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Changes in soil enzymes related to C and N cycle and in soil C and N content under prolonged warming and drought in a Mediterranean shrubland

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ABSTRACT

In a Mediterranean shrubland, we investigated the effects of the projected warming and drought on soil urease, protease and β-glucosidase activities and the relation of the possible changes in the activities of these enzymes with the observed changes in soil moisture, soil pH and in C and N stocks in soils, leaves and leaf litter during 1 year (April 2004–May 2005). This investigation was conducted in a long-term experiment of warming and drought manipulation that began in 1999 and is lasting until now. Warming increased soil urease activity by 10% in the study period, mainly by increasing soil urease activity 30% in winter and 10% in spring, and increased β-glucosidase activity 38% in spring. Soil urease and β-glucosidase activities were positively correlated with soil temperatures in winter and negatively in summer. Warming increased soil enzyme activities in winter when soil moisture was highest and in spring coinciding with the greatest biological activity. Warming decreased NH_4^+ soil concentration in the spring of 2004 (by 30%) and 2005 (by 72%), in consonance with the increase in N uptake by plants. Warming decreased N concentration in *Globularia alypum* leaf litter, increasing C/N leaf ratio by 30% showing an increase in N mobilization and contributing to a greater total N accumulation in plants. However, the greater NO_3^- availability in soil observed under warming, probably by an increase in nitrification, may lead to a net N loss by leaching under the torrential rainfalls typical of the Mediterranean climate regions. Drought reduced soil protease activity (9%) in the study period, mainly by decreasing it in spring by 13–21%, but did not affect N soil contents because N turn-over reduction was counterbalanced by a decrease in N leaf concentrations. Soil protease activity was positively correlated with soil water content showing a strong dependence of this enzyme on soil water content. Drought did not affect β-glucosidase activity but tended to increase C contents in soils, which together with the increase in C/N in leaves indicate a reduction of C turn-over and a trend to increase C stocks in soil at long term. The effects of warming and drought on soil enzyme activities were due to a direct effect on soil temperature and soil water content, respectively, and not to changes on soil organic matter quantity and nutritional quality.

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1. Introduction

Current climate and ecophysiological models such as Gotilwa (IPCC, 2001; Sabaté et al., 2002; Peñuelas et al., 2005) predict increased warming and drought in the coming decades in the Mediterranean Basin. Over the last century, temperatures in this region have already shown warming trends (Kutiel and Maheras, 1998; Peñuelas et al., 2002, 2005; Peñuelas and Boada, 2003). Precipitation has already begun to exhibit either a long-term downward trend, mainly in the dry season (Kutiel et al., 1996; Esteban-Parra et al., 1998), or no significant change (Piñol et al., 1998; Peñuelas et al., 2002, 2005), although in all cases a rise in the potential evapotranspiration has led to increased aridity (Piñol et al., 1998; Peñuelas et al., 2005). Such changes in climate are predicted to influence C cycling (Cox et al., 2000).

Water availability is not the only limiting factor in Mediterraneaan ecosystems. Nutrient supplies have often been shown to be an important factor in the growth, structure and distribution of these communities (McMaster et al., 1982; Sardans et al., 2006a). Along with phosphorus, nitrogen is a limiting factor in Mediterranean ecosystems (Villar-Salvador et al., 2004; Fernández et al., 2006). In these Mediterranean ecosystems, the effects of climate change on plant growth and ecosystem functioning may be mediated by the effects on nitrogen supply (Minerbi, 1987; Sardans and Peñuelas, 2005).

If we want to understand the overall effect of the warming and drought predicted by IPCC models, it is essential to investigate how warming and drought affect the activity of the soil enzymes involved in C and N mineralization in the mid and long term. To test warming and drought effects on Mediterranean shrublands, an experiment of warming and drought simulation has been conducted in the Garraf mountains (Catalonia, north-east Spain) since 1999. We have observed that warming and drought have changed some soil enzyme activities related to P turn-over in some year seasons (Sardans et al., 2006b). Unfortunately, there is a lack of information on the effects of prolonged warming and drought on the activity of soil enzymes related to C and N cycle, on C and N soil and plant contents and on N availability in soil. In a Mediterranean forest we have also observed that drought decreased urease, protease and β-glucosidase activities in spring and autumn in some cases (Sardans and Peñuelas, 2005). But, we do not know whether these changes are the direct results of warming and drought, or indirect effects through changes in quantity and nutritional quality of soil organic matter or of soil pH. In a Mediterranean area, when the environment evolves towards drier conditions, sclerophylly can increase (Dunn et al., 1977; Oliveira et al., 1994), increasing the proportion of structural compounds in litter, and consequently the C/N ratios in soil organic matter and litter and decomposition rates. On the other hand, drought can increase litter production and/or soil organic matter stimulating soil enzyme activity (Zaman et al., 1999; Dodor and Tabatabai, 2003). Finally, other important questions are whether the different soil enzymes are affected more by changes in soil temperature or by changes in soil moisture, and whether the possible effects are similar or not throughout the different year seasons.

The effects of warming and drought on soil nutrient availability can change the growth capacity (Martínez-Mena et al., 2002) and therefore the capacity of the ecosystem will sequester carbon. N soil status and consequently, the capacity of N capture by plants also affect water use efficiency (WUE) because WUE is positively correlated with N leaf concentration (Sing et al., 2000; Ruiz-Lozano et al., 2001). A decrease in nitrogen supply was found to increase stomatal conductance and to increase water loss, and hence to lower WUE whereas N fertilization reduced water loss and increased WUE (Fernández et al., 2006). N soil availability may offset positive plant growth responses under elevated CO_2 in Mediterranean ecosystems (Peñuelas and Matamala, 1990; Cruz et al., 2003) having an important role in C sequestration capacity.

Factors influencing soil microbial activity exert control over soil enzyme production and nutrient availability (Sinsabaugh et al., 1993). Soil enzyme activities are ''sensors'' of soil microbial status and soil physico-chemical conditions (Aon and Colaneri, 2001; Baum et al., 2003). They are used as sensors in studies on the influence of soil treatments on soil fertility (Chen et al., 2003). They may correlate well with nutrient availability (Asmar et al., 1994). Bacteria and fungi synthesize and secrete enzymes such as proteases, ureases and pectinases extracellularly. Those microbial-secreted enzymes constitute an important part of the soil matrix as extracellular enzymes, also called abiotic enzymes (Sinsabaugh, 1994).

