations indicating relatively deeper roots (Tognetti *et al.* 2000*b*) which would allow this species to use N from more N-saturated soil layers (Lloret *et al.* 1999).

There was not strong variation in C stable isotope composition amongst the leaves of *E. arborea*, *M. communis* and *J. communis*, which showed for plants at the control site values similar to those reported for other C_3 species of macchia vegetation (Valentini *et al.* 1992). The variation between sites seemed more important in determining leaf C stable isotope composition than N stable isotope composition ($\delta^{13}C$ values of the source CO_2 were similar between sites compared to the differences seen in plants, environmental factors being the same except for [CO_2], see Materials and methods). In the three species studied $\delta^{13}C$ values for CO_2 spring plants were more negative than for plants at the control site, and therefore elevated [CO_2] has resulted in "consequent" lower $\delta^{13}C$-derived water use efficiency values. The depletion of ^{13}C, as measured in the products of photosynthesis, is modulated by the rate at which CO_2 diffuses into the leaf and by the rate at which CO_2 is fixed by ribulose-1,5-bisphosphate carboxylase/oxygenase (Farquhar *et al.* 1989). Provided that stomatal conductance of plants grown at the CO_2 spring site was lower than in control plants (Tognetti *et al.* 2000*b*), stable isotope analysis suggests photosynthetic acclimation under long-term CO_2-enriched atmosphere (Miglietta *et al.* 1998). Previous studies on gas exchange and growth of evergreens grown at CO_2 springs fit with $\delta^{13}C$ data, as reported in this study, and support this hypothesis. The down-regulated photosynthetic rates might reduce the usually observed benefit of increased water use efficiency associated with growth under elevated [CO_2] (Eamus 1991).

The Mediterranean sclerophylls studied in this experiment display an intrinsic growth strategy that highly prioritizes water saving over C uptake. Long-term water use efficiency, however, might be affected by different proportion of respired *versus* fixed C between CO_2 spring and control plants, when derived by C stable isotope analysis. Several studies have shown that contents of C-based secondary compounds increase in plants grown under elevated [CO_2] (*e.g.* Lavola *et al.* 1994), while others have found no significant response (*e.g.* Johnson and Lincoln 1990). Due to their biosynthetic pathway, C-based secondary metabolites are characterized by lower $\delta^{13}C$ than carbohydrates. Indeed, changes in concentration of phenolic compounds and total non-structural sugars and emission of volatile organic compounds have been found for leaves of *Quercus* species grown in another CO_2 spring, though differences between species in magnitude and direction of such changes were observed (Tognetti *et al.* 1998).

The plot of leaf $\delta^{15}N$ *versus* leaf N concentration did not indicate clear changes in N availability with variations in N-loss activity, regardless of species and site. In plants, $\delta^{13}C$ has been used as an index of stress tolerance (water loss *versus* carbon gain) and $\delta^{15}N$ is genetically and environmentally determined (rather than being simply a function of the isotopic signature of external N source). The plot of leaf $\delta^{15}N$ *versus* $\delta^{13}C$ values separates the two sites well, regardless of the species, and the separation is due to differences in $\delta^{13}C$. The variation in the isotopic signature reveals different strategies with respect to water use (and N use in *E. arborea*) between plants at the CO_2 spring and control site. Environmental factors, which decrease the proportional flux of plant N into organic matter storage pools, may push the plant N toward ^{15}N-enrichment, relative to atmospheric N.

In conclusion, reduced N concentration and related changes in C/N ratio are not a general response to long-term exposure to elevated [CO_2] in these Mediterranean shrubs. Instead, acclimation to elevated [CO_2] of C metabolism, with relative lack of excess C, may result in different water and N use. Minimal or species-specific time-dependent changes in leaf chemical composition under elevated [CO_2] of plants at CO_2 spring sites emphasize the importance of conducting experiments in "natural" environments with plants under elevated [CO_2] and caution in interpreting isotopic signatures from experiments conducted in highly manipulated environment. The long-term effects of elevated [CO_2] on this Mediterranean-type ecosystem and its vegetation are reflected in the carbon-nutrient-water relationships. Plant growth adjustment (C sink) under different interacting environmental conditions or resource availabilities, and under seasonal and species-related variation may integrate acclimations processes to elevated [CO_2].

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