Carbon-based secondary and structural compounds in Mediterranean shrubs growing near a natural CO₂ spring

J. PEÑUELAS*, E. CASTELLS*, R. JOFFRE† and R. TOGNETTI‡

*Unitat d'Ecofisiologia CSIC-CREAF, Centre de Recerca Ecològica i Aplicacions Forestals (CREAF), Facultat de Ciències, Universitat Autònoma, Bellaterra 08193 (Barcelona), Spain, †DREAM Unit, Centre d'Ecologie Fonctionnelle et Evolutive-CNRS, 1919 route de Mende, 34293 Montpellier Cedex 5, France, ‡Dipartimento di Scienze Animali Vegetali e dell'Ambiente (SAVA), Università degli Studi del Molise, via De Sanctis I-86100 Campobasso, Italy

Abstract

We studied carbon-based secondary and structural compounds (CBSSCs) in *Myrtus communis*, *Erica arborea*, and *Juniperus communis* co-occurring in a natural CO₂ spring site and in a nearby control site in a Mediterranean environment. Leaf concentrations of phenolics and CBSSCs, such as lignin, cellulose, and hemicellulose, total nonstructural carbohydrates (TNCs), and lipids were measured monthly (phenolics) and every two months (the other compounds) throughout a year. There was a slight seasonal trend towards maximum concentrations of most of these CBSSCs during autumn–winter and minimum values during the spring season, particularly in *Myrtus communis*. For most of the CBSSCs and species, there were no consistent or significant patterns in response to the elevated [CO₂] (c. 700 μmol mol⁻¹) of the spring site. These results were not due to a dilution effect by increased structural or nonstructural carbon. Therefore, in contrast to many experimental studies of CO₂ enrichment, mainly conducted for short periods, there were no greater concentrations of phenolics, and, as in many of these studies, there were neither greater concentrations of the other CBSSCs. These results do not agree with the predictions of the carbon source-sink hypotheses. Possible causes of this disagreement are discussed. These causes include the complex heterogeneous environmental conditions and the variability of resource availabilities in the field, photosynthetic down-regulation, and/or the homeostatic and evolutionary nature of organisms. These results suggest evolutionary adaptive responses to changes in CO₂. They also suggest caution in attributing increased CBSSC concentrations to elevated [CO₂] at long-term scale in natural conditions, and therefore in their implications for plant–herbivore interactions and for decomposition.

Keywords: cellulose, *Erica arborea*, *Juniperus communis*, lignin, lipids, *Myrtus communis*, phenolics, total nonstructural carbohydrates

Introduction

Uncertainties regarding the effects of elevated [CO₂] on plant ecophysiology in general, and on plant chemical composition in particular, rise because predictions have mainly been extrapolated from studies on short-term exposure of plants in manipulated environments (greenhouses and growth chambers), without considering factors, such as long-term acclimation, species and genotype responses, and the complex environmental field conditions (including heterogeneous resource availabilities). Moreover, vegetation is not likely to be suddenly subjected to a dramatic increase in atmospheric [CO₂] such as the one typically imposed in short-term CO₂-enrichment experiments. Various physiological processes could adjust or acclimate to gradual increases in CO₂ at different rates over periods ranging from hours to years, to generations (Eamus & Jarvis 1989; Saxe *et al.* 1998). However, despite the potential long-term acclimation, few studies on CO₂ have extended beyond a single growing season. Interestingly, in some of the few

Correspondence: Prof. Josep Peñuelas, fax +34/93-5811312, e-mail Josep.Penuelas@uab.es

relatively long studies the initial reductions in the leaf concentrations of nitrogen (N) and other elements have been found to gradually disappear over the forthcoming years (Peñuelas & Matamala 1990; Peñuelas *et al*. 1997). Natural CO_2 springs offer an opportunity to overcome these sources of uncertainty. They allow us to study long-term responses of entire plant communities to enriched [CO_2] in complex field conditions (Miglietta *et al*. 1993; Körner & Miglietta 1994; Hättenshwiler *et al*. 1997; Peñuelas *et al*. 2001).