Between the most important enzymes involved in organic matter mineralization in soil and in N and C cycle there are proteases, urease and β-glucosidase. Proteases are involved in the first phase of the release of N by hydrolyzing the peptide bond between amino acids. The release of amino acids is the first phase of N mineralization and an indispensable step for N uptake by plants. Ureases release N-NH_4^+ through urea hydrolysis and are essential in the chain of hydrolysis of amino compounds which are supplied to the soil from plants and to a lesser extend from animals and microorganisms. Soil ureases are microbial products that can accumulate in cell-free forms in the soil because they are highly resistant to environmental degradation (Zantua and Bremner, 1977). β-Glucosidase is one of the enzymes that break down labile cellulose and other carbohydrate polymers. Its action is fundamental in order to liberate the nutrients of organic compounds through its role in the first phases of degradation of organic compounds that reduce the molecular size and produce smaller organic structure, and thus facilitate future microbe enzyme activities.

The climate change scenarios for the next decades by IPCC (2001, 2007) and several ecophysiological models, such as, e.g. GOTILWA project a decrease in soil water content of 15–20% for the next three decades in Mediterranean areas. We hypothesized that this decrease in soil water contents when the temperatures are higher and water availability is lower (i.e. in summer), which can be critical, but that this decrease of soil water availability when the natural levels are higher and temperatures lower (i.e. in winter) probably should be negligible. Thus, drought can have different effects on soil enzyme activities depending on the annual season. Regarding changes in temperature, we hypothesized that a reduction in soil enzyme activity is expected in response to a temperature increase of 1–2 °C in summer when temperatures are higher and soil water content is lower, whereas higher soil enzyme

activity is expected in winter when the environmental temperatures are lower and water availability higher. These effects of drought and warming can be different depending on the different soil enzymes because some are more sensitive to changes in temperatures and others to soil water content. Indirect effects such as changes in soil organic matter, litter composition or soil pH changes due to variations in soil temperature or soil water content can also affect soil enzyme activity. We also aimed to gain knowledge on whether drought or warming effect on soil enzyme activity is mainly related to a direct effect on soil water content or to indirect effects, e.g. by changing litter and soil organic matter quality or soil pH. To test these hypotheses, in a long-term field experiment of warming and water availability manipulation, we conducted a study of the effects of the warming and drought forecasted for the next decades by climatic and ecophysiological models (Sabaté et al., 2002; Peñuelas et al., 2005; IPCC, 2001, 2007) on the activities of three enzyme groups linked to N and C cycle: proteases, ureases and β-glucosidases, soil pH, N soil availability and on C and N soil, litter and leaf contents during 1 year (spring 2004–spring 2005).

2. Material and methods

2.1. Study site

The study was carried out in a natural Mediterranean calcareous shrubland on a south-facing slope in the Garraf mountains in southern Catalonia (NE Spain) (41°18′N, 1°49′E) at 210 m above the sea level. The site is located on formerly cultivated terraces – abandoned approximately a century ago – with a *Petrocalcic calcixerepts* (Soil Survey Staff, 1998) soil lying on bedrock of sedimentary limestone. During the study period (1999–2005) the average annual temperature was 15.1 °C (7.4 °C in January and 22.5 °C in July) and the average annual rainfall 580 mm. The summer drought is pronounced and usually lasts for 3 months. The total vegetation cover is 70% and consists of a calcareous shrubland with plants about 1 m high dominated by the shrubs *Globularia alypum*, *Erica multiflora*, *Dorycnium pentaphyllum*, *Rosmarinus officinalis*, *Ulex parviflorus,* and *Pistacia lentiscus*. Aleppo pines *Pinus halepensis* were air seeded after the last forest fire in 1994 and are today gaining ground. The undergrowth is dominated by small shrubs such as *Fumana ericoides* and *Fumana thymifolia*. Grasses represent only a 2% of the total vegetation cover.

2.2. Experimental design

We conducted field-scale night-time warming, drought and control treatments (Beier et al., 2004; Peñuelas et al., 2004). Each of the three treatments (control, warming and drought) was applied in three plots. Each plot occupied an area of 4 m × 5 m, to avoid the effect of edge disturbance, samples were only taken from an internal area of 3 m × 4 m. Manipulation started in March 1999 and has continued up to the present day.

The warming treatment consisted of increasing night-time temperatures by covering the vegetation with an aluminum curtain coiled on a beam and connected to a motor controlled by light sensors that automatically covered the vegetation at night. This curtain reflected long-wave infrared radiation back into the vegetation, resulting in a temperature increase in relation to untreated plots. In order to avoid interfering with the hydrological cycle, roofs were automatically removed when it rained. This warming treatment has been applied since March 1999. The control plots were equipped with the same scaffolding as the treatment plots are without the roof.

The drought treatment reduced spring and autumn rainfall input. This was achieved by automatically covering vegetation with a transparent plastic curtain during periods of rain by means of automatic rain sensors. Once the rain stopped, the curtain was automatically removed. As in the warming treatment, the drought roof was removed if the wind speed exceeded 10 m s^{-1}. Roofs were also removed if wind speed exceeded 10 m s^{-1} in order to avoid structural damage. Given that plots were located on a slope, pipes were laid along the upper edges of the drought plots to minimize the entrance of runoff water. In summer and winter (outside the drought period), the treatment was not applied and drought plots were allowed to develop under the same conditions as the control plots.

Environmental conditions were monitored in all plots. Soil moisture was measured biweekly by TDR (time domain reflectometry) model Tektronix 1502C (Tektronix, Beaverton, OR, US) using three installed in each plot. The air and soil temperatures were recorded from every plot by temperature sensors (RTD Pt 100 1/3 DIN, Desin Instruments, Barcelona, Spain) located at depths of −10 and −5 cm in the soil and at 20 cm above the soil. Air and soil temperatures, as well as the correct functioning of the motorized curtain (magnetic sensors were installed at the end of the curtain track), were recorded every half an hour by a datalogger (Campbell Scientific, Inc. Logan, Utah, USA). Precipitation was measured by standard rain gauges and all the water entering the plots was collected biweekly.

2.3. Sampling process

2.3.1. Biomass

We sampled the leaf biomass 6 years after treatment application, at January 2005. We sampled five plants per plot and for each one of the three dominant shrub species, *G. alypum* and *E. multiflora*. Leaf life is longer in *E. multiflora* than in *G. alypum* and the leaf population consisted of current-year leaves and one-year-old leaves and so for this species we analyzed two different leaf-year cohorts (current-year and one-year old). In *G. alypum* only current-year leaves were present during the sampling campaign and therefore only one cohort was considered.

2.3.2. Litterfall

The litterfall of 4–8 plants of *E. multiflora* and 9–12 plants of *G. alypum* per plot was monitored during 1999 and 2004. Plant litterfall was collected bimonthly by means of open collectors (4.4 cm diameter) located under each selected plant. Samples were dried to constant weight and afterwards separated from different dominant species (*E. multiflora*, *G. alypum* and *D. pentaphyllum*) and weighed.

