

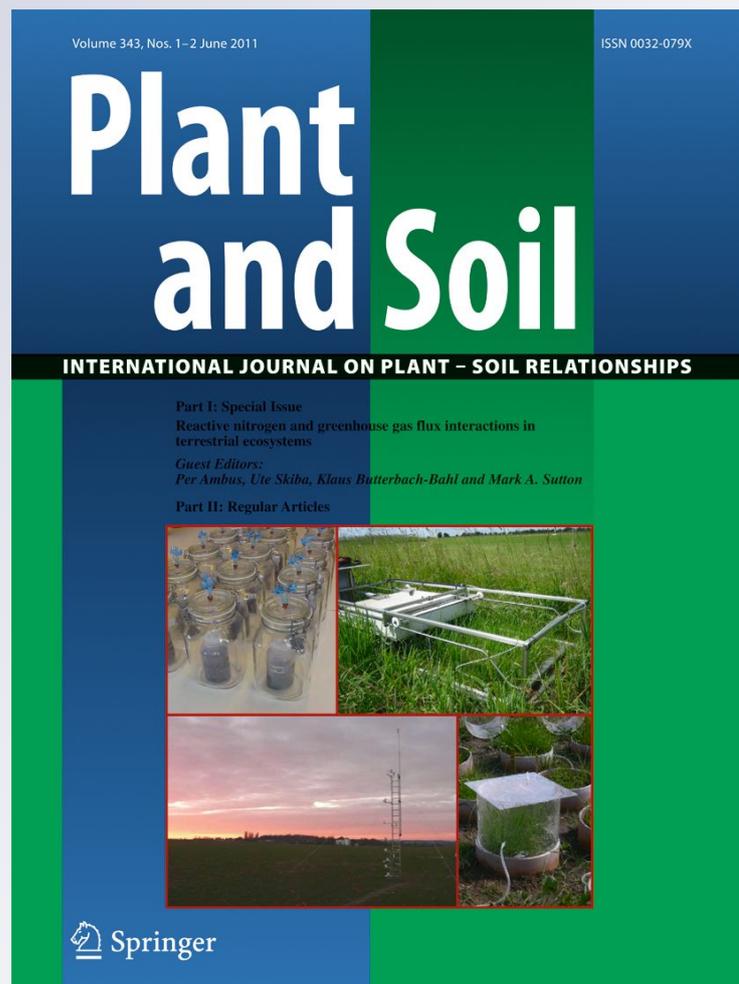
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Patterns of nitrate reductase activity vary according to the plant functional group in a Mediterranean maquis

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Abstract Since little is known about how the Mediterranean Basin ecosystems are affected by nitrogen deposition, we aimed to understand the use of nitrogen by distinct plant functional groups (PFG: summer semi-deciduous and evergreen sclerophylls) present in the Mediterranean maquis in order to assess which may be more affected by changes in nitrogen availability. The availability of soil inorganic nitrogen, leaf nitrate concentrations and nitrate reductase activity (in vivo and in vitro) were measured during the year in three plant species from each PFG. The patterns of in vitro NRA along the shoot and through the day were also determined. Although summer semi deciduous species occupied soil patches richer in nitrate, their leaf NRA were significantly lower than that of evergreen sclerophylls species. The pattern of nitrate and ammonium

availabilities along the year also distinguished the PFG. Results show that each PFG is composed of a number of physiologically similar species. Patterns of NRA varied according to the PFG, which may represent distinct specializations of co-occurring species to access nitrogen. Therefore, the NRA can be used as an indicator of the nitrate availability taking into consideration the time of the year, the plant species and its PFG.

Keywords Mediterranean · Plant functional groups · Nitrate · Ammonium · Pattern of nitrate reductase activity · Nitrogen strategies

Introduction

Anthropogenic changes in ecosystem functioning threaten human well-being (Sala et al. 2000; Cardinale et al. 2007). Thus, Rockström et al. (2009) identified three main interdependent pressures on global sustainability (changes in the global nitrogen cycle, biodiversity loss, and climate change), with the role of increased nitrogen deposition in biodiversity loss (for review see Bobbink et al. 2010) and in alterations of ecosystem functioning (Gruber and Galloway 2008; Rockström et al. 2009) being well established. According to Bobbink et al. (2010) increased availability of reactive nitrogen is threatening the biodiversity of ecosystems around the world.