The steady increase in atmospheric CO_2 concentrations is likely to affect biota by producing changes not only in plant growth and allocation, but also in plant-tissue chemical composition (Peñuelas & Estiarte 1998; IPCC 2001). Among such composition changes, most source–sink hypotheses assume that elevated CO_2 concentrations promote a relative increase of carbon availability that is accumulated in TNCs and CBSSCs when the provided carbon amounts exceed growth requirements (when the source–sink ratio raises) (Bryant *et al*. 1983; Herms & Mattson 1992; Peñuelas & Estiarte 1998). These hypotheses thus predict a larger accumulation of carbon-based secondary and structural compounds (CBSSCs), like phenolics, terpenes, or structural polysaccharides (cellulose, hemicellulose, and pectin), at elevated CO_2 concentrations (Peñuelas & Estiarte 1998). Such changes could have significant consequences for ecosystem functioning, e.g. through plant–herbivore interactions (Lincoln *et al*. 1993; Lindroth *et al*. 1993) and plant litter decomposition (O'Neill & Norby 1996).

However, experimental results on leaf chemistry only provide evidence of increases in concentrations of soluble phenolics and condensed tannins, but not in concentrations of other CBSSCs with various metabolic pathways (Peñuelas & Estiarte 1998), and overall effects on litter quality appear smaller than it was initially thought (Ball 1997). The changes in soluble phenolics and condensed tannins seemed more evident when the concentrations were expressed on structural dry weight basis because the expression of concentration on a dry weight basis can mask or diminish the changes in the concentration of compounds. This happens especially in high-CO_2 grown plants that typically have large increases in total nonstructural carbohydrates (TNCs) (Poorter *et al*. 1997; Koricheva *et al*. 1998), and specially in organs such as leaves with large daily TNC fluctuations due to TNC accumulation during light period and TNC export at night (Hendrix & Grange 1991).

Plant communities of Mediterranean-type ecosystems are expected to be particularly sensitive to ongoing increases in concentrations of atmospheric CO_2 (IPCC 2001). These Mediterranean plant communities are likely to face more severe drought conditions in the future due to the increase in mean temperature and potential evapotranspiration and to the concurrent decrease in precipitation at Mediterranean latitudes as forecasted by General Circulation Models (Kattenburg *et al*. 1996).

In this study, we compared leaf concentrations of phenolics and other CBSSCs, such as lignin, cellulose, and hemicellulose, TNCs, and lipids in *Myrtus communis* L., *Erica arborea* L., and *Juniperus communis* L.—Mediterranean shrubs growing in the proximity of a natural CO_2 spring site—with those of comparable shrubs of the same species grown in a nearby control site exposed to ambient [CO_2]. The objective of our research was to study changes in leaf concentrations of phenolics and other CBSSCs in response to CO_2 enrichment during long-term growth in natural field conditions. Our questions were: (1) whether increased CBSSC concentrations occur at elevated [CO_2] or not (i.e. whether CBSSC concentrations follow the source-sink hypotheses or, alternatively, there are long-term adjustments or 'acclimation' of leaf CBSSC concentrations over a period of several generations growing on complex environmental natural conditions; and (2) if CBSSC concentrations do increase, whether they are general or particular for various compound classes and various plant species, and whether they are linked to changes in concentrations of total nonstructural carbohydrates (TNCs) or lipids.