2.3.3. Soil

We sampled the soil in spring 2004 (April), summer 2004 (July), autumn 2004 (November), winter 2004–2005 (January) and spring 2005 (May), i.e. 5–6 years after treatments were started. In each plot and date, we sampled three soil cores (ϕ = 5 cm) from the top 10 cm of soil profile. Each soil sample was sieved in the laboratory and the fraction <2 mm was selected for measurements of soil moisture, soil enzyme activity, extractable NH_4^+ and NO_3^-, and total C and N soil content measurements.

2.4. Chemical analyses

2.4.1. Sample preparation

All the samples were taken to the laboratory and stored at 4 °C until analysis began in the next 6 days. All biomass samples were dried in an oven at 60 °C to a constant weight and then ground in a CYCLOTEC 1093 (Foss Tecator, Höganäs, Sweden). One aliquot of each soil sample was dried at 105 °C for soil water content determination. A second soil aliquot was stored at 4 °C for soil ammonium, nitrate and total soil organic C analyses. A third soil sample aliquot was dried at 105 °C for total soil N and C analyses. This soil aliquot was ground using a FRITSCH Pulverisette (Rudolstadt, Germany). The rest of each soil sample was kept without drying at 4 °C (during the next 6 days) until soil enzyme determination.

2.4.2. Total C and N in biomass and total N in soil

For C and N concentration determination in biomasses and litter and for N determination in soils sampled in winter 2004–2005, 1–2 mg of fine sieved, ground and dried sample plus 2 mg of V_2O_5 (as oxidant) was used. C biomass concentrations were analyzed by organic elemental analysis by the combustion coupled to gas chromatography. We used a Thermo Electron Gas Chromatograph model NA 2100 (C.E. instruments-Thermo Electron, Milano, Italy). In order to assess the accuracy of the biomass digestion and analytical procedures, we used standard certified biomass (DC73351). For soil analyses, the analytical precision – as verified by parallel analyses to an international (GSR-6) standard – was better than 5% for N and C analyses.

2.4.3. Soil organic carbon

For the determination of total soil C in soil samples, we used the Walkley–Black Method (Walkley and Black, 1934). Briefly, 10 ml of 1N of potassium dichromate + 20 ml of concentrated H_2SO_4 solution were added to 0.1 g of sieved and dried soil, then they were mixed by gentle rotation for 1 min and treated at 150 °C during 10 min and afterwards cooled until room temperature. Afterwards they were diluted to 200 ml with deionized water and 10 ml of phosphoric acid, 0.2 g of ammonium fluoride and 10 drops of diphenylamine indicator were added. Finally, the excess of dichromate was titrated with Morh salt solution (0.5N SO_4FeNH_4 and 0.1N H_2SO_4). We determined the %C in the soil samples of all the five season samplings.

2.4.4. Soil KCl-extractable-ammonium and soil KCl-extractable-nitrate determination

We analyzed soil available ammonium and nitrate in all soil samples of all five sampling seasons. We extracted ammonium and nitrate with a 2 M KCl extracting water solution. We analyzed both ammonium and nitrate by colorimetric analyses using a spectrophotometer Spectronic 20 Genesys (Spectronic Instruments Inc., Rochester, NY, USA) against the reagent blank. We analyzed ammonium in water extracts by a modified Berthelot reaction (Schinner et al., 1996) and nitrate by cadmium reduction method (U.S. EPA, 1979).

2.5. Enzyme activity measurements

We determined the soil protease, urease and β-glucosidase activities in the soil samples of the five sampling seasons. For protease activity determination we used the method developed by Ladd et al. (1976). 5 ml of the substrate solution (casein solution, 2%, w/v) was added to 1 g of field-moist soil. We added 5 ml of Tris (Tris–hydroxymethyl–aminomethane) buffer (0.05 M, pH 8.1), and then incubated for 2 h at 50 °C. After incubation, the remaining substrate was precipitated after the addition of thichloroacetic acid. Thereafter, samples and controls were filtered immediately. For photometric analysis, we pipetted 5 ml of filtrate and 7.5 ml of alkali reagent into a test tube, mixed well, added 5 ml of Folin–Ciocalteu's phenol reagent, and mixed again. Before color measurements, samples, controls and the standards were filtered (0.45 μm HA nitrocellulose, Millipore) in order to prevent the interference of precipitates formed by the casein remainders, and then they were allowed to stand at room temperature for exactly 90 min for color development. Within the following 90 min, we measured the extinction at 700 nm with a spectrophotometer Spectronic 20 Genesys (Spectronic Instruments Inc., Rochester, NY, USA) against the reagent blank. We calculated the tyrosine content by referring to a calibration curve obtained with standards containing 0, 100, 250, 1000 and 1500 μg of tyrosine. Protease activity was expressed as μg tyrosine equivalents per gram dry matter and incubation time. Tyrosine equivalents were calculated from the calibration curve.

For urease activity determination, we used the Kandeler and Gerber (1988) method. After the addition of an aqueous (controls) or a buffered urea solution (samples) to 5 g of soil samples, they were incubated for 2 h at 37 °C. Released ammonium was extracted with potassium chloride solution, and determined by a modified Berthelot reaction (Schinner et al., 1996). The solutions were shaken for 30 min and filtered (0.45 μm HA nitrocellulose, Millipore) in order to prevent the interference of possible precipitates. The determination was based on the reaction of sodium salicylate with NH_4^+ in the presence of sodium dichloroisocyanurate which forms a green-colored complex under alkaline pH conditions, being the extinction measured at 690 nm with a spectrophotometer Spectronic 20 Genesys (Spectronic Instruments Inc., Rochester, NY, USA) against the reagent blank. We calculated the ammonium content by referring to a calibration curve obtained with standards containing 0, 1, 1.5, 2 and 2.5 μg NH_4^+ ml^{-1}. Sodium nitroprusside was used as a catalyst. It increased the sensitivity of the method about 10-fold.

The method used to determine β-glucosidase was based on the colorimetric determination of saligenin released by β-glucosidase when 5 g of soil was incubated 3 h at 37 °C with acetate buffer (pH 6.2), and salicin (β-glucosido-saligenin) (Tabatabai, 1994). The solutions were filtered (0.45 μm HA

nitrocellulose, Millipore) in order to prevent the interference of possible precipitates. Saligenin released from the substrate was determined colorimetrically after coloring with 2,6-dibromomchinon-4-chlorimide with borate buffer. At pH values above 9, saligenin forms a blue indophenol dye with 2,6-dibromchinon-4-chlorimide which was read at 578 nm in a spectrophotometer Spectronic 20 Genesys (Spectronic Instruments Inc., Rochester, NY, USA). We calculated the saligenin content by referring to a calibration curve obtained with standards containing 0, 10, 20, 50 and 100 μg of saligenin. β-glucosidase activity was expressed as the amount of saligenin released per gram dry matter and incubation time.

2.6. Soil pH

We measured soil pH in a 1:2.5 soil solution (in both water and 0.1 M KCl) using a glass electrode (ORION 960 Autochemistry System) in all the soil samples.

2.7. Statistical analyses

We analyzed soil enzyme activities and C and N content and N soil available forms in soil, litter and leaves by using a one-way ANOVA with treatments: C = Control, D = drought and W = warming. We compared the different treatments through Bonferroni/Dunn post-hoc ANOVA tests. To calculate the differences of soil enzyme activities between different treatments along the seasons we used a repeated measures ANOVA. To test the whole effect of soil enzymes, we conducted repeated measures ANOVA using the data from five seasons. We also tested the block effect by factorial ANOVA (treatments × block). The Statistica 6.0 package (SAS Institute Inc., Core, NC, USA) was used for all statistical tests.

We correlated the seasonal values of soil enzyme activities with the corresponding mean of soil moisture obtained gravimetrically in an aliquot of each soil sample and of soil temperature of each control, drought and warming plots, i.e. nine points (3C + 3D + 3W) in each correlation. Each value of soil urease or protease or β-glucosidase activity was the mean of five samples per plot, whereas the soil moisture was the mean of five different TDR values of soil water content per plot and soil temperature the mean of two sensors per plot.

3. Results

3.1. Temperatures, precipitation, soil water content, and soil pH

Drought treatment reduced the soil water content of the soil samples by 28% in autumn 2004 (Table 1). In the TDR measurements a significant decrease in soil water content was observed in drought plots in the periods May–June 2004 and November–December 2004 (Fig. 1). An increase of soil T (°C) was observed in warming plots in soil (at − 5 cm) during the period November 2004–March 2005 (Fig. 1), and in air (at 20 cm above soil) in the periods April–May in 2004, July–September in 2004, November 2004 and February–April in 2005 (Fig. 1).

Warming did not significantly change soil water content either in the sampling days or through the 6 years of treatment. Therefore, the observed effects of warming on soil enzyme activities, C and N soil content and on C and N content in leaves and leaf litter were directly attributable to the effects of warm-stimulating microbial and plant activities, or by drought effects on enzyme activity, by optimal temperature or energy of activation. No significant effects of treatments were observed on soil pH, neither in water (7.7 throughout the year and in all treatments) nor in 0.1 M KCl (7.5 throughout the year in all treatments).

Table 1 – Temperature (°C) in soil at 5-cm depth and in air at 20 cm of height in the 30 previous days to the sampling day, and soil water content (%, w/w) obtained gravimetrically in the samples where the soil analyses were performed

Mean T in the previous 30 days	Season	Treatment		
		Control	Drought	Warming
Soil at 5-cm depth	Spring 2004	**8.37 ± 0.12 b**	**7.85 ± 0.04 b**	**9.58 ± 0.26 a**
	Summer	26.7 ± 0.5	26.5 ± 0.3	26.7 ± 0.7
	Autumn	17.4 ± 3.2	14.1 ± 0.2	15.1 ± 0.4
	Winter	**6.63 ± 0.15 b**	**5.95 ± 0.18 b**	**7.78 ± 0.43 a**
	Spring 2005	20.5 ± 0.74	21.1 ± 1.0	21.0 ± 0.4
Air at 20 cm	Spring 2004	**7.48 ± 0.88 b**	**7.38 ± 0.06 b**	**8.00 ± 0.12 a**
	Summer	**24.0 ± 0.3 b**	**24.1 ± 0.2 ab**	**24.8 ± 0.2 a**
	Autumn	**13.1 ± 0.1 ab**	**12.9 ± 0.2 b**	**13.6 ± 0.1 a**
	Winter	**5.99 ± 0.13 ab**	**5.66 ± 0.19 b**	**6.37 ± 0.15 a**
	Spring 2005	**17.7 ± 0.2 b**	**18.1 ± 0.1 b**	**18.7 ± 0.2 a**
Soil water content (%, w/w)	Spring 2004	19.5 ± 1.2	19.5 ± 1.4	19.7 ± 0.4
	Summer	6.5 ± 0.4	5.9 ± 0.6	6.6 ± 0.7
	Autumn	**12.1 ± 0.6 a**	**8.7 ± 1.0 b**	**13.6 ± 1.4 a**
	Winter	14.7 ± 1.1	14.9 ± 1.3	14.2 ± 0.5
	Spring 2005	9.1 ± 0.7	9.1 ± 1.0	8.8 ± 0.9

Values are means ± S.E. ($n = 3$ plot means of 5 replicates per plot) (different letters indicate significant differences at $p < 0.05$ among treatments; they are highlighted in bold type).

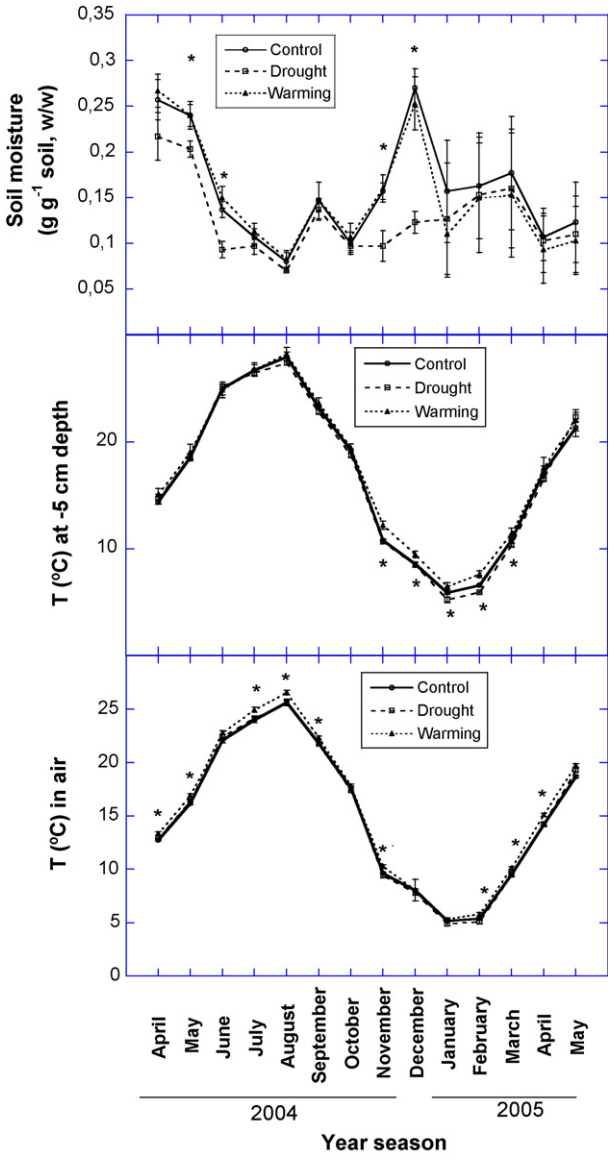

Fig. 1 – Soil moisture obtained with TDR (%, v/v) and soil and air temperatures (°C) (mean ± S:E.) during the period April 2004–May 2005 in the different treatments ($n = 3$ means of $n = 5$ replicates per plot). *Significant differences at $P < 0.05$ among treatments).