Materials and methods

Site description and plant material

Leaf samples were obtained from *Erica arborea, Myrtus communis,* and *Juniperus communis—the* three common and widespread Mediterranean macchia shrub species growing near a natural CO_2 spring called 'I Borboi' in Lajatico (Pisa, Italy) (43°26′N, 10°42′E) that has been active for centuries (city council archives). A full description of the geology of the site can be found in Panichi & Tongiorgi (1975). The CO_2-enriched area extends over 0.7 ha. The studied coppiced stand is dominated by *Quercus ilex* L., *Quercus pubescens* Willd., and *Arbutus unedo* L. Several other tree species (e.g. *Quercus cerris* L. and *Fraxinus ornus* L.) are represented by scattered individuals. Shrubs include the species studied here, as well as *Smilax aspera* L., *Cytisus scoparius* L., *Cistus salvifolius* L., *Genista* sp., *Ligustrum vulgare* L., *Pistacia lentiscus* L., and *Phillyrea latifolia* L. The CO_2 spring site is located on the north-facing slope (20%) of a hill near the bottom of a small valley about 200 m above sea level (Raiesi 1998a,b; Tognetti 1999). Almost pure CO_2 emissions occur from a series of vents located along a narrow seasonal creek, and the [CO_2] tends to decrease upslope; see Tognetti *et al*. (2000a) for a map of the site. The vents emit small amount of H_2S (hydrogen sulfide), but their concentrations never exceed a level of 0.04 μmol mol^{-1}, which is not

considered harmful to plants (Raiesi 1998a,b; Schulte 1998). Plants around the CO_2 spring site are exposed to daytime $[CO_2]$ of about $700\ \mu mol\ mol^{-1}$ throughout the year, with short-term variations between 500 and $1000\ \mu mol\ mol^{-1}$ depending on wind speed and convective turbulence. The $[CO_2]$ varies little between different heights within the canopy (Hättenshwiler *et al.* 1997).

Leaf samples were collected from individuals growing close to the CO_2 spring site. Control measurements were made at a site chosen along the same creek, about 150 m upstream; thus root systems of the sampled plants experienced similar soil environment. The area has noncalcareous, brown loamy clay soils developed from calcareous marl (pH 6–7), with total soil N and C/N ratio in the forest floor and mineral horizon (0–10 cm) being comparable in both the spring and control sites (Raiesi 1998a,b). The climate is typical Mediterranean, with cool, wet winters and hot, dry summers (Tognetti 1999). At both sites, six replicate shrubs of similar exposure were selected for each species and sampling month. Measurements were made on current year well-developed leaves from sunny shoots in the upper third of the canopy. The mean leaf longevity of the three species is 2–3 years, buds break in April–May, and leaf abscission also takes place in April–May. To avoid possible CBSSC differences due to phenological effects, we sampled plants and leaves of the same stage of development in the two sites.

Leaf chemical composition

Every month from October 1996 to September 1997, shoots were collected from six individuals (not necessarily the same ones every month) of each species (*Erica arborea*, *Myrtus communis,* and *Juniperus communis*) at each site (CO_2 spring and control), early in the morning. The shoot material was oven-dried at $60\ ^{\circ}C$ to constant weight (dry weight, DW). Thereafter, 10–12 leaves of the same stage of development were bulked for each plant at each sampling date and then ground to a fine powder in a mill. Phenolics were analysed every month and the rest of CBSSCs, TNCs, and lipids every two months.

Leaf concentrations of phenolics were measured on subsamples (about 50 mg) of ground leaves. Total phenolic compounds were analysed by the Folin–Ciocalteu method, improved by using a blank of polyvinylpolypyrrolidone (PVPP) (Marigo 1973). Polyvinylpolypyrrolidone removes phenolic compounds from the solution, and avoids overestimation of total phenolics due to nonphenolic Folin–Ciocalteu reactive compounds. Gallic acid was used as a standard to estimate concentrations of phenolic compounds.