3.2. *Soil enzyme activities*

Analyzing the treatment effects on soil enzyme activities of each season, we observed as warming increased soil urease activity 30% in winter 2004–2005 ($P = 0.011$) and marginally increased it 10% in spring 2004 ($P = 0.067$). Warming increased β-glucosidase 38% in spring 2004 ($P = 0.022$) (Table 2). Drought decreased soil protease activity 21% in spring 2004 ($P = 0.040$) and 13% in spring 2005 ($P = 0.045$) (Table 2).

The repeated measures ANOVA showed that warming increased urease activity with respect to control ($P = 0.039$) and drought plots ($P = 0.048$). Drought-decreased protease activities both with respect to control soils ($P = 0.030$) and warming soils ($P = 0.042$). We did not observe effects of treatments on β-glucosidase activity in repeated measures ANOVA (data not shown). We did not observe effects of blocks on soil enzyme activity (data not shown).

The correlation between soil enzyme activities and soil temperature tended to be negative in summer and positive in winter (Figs. 2–4). The enzyme activity was positively correlated with soil water content in summer 2004 for urease and protease, in winter 2005 for protease and β-glucosidase and in spring 2005 for protease (Figs. 2–4). In autumn and spring no clear correlations of soil enzyme activities with soil temperature and water content were found, except in the case of protease activity in the dry spring 2005 when it was negatively correlated with soil temperature and positively with soil water content (Fig. 4).

3.3. *C and N in soil*

Warming decreased soil concentration of extractable N-NH_4^+ 28% in spring 2004 and 65% in spring 2005 but increased it 24% in winter (Table 3). Warming increased ca. three times soil concentration of extractable N-NO_3^- in summer 2004 and ca. twice in autumn 2004 (Table 3).

In winter 2004–2005, total concentration of soil organic C was greater in drought soils (Table 4). No differences were observed between different treatments neither on soil N nor on soil C/N total content ratio (Table 4). We did not observe effects of blocks on soil N contents (data not shown).

3.4. *C and N in leaves and leaf litter*

Warming decreased 12% N concentration in current-year leaves of *E. multiflora* (Table 4). Drought increased C content 3% and decreased N content 14% in current-year leaves of *E. multiflora*, increasing the C/N content 16% (Table 4). No effects of drought were observed in 1-year old nor in leaf litter of *E. multiflora* (Table 4).

Warming and drought decreased N concentration 24 and 19%, respectively, and increased C/N concentration ratio in leaves of *G. alypum* 32 and 33%, respectively (Table 4). Leaf litter of *G. alypum* had 2.5% lower C contents in warming plants than in drought plants (Table 4).

4. Discussion

Warming increased soil urease in winter 2004–2005 and in spring 2005, and soil β-glucosidase activities only in spring 2004, whereas drought decreased soil protease activity, mainly by reducing it in spring 2004 and spring 2005. The repeated measures ANOVA confirmed that urease is more sensible to warming changes whereas protease is more sensible to drought change. Increases of the soil enzyme activities in response to higher temperatures have also been observed in several experiments in tropical and temperate non-Mediterranean ecosystems (Tscherko et al., 2001; Fey and Conrad, 2003; Fenner et al., 2005), the increase depending on the changes of water soil content through the different year seasons (Fenner et al., 2005). In our case, the great increase in the activity of soil urease was observed in winter when the soil temperatures were low and warming increased the soil

Table 2 – Activities of soil urease, protease and β-glucosidase in spring, summer and autumn 2004 and in winter and spring 2005, after 5–6 years of treatment

Enzyme	Season	Treatment		
		Control	Drought	Warming
Urease activity (mg N g^{-1} h^{-1})	Spring 2004	0.36 ± 0.02	0.37 ± 0.03	0.37 ± 0.02
	Summer	0.44 ± 0.02	0.40 ± 0.02	0.43 ± 0.03
	Autumn	0.27 ± 0.02	0.25 ± 0.01	0.27 ± 0.05
	Winter	**0.38 ± 0.02 b**	**0.40 ± 0.03 ab**	**0.50 ± 0.06 a**
	Spring 2005	**0.32 ± 0.01 (b)**	**0.33 ± 0.01 (ab)**	**0.36 ± 0.01 (a)**
Protease activity (mg tyr g^{-1} h^{-1})	Spring 2004	**38.6 ± 2.2 a**	**30.5 ± 2.6 b**	**38.6 ± 2.0 a**
	Summer	22.9 ± 5.5	23.6 ± 5.6	24.8 ± 3.7
	Autumn	51.1 ± 2.4	51.2 ± 1.5	51.0 ± 0.9
	Winter	58.8 ± 0.7	54.1 ± 4.1	52.2 ± 5.2
	Spring 2005	**64.1 ± 4.0 a**	**56.5 ± 1.1 b**	**61.6 ± 4.8 ab**
β-glucosidase (mg saligenin g^{-1} h^{-1})	Spring 2004	**0.98 ± 0.05 b**	**0.93 ± 0.07 b**	**1.38 ± 0.18 a**
	Summer	1.65 ± 0.20	1.87 ± 0.16	1.64 ± 0.15
	Autumn	2.80 ± 0.21	2.91 ± '0.15	2.51 ± 0.22
	Winter	2.91 ± 0.31	2.55 ± 0.19	2.73 ± 0.20
	Spring 2005	3.92 ± 0.41	3.91 ± 0.31	3.51 ± 0.32

Values are means ± S.E. ($n = 3$ plot means of 5 replicates per plot) (different letters indicate significant differences at $p < 0.05$ among treatments; when between brackets they indicate significant differences only at $p < 0.10$; they are highlighted in bold type). Urease activity was measured as mg N g^{-1} soil h^{-1}, protease activity as mg tyr g^{-1} soil h^{-1} and β-glucosidase activity was measured as saligenin g^{-1} soil h^{-1}

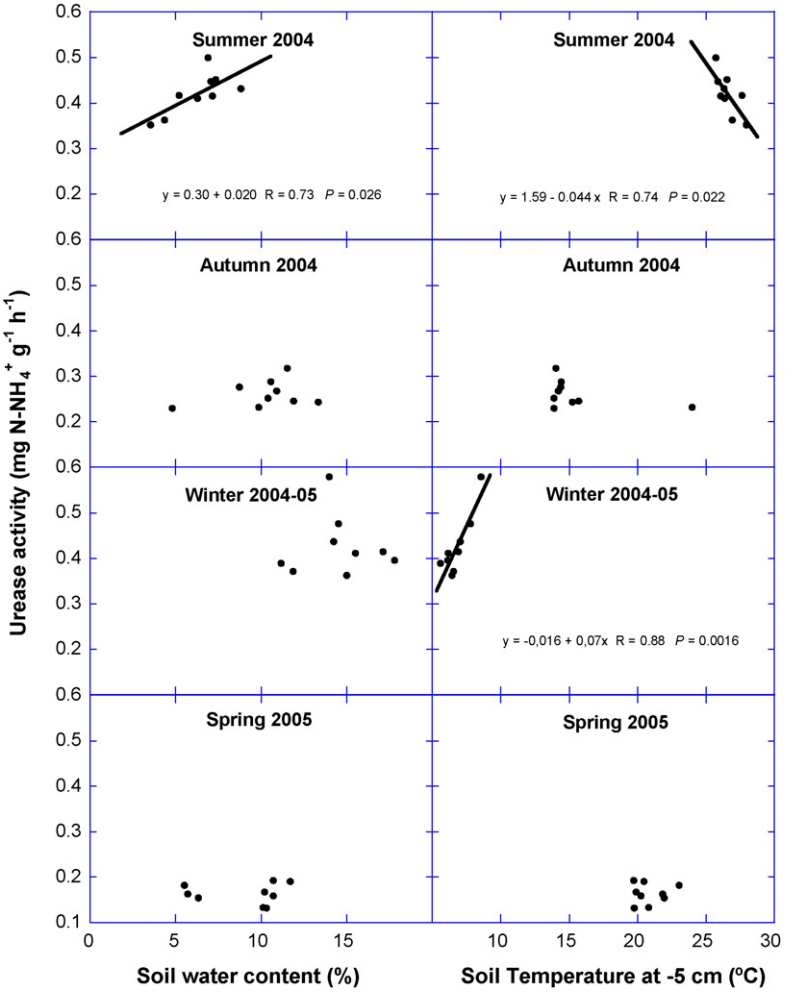

Fig. 2 – Relationships of urease activity (mg N-NH_4^+ g^{-1} h^{-1}) with soil water content (%) and soil temperatures (°C at 5 cm depth) in the day of sampling ($n = 9$ plot means of $n = 5$ replicates per plot).

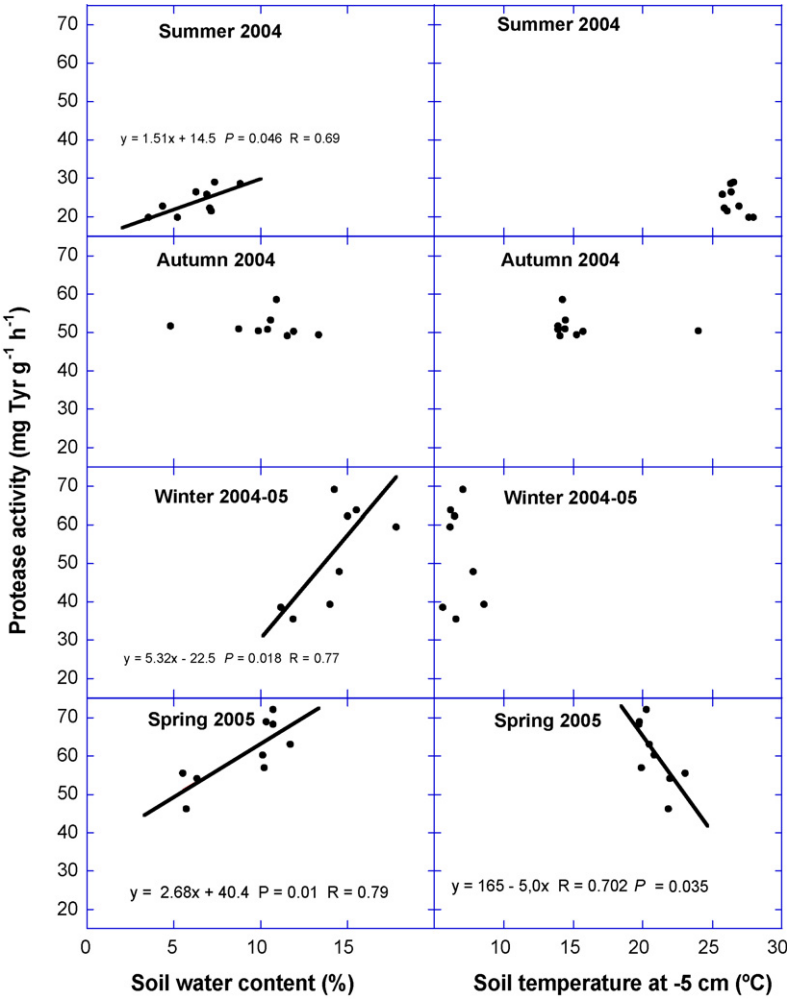

Fig. 3 – Relationships of protease activity (mg tyr g^{-1} h^{-1}) with soil water content (%) and soil temperatures (°C at 5 cm depth) in the day of sampling (n = 9 plot means of n = 5 replicates per plot).

temperature significantly. In this season, we observed a significant and positive correlation between temperature and urease activities. On the contrary, in summer, the correlation between soil temperature and urease activity was negative. This contrasting response of soil urease activity in summer and winter was also observed in β-glucosidase. Thus, these results showed as warming increased urease activity, mainly by increasing the activity from autumn to spring when the natural soil water content is higher.

Drought reduced soil protease activity in spring 2004 and in spring 2005. On the other hand, soil protease activity was positively correlated with soil water content in three of the 4 year seasons. Several experiments in laboratory and in field conditions have shown that soil protease activity is related mainly to soil water content and less affected by temperature (Tscherko et al., 2001; Sierra, 2002).