Leaf concentrations of lignin, cellulose, hemicellulose, TNCs, and total lipids were determined on subsamples of ground leaves using near-infrared reflectance spectroscopy (see Joffre *et al.* 1992 and Damesin *et al.* 1997 for a description of the procedure). All samples were scanned with an NIRSystem 6500 spectrophotometer. The spectral and wet chemical database used to build calibration equations comprises leaves of 25 species, representing the diversity of the Mediterranean woody species collected by us from the Mediterranean areas of Portugal, Spain, France, and Italy, and includes part of the database of Meuret *et al.* (1993). The concentrations of fibre, total lipids, and TNCs in these calibration-set samples were determined using wet chemistry methods. Fibre fractions were determined using the Fibertec procedure (Van Soest & Robertson 1985), total lipids were obtained by weighing the residue extracted by a chloroform–methanol mixture (Allen 1989) and TNC analysis was carried out following the method of Farrar (1993).

Statistical analyses

Within each species, two-way analyses of variance (ANOVA) with sampling date and site (with different $[CO_2]$) as the main effects were conducted for all compound concentrations after testing for normality and homogeneity of variance. A repeated-measures analysis was not considered appropriate because we did not sample the same shrubs over the sampling time period. Differences amongst the three species were also tested with ANOVA. Statistical analyses were conducted using Statview 4.5 (Abacus Concepts Inc., Berkeley, CA, USA) and SYSTAT 5.2 (SYSTAT Inc., Evanston, IL, USA).

Results and discussion

There were significant differences amongst the species for most CBSSCs ($P < 0.01$, ANOVA) (Figs 1 and 2). *Myrtus communis* had the greatest leaf concentrations of phenolics and the lowest leaf concentrations of hemicellulose (Figs 1 and 2). *Erica arborea* had the greatest leaf concentrations of hemicellulose, and the lowest leaf concentrations of TNCs. *Juniperus communis* had the greatest leaf concentrations of lignin, cellulose, and lipids, and the lowest leaf concentrations of phenolics (Figs 1 and 2).

There were various responses to site (and, therefore, $[CO_2]$), depending on compounds and species. Foliar phenolic concentrations were 28% lower in the CO_2 spring site than in the control site in *Erica arborea*. Foliar TNC concentrations were 13% greater in *Myrtus communis,* but on the contrary, 12% lower in *Juniperus communis*. Foliar lignin concentrations were 5% greater in *Juniperus communis*. Leaf concentrations of hemicellulose were 19% greater in *Myrtus communis*. For all other compounds and species there were no significant patterns in response to the elevated $[CO_2]$ of the spring site (Figs 1 and 2).
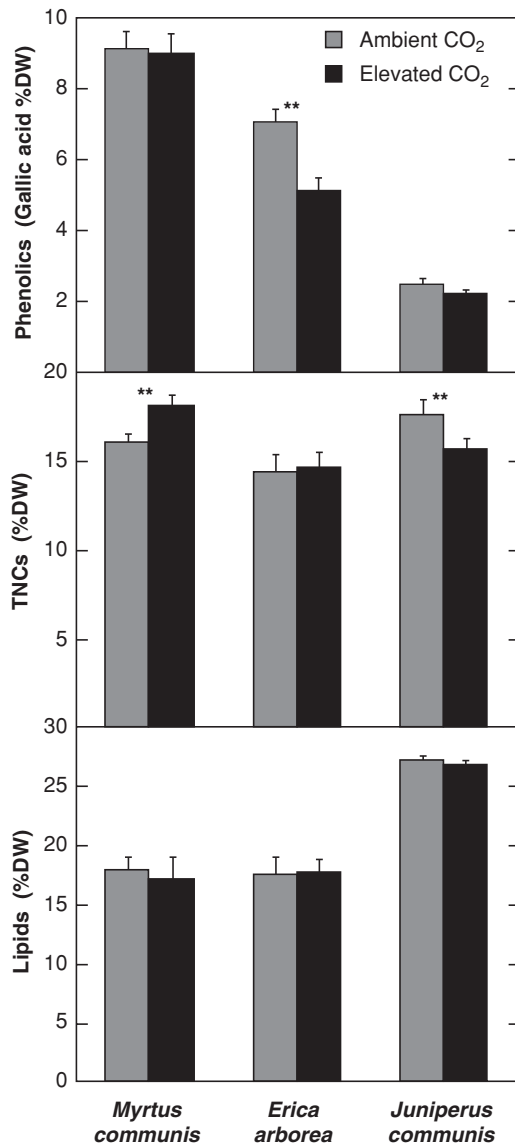
Fig. 1 Effects of elevated $[CO_2]$ on annual average leaf phenolics (top), total nonstructural carbohydrates (TNCs) (middle), and lipid concentrations (bottom). Data presented as percent dry weight (%DW). Error bars indicate $\pm$ SEM. $n = 6$ month averages, calculated on three plants (10–12 leaves pooled together for each plant). $**p < 0.01$, ANOVA.

Fig. 2 Effects of elevated $[CO_2]$ on annual average leaf lignin (top), cellulose (middle), and hemicellulose (bottom). Data presented as percent dry weight (%DW). Error bars indicate $\pm$ SEM. $n = 6$ month averages, calculated on three plants (10–12 leaves pooled together for each plant). $*p < 0.05$, $**p < 0.01$, ANOVA.

The concentrations of many CBSSCs differed significantly by sampling month (Fig. 3 for phenolics) as expected for leaves in various developmental stages (Peñuelas & Estiarte 1998). However, the three shrub species did not display consistent seasonal changes for most CBSSCs, except for a slight trend towards minimum values in spring (youngest leaves) and maximum values in autumn–winter (mature leaves) (Fig. 3). This trend was the strongest for *Myrtus communis*. The analyses revealed some statistically significant interactions between CO_2 enrichment and month (Fig. 3).
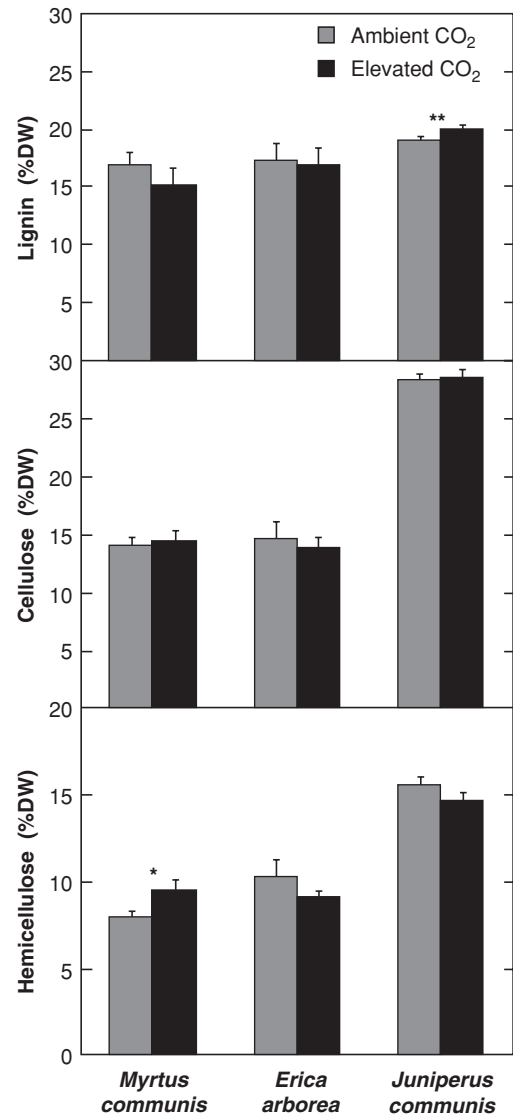
Absence of increased CBSSC concentrations at elevated $[CO_2]$