In the Mediterranean ecosystems, high temperatures coincide in summer with low or absent precipitation generating a deep water stress. In contrast, the coldest season, winter, coincides with greater levels of water availability. Thus, moderate increase in soil temperatures can enhance or hinder the enzyme activities depending on soil water content. Our results confirm that a moderate increase of soil temperature in spring, autumn or winter, when soil water content is greater can imply a great increase in soil enzyme activity in comparison with the changes among different seasons where great changes in soil temperature have only moderate effects on soil enzyme activity. For example there was only a little difference between soil enzyme activities in summer and winter in spite of the great difference in soil temperature between these two seasons. The low soil water content in summer did not permit an enhancement of the enzyme activity in a response to warming. On the other hand, probably different soil microbial communities with different temperature and soil moisture optimum operated at different times throughout the year. Coinciding with these results pointing to a decrease of some enzymes activity in drought conditions, there has also been a decrease of soil respiration in drought soils during the same period (Asensio et al., unpublished data). No changes in soil respiration were observed in warming soils (Asensio et al., unpublished data).

The increases of soil urease activity in warmed soils were not accompained by an increase in soil extractable NH_4^+ concentration, that decreased in spring in warming plots,

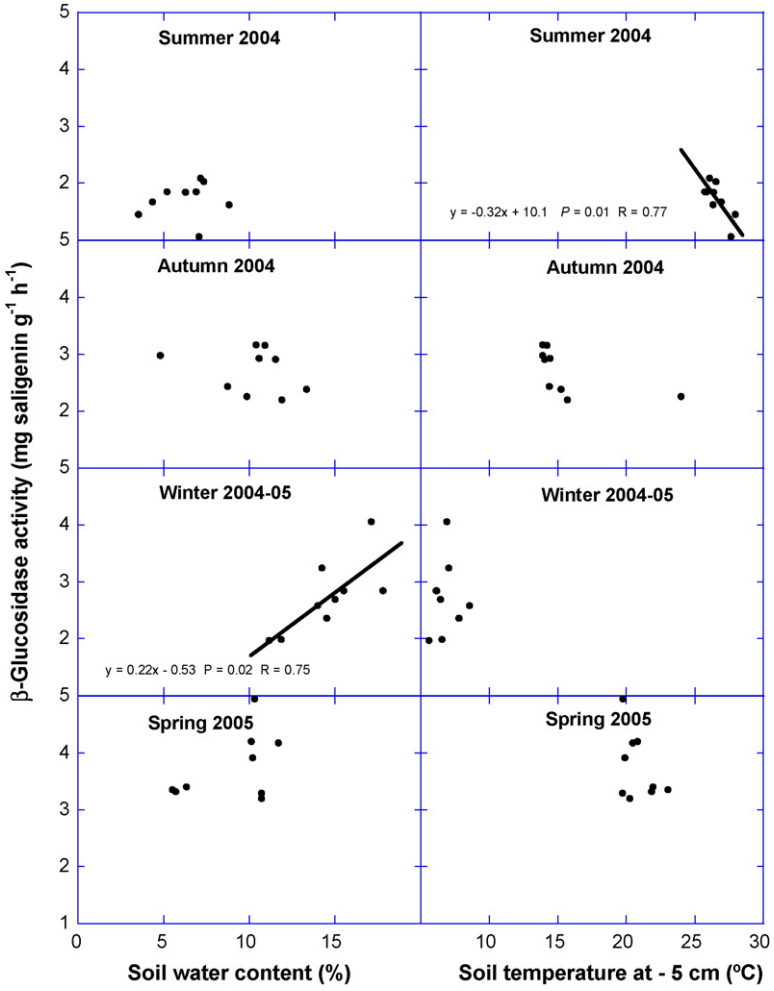

Fig. 4 – Relationships of β-glucosidase activity (mg saligenin g^{-1} h^{-1}) with soil water content and soil temperatures (°C at 5 cm depth) in the day of sampling (n = 9 plot means of n = 5 replicates per plot).

neither with soil organic C, that did not change significantly in warming plots. These decreases in soil extractable NH_4^+ in spring are related to the higher N plant capture by the enhancement of growth observed in warming plants in the growing season (spring) of some years during the period 1999–2005 (Llorens et al., 2003; Prieto et al., unpublished data). As in other reports (García et al., 2002), in this study no clear correlation was observed between reduction in soil urease and available ammonium. On the other hand, soil extractable NO_3^- concentrations increased in warming plots in summer and in autumn, as expected from temperature being positively related with nitrification, as observed in temperate non-Mediterranean ecosystems (Ryan et al., 1998; Wang et al., 2006) and in tropical ecosystems (Gregal et al., 1999; Sierra, 2002). The increases of soil phosphatases activities and of P mobilization in soil observed in warming plots (Sardans et al., 2006b) could also favor a greater nitrification since nitrification has been reported to be dependent on P availability (Sierra et al., 2003). On the other hand, the increases of temperature favor the environment oxidation capacity, which has also been shown to be related with soil nitrification process (Brzezinska et al., 1998). The increases in NO_3^- soil contents observed in warming plots might facilitate the losses of N in Mediterranean areas with high frequency of torrential rainfalls. Warming also decreased C contents in leaves and in leaf litter of *G. alypum*, but did not affect its C/N concentration ratio nor C and N content in soil organic matter and leaf litter. Thus, the changes observed in soil enzyme activities were more likely related to a direct effect of warming stimulating biological activity than to an indirect effect of enhancement of the nutritional quality of soil organic matter (C/N ratio) or its amounts.

The reduction in soil protease activities in drought plots were not accompanied by decreases in soil NH_4^+ availability, probably because the reduction of soil protease activity in drought plots was counterbalanced by a reduction in N leaf concentrations (and C/N concentrations ratio) in drought plants, in an enhancement of sclerophylly typical of Mediterranean shrub plants under drier conditions (Sardans et al., 2006c). Drought did not change soil soluble NO_3^- in agreement with results observed in non-Mediterranean temperate and boreal ecosystems where no clear nitrification pulses were observed in drought conditions neither on rewetting periods nor after prolonged drought periods (Lamersdorf et al., 1998; Ryan et al., 1998).

Table 3 – Soil NH_4^+ concentration (mg kg^{-1} soil), soil NO_3^- concentration (mg kg^{-1} soil) and soil organic C (%, w/w)

Season	Treatment		
	Control	Drought	Warming
Soil NH_4^+			
Spring 2004	**1.04 ± 0.10 a**	**0.94 ± 0.17 ab**	**0.73 ± 0.19 b**
Summer	1.50 ± 0.35	1.20 ± 0.21	1.64 ± 0.20
Autumn	1.25 ± 0.10	1.14 ± 0.14	1.37 ± 0.17
Winter	**1.28 ± 0.09 (b)**	**1.51 ± 0.10 (ab)**	**1.56 ± 0.12 (a)**
Spring 2005	**1.63 ± 0.26 a**	**1.45 ± 0.31 a**	**0.45 ± 0.09 b**
Soil NO_3^-			
Spring 2004	2.5 ± 1.8	6.2 ± 3.9	3.3 ± 1.4
Summer	**7.5 ± 4.3 b**	**11.1 ± 3.9 ab**	**22.1 ± 6.8 a**
Autumn	**8.5 ± 2.3 b**	**4.1 ± 1.7 b**	**18.4 ± 4.2 a**
Winter	17.8 ± 3.5	25.9 ± 6.1	24.8 ± 2.9
Spring 2005	10.6 ± 6.8	9.1 ± 2.6	7.6 ± 3.0
Soil organic-C			
Spring 2004	2.0 ± 0.3	2.3 ± 0.3	2.3 ± 0.2
Summer	2.2 ± 0.3	2.3 ± 0.3	2.2 ± 0.2
Autumn	2.1 ± 0.3	2.3 ± 0.4	2.3 ± 0.2
Winter	**2.5 ± 0.3 ab**	**2.9 ± 0.3 a**	**2.1 ± 0.2 b**
Spring 2005	2.1 ± 0.3	2.7 ± 0.3	2.5 ± 0.5

Values are means ± S.E. ($n = 3$ plot means of 5 replicates per plot) (different letters indicate significant differences at $p < 0.05$ among drought treatments. Different letters between brackets indicate significant differences only at $p < 0.1$; they are highlighted in bold type).

4.1. *Final remarks*

Warming increased soil urease activity, mainly by increasing soil temperature in the seasons with high soil moisture, whereas it had no significant effects in summer, when temperatures are highest and soil moisture is lowest. Warming also increased β-glucosidase activitiy in spring 2004 and decreased foliar N concentrations, increasing foliar C/N

Table 4 – %C, %N and C/N ratio of soil and leaves of *Erica multiflora* and *Globularia alypum*, in the different treatments in samples of January 2005

	Variable	Treatment		
		Control	Drought	Warming
Soil	%C	**2.5 ± 0.2 ab**	**2.9 ± 0.2 a**	**2.1 ± 0.1 b**
	%N	0.15 ± 0.02	0.17 ± 0.02	0.16 ± 0.02
	C/N	17.6 ± 1.4	20.6 ± 4.1	14.9 ± 2.3
Erica multiflora				
Current-year leaves	%C	**57.5 ± 0.11 b**	**59.0 ± 0.4 a**	**58.8 ± 0.6 ab**
	%N	**0.67 ± 0.04 a**	**0.58 ± 0.03 b**	**0.60 ± 0.02 ab**
	C/N	**90.0 ± 5.0 b**	**105.0 ± 5.0 a**	**100.0 ± 5.0 ab**
One-year-old leaves	%C	57.6 ± 0.5	57.1 ± 0.8	59.7 ± 1.4
	%N	0.59 ± 0.02	0.61 ± 0.08	0.61 ± 0.06
	C/N	101.0 ± 5.0	103.0 ± 8.0	113.0 ± 9.0
Leaf litter	%C	57.4 ± 0.1	57.9 ± 0.3	57.2 ± 1.5
	%N	0.81 ± 0.04	0.78 ± 0.05	0.76 ± 0.03
	C/N	71.2 ± 2.9	74.5 ± 4.7	75.8 ± 4.1
Globularia alypum				
Leaves	%C	50.5 ± 0.3	50.3 ± 0.3	49.8 ± 0.2
	%N	**0.84 ± 0.02 a**	**0.68 ± 0.05 b**	**0.64 ± 0.02 b**
	C/N	**60.7 ± 2.2 b**	**80.5 ± 7.8 a**	**79.5 ± 2.4 a**
Leaf litter	%C	**50.4 ± 0.2 ab**	**50.5 ± 0.3 a**	**49.3 ± 0.5 b**
	%N	1.33 ± 0.03	1.37 ± 0.10	1.33 ± 0.10
	C/N	37.8 ± 1.1	37.3 ± 2.8	37.6 ± 2.5

Values are means ± S.E. ($n = 3$ plot means of 5 replicates per plot). Different letters indicate significant differences at $p < 0.05$ among drought treatments; they are highlighted in bold type.%C, %N and C/N ratio in leaf litter of *Erica multiflora* and *Globularia alypum* collected during the whole 2004 year

concentrations ratio, in one of the two dominant shrub species, but did not change C and N content in leaf litter and in soil organic matter with respect to control. These results show that the changes in soil enzyme activity in warming soils were not related to an indirect effect of nutritional quality changes of soil organic matter and litter, other factors such as optimal temperature or energy of activation for soil enzymes may also explain this effect. However, in warming plots the decrease in C/N ratios in *G. alypum* leaf litter can contribute to microbe stimulation to synthesize more enzymes to maintain adequate levels of nutrient supply. Warming also increased NO_3^- availability in soil in summer and autumn, favoring the N uptake by plants, which is in agreement with the increases in their photosynthetic capacity and growth (Llorens et al., 2003, 2004) and the greater accumulation of total N in aboveground biomass (Sardans et al., unpublished data). However, a greater NO_3^- availability in soil can favor a net N loss by leaching under the torrential rainfalls typical of the Mediterranean climate regions.

Drought decreased soil protease activities but did not affect N soil contents because N turn-over reduction was counterbalanced by a decrease in plant uptake capacity and N concentration and accumulation in aboveground biomass. Thus, protease decrease activity was mainly correlated with the changes in soil water contents more than the changes in soil organic matter quantity and quality. Drought did not affect β-glucosidase activity but tended to increase C contents in soils, effect related to the tendency of C concentrations to increase in leaf litter of *G. alypum*, in spite of the absence of significant changes in litter production. This fact, together with the C/N increases in leaves and growth reduction point to a reduction of C turn-over and to a decrease of C and N stocks in aboveground biomass accompanied by an increase of C and N stocks in soil at long term.

Thus, warming affected mainly soil urease activity whereas drought affected soil protease activity. The climate change effects on C and N soil dynamics and stocks, and N soil availability in Mediterranean shrublands will thus depend on whether the main component of climate change is warming or it is drought.

The results highlight a positive correlation between soil water content and soil enzyme activities in this Mediterranean shrubland. Thus, all the management activities that contribute to maintain soils and to prevent soil water losses by direct soil water evaporation, such as for example control of livestock grazing intensity, better fire prevention and extinction or human driven acceleration of post-fire regeneration of vegetation, will favor a better soil nutrient status.

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