The most important finding of this study is that although long-term growth at high concentrations of atmospheric CO_2 generated some differences in the CBSSC composition of plant leaves depending on species and compounds, the overall CBSSC concentrations did not increase in the CO_2 spring site. Even lower phenolic concentrations in plants grown at the CO_2 spring site than those in plants grown at the control site were found for one of the studied species—*Erica arborea*.
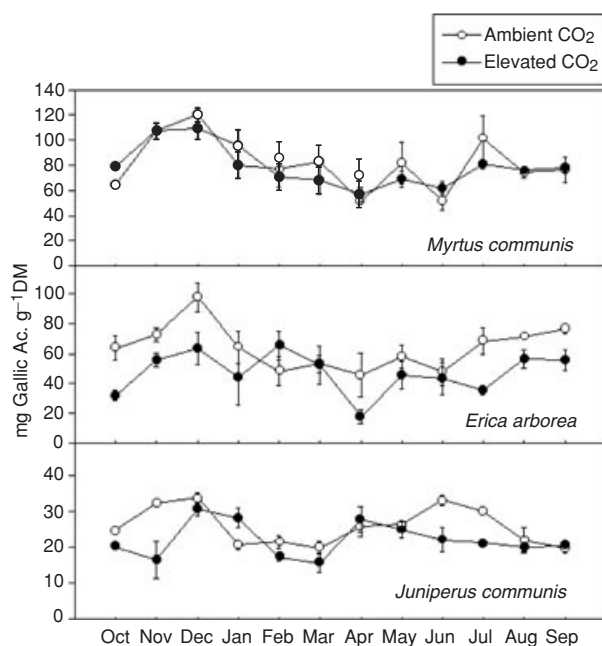
Fig. 3 Seasonal trends in total phenolics concentration (expressed as gallic acid equivalents) measured in leaves of *Myrtus communis*, *Erica arborea,* and *Juniperus communis* plants during 1996–97. Vertical bars indicate standard error of the mean. $n = 3$ plants (10–12 leaves pooled together for each plant).

Moreover, these results were not due to a dilution effect because there was neither increased structural or nonstructural carbon nor lipid concentrations (Fig. 1).

These total phenolic data contrast with several previous CO$_2$ experimental studies that were conducted in less natural shorter-term conditions (Peñuelas & Estiarte 1998). However, the results for lignin and structural polysaccharides, both carbon-based structural compounds that are linked to cell wall, are in agreement with available data (Peñuelas & Estiarte 1998). All the data are in apparent disagreement with carbon source–sink hypotheses that predict increased CBSSC concentrations at elevated CO$_2$ concentrations (Peñuelas & Estiarte 1998). These hypotheses assume that elevated CO$_2$ concentrations promote a relative increase of carbon availability that is accumulated in TNCs and CBSSCs when the provided carbon amounts exceed growth requirements (when the source–sink ratio raises) (Bryant *et al*. 1983; Herms & Mattson 1992; Peñuelas & Estiarte 1998).

Interspecific and environmental heterogeneity

It is difficult to ascertain the specific cause or causes of the absence of elevated CO$_2$ effect on CBSSC concentrations from the available data. Several explanations can be proposed. A likely explanation for the lack of CO$_2$ sensitivity may be related to the various carbon investment

strategies available to various plant species, including growth, storage, structural compound, and defense components. The interspecific variability of our data is not surprising; it is also found in many other ecophysiological variables including growth, whose response to elevated [CO$_2$] can vary greatly even in co-occurring species of the same functional type and in interaction with abiotic and biotic factors (Bazzaz 1990; Körner 2000). Diverse interspecific responses have also been found for concentrations of several elements in these same plants. Under the elevated [CO$_2$] of the spring site, both greater (e.g. in Ca, K, S, Mg, Mn, Al, Fe, P, and Ti) and lower (e.g. in C, Ba, Co, N, Cr, Sr, P, and B) leaf concentrations have been reported. Apart from interspecific differences, there were also different CO$_2$ responses among the various elements and seasons of the year (Peñuelas *et al*. 2001). These interspecific and seasonal differences show that different plant species may use various available resources within the Mediterranean sites and seasons (Tognetti & Peñuelas 2001). In fact, the complex natural conditions of the field make it difficult to find exactly identical sites for resource availabilities or environmental conditions, and under such natural conditions, variability in characteristics within and between plant populations can exceed any response to CO$_2$ (Van Gardingen *et al*. 1997).

Long-term acclimation

Another explanation for these results comes from a likely long-term adjustment or acclimation of photosynthetic carbon uptake, and finally of most leaf CBSSC concentrations to elevated CO$_2$ over many generations of plant development. Plants have grown in such CO$_2$-enriched environment at least for several centuries (city council archives). Provided that stomatal conductance of plants grown at the CO$_2$ spring site was lower than that of control plants (Tognetti *et al*. 2000b), the studies of the ^{13}C composition of these plants suggest photosynthetic acclimation under long-term CO$_2$-enriched atmosphere (Miglietta *et al*. 1998; Tognetti & Peñuelas 2001), leading to decreased photosynthetic capacity (Miglietta *et al*. 1998). We have no photosynthetic data for these shrubs, but at the same site, the evergreen *Quercus ilex* showed significant down-regulation and homeostatic adjustment to elevated [CO$_2$] (W.C. Oechel & C.L. Hinkson, personal communication) in accordance with the results of Miglietta *et al*. (1995) and Oechel & Vourlitis (1996). The down-regulated photosynthetic rates may have been enhanced by water stress, which may have been sufficient to decrease carbon uptake relative to the other elements (i.e. restrict growth more than nutrient uptake). The Mediterranean sclerophylls studied in this experiment may have an intrinsic growth strategy that highly prioritizes water saving over carbon uptake. In fact, other

forest tree species of these sites did not exhibit any increase in above-ground productivity. Possible acclimations to the high CO_2 or/and nutrient limitations have been suggested to counteract the positive effect of CO_2 under drought stress (Tognetti *et al.* 2000a).

Complex space and time conditions

These results showing minimal or species-specific time-dependent changes are especially valuable because they were obtained accounting for the complexity of interactions in real life throughout time and space (plant age, neighbours, microbial partners, soil processes, atmospheric conditions...) (Körner 2000). These results showed that increased CBSSC is not a general response to long-term exposure to elevated [CO_2] in these shrubs. This conclusion is in apparent disagreement with the carbon source–sink hypotheses. Some of the premises of such hypotheses may be unaccomplished under natural field conditions (e.g. microhabitats are heterogeneous for resource availabilities). Moreover, these hypotheses partially ignore the homeostatic nature of organisms and their evolutive history (Hamilton *et al.* 2001). Our data are in support of acclimation to elevated [CO_2] of carbon metabolism, with relative lack of carbon excess. This acclimation in leaf chemical composition under elevated CO_2 concentration of spring site emphasizes, once more, the importance of conducting natural (field conditions) long-term experiments with plants under elevated [CO_2]. Long-term studies allow the record of homeostatic plant mechanisms and possibly adaptive evolutionary histories. Different growth rates (carbon sink) under various interacting environmental conditions or resource availabilities, and under seasonal and specific variation must be considered, apart from [CO_2] itself, to study the carbon–nutrient–water relationships under elevated [CO_2] in shrubs of the Mediterranean environments.

Caution on possible implications for herbivory and decomposition

Many authors have noted the potential significance of CO_2-induced changes in leaf nutrient quality for herbivore feeding and development (Fajer *et al.* 1989; Lincoln *et al.* 1993; Lindroth *et al.* 1993; Arnone *et al.* 1995; Peñuelas & Estiarte 1998), and for litter decomposition and carbon sequestration in soils (Strain 1985; Rastetter *et al.* 1992; Comins & McMurtrie 1993; Schimel 1995; Peñuelas & Estiarte 1998). Plant digestibility and litter decomposition would be hindered by increased CBSSC concentrations under elevated [CO_2]. However, the results of this investigation suggest caution in attributing increased CBSSC concentrations to elevated [CO_2] and, in turn, consequences for herbivory and decomposition under long-term CO_2 exposure, which is especially relevant to the context of the global carbon budget.

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