Most empirical research linking biodiversity with ecosystem functioning has focused on terrestrial

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systems with low structural complexity (Maestre and Reynolds 2006; Montès et al. 2008). This limits our ability to make confident generalizations on the functional role of biodiversity, and/or to extrapolate the results obtained so far to other communities (Montès et al. 2008). Mediterranean-type ecosystems are plant biodiversity hotspots (Phoenix et al. 2006) with high structural complexity. The relationship between species richness, functional characteristics and spatio-temporal distribution could be examined in these ecosystems. Moreover, decades of research on these ecosystems have highlighted the importance of plant-plant interactions and environmental heterogeneity (Válladares and Gianoli 2007), both potentially altered by increased nitrogen deposition (Ochoa-Hueso et al. 2011). However, the relationship between nitrogen availability and biodiversity has been poorly studied in this type of ecosystem, especially among those located in the Mediterranean Basin (Phoenix et al. 2006; Bobbink et al. 2010; Ochoa-Hueso et al. 2011). Mediterranean ecosystems are thought to be currently experiencing increases in nitrogen deposition (Galloway et al. 2004; Phoenix et al. 2006) and the greatest proportional biodiversity change (Sala et al. 2000; Phoenix et al. 2006). Evidence of the link between biodiversity and increased reactive nitrogen availability is starting to emerge: changes in plant (Dias et al. 2011) and epiphytic lichen diversity (Pinho et al. 2009, 2011). Most semi-natural ecosystems have evolved under nitrogen limitation and Mediterranean ecosystems are no exception (Cowling et al. 1996). Thus, plant species are expected to be adapted to the nitrogen source available to them (Chapin et al. 2002; Craine 2009). Given that nitrate is the most common form of reactive nitrogen in the Mediterranean Basin we would expect that the enzyme responsible for processing nitrate, nitrate reductase (NR) will show considerable variability in its activity between plant functional groups.

However, nitrate reductase activity (NRA) is regulated by multiple environmental stimuli (Kaiser et al. 2000, Kaiser and Huber 2001). For instance, when plants are deprived of nitrate, NRA falls rapidly (in less than 24 h—Kaiser and Spill 1991); if nitrate is added to the soil around plants growing in natural communities, many plants may increase or induce NRA (Kaiser et al. 2002; Arslan and Güleriyüz 2005). Substrate induction increases the flexibility within the metabolic systems of many plant species. The

inducibility of NRA gives flexibility to the metabolic systems of many plant species, constituting an 'ecological' response to the wide variation in nitrate supply that occurs under natural conditions (Cruz et al. 2003; Sakar et al. 2010), especially in Mediterranean ecosystems (Gallardo et al. 2006; Cruz et al. 2008). This, combined with the NR genetic differences between species (Havill et al. 1974; Arslan and Güleriyüz 2005; Arslan et al. 2009), can contribute to the coexistence of plant species.

The plant species pool of the Mediterranean maquis offers an opportunity to assess the capacity to use nitrate by co-existing species as an indicator of the nitrate status of the ecosystem. But can the NRA of all plant species be used or only that of some? There is growing literature suggesting that focusing on functional traits, rather than species (McGill et al. 2006), is more relevant for the assessment of how ecosystems function (Naeem 2009), and a more practical approach for biodiversity hotspots such as Mediterranean ecosystems. The vegetation of Mediterranean ecosystems may be grouped into two plant functional groups (PFG): summer semi-deciduous and evergreen sclerophylls. Each group has been characterized on the basis of its phenology, water relations, carbon exchange properties and abundance during different successional stages (Correia 1988; Werner et al. 1999, 2001). Our working hypothesis is that the co-existing summer semi-deciduous and evergreen sclerophylls species show distinct patterns of NRA associated with the availability of nitrogen in their ecological niches (Cruz et al. 2008) and their phenological and root traits. In order to test our hypothesis, we related leaf *in vivo* and *in vitro* NRA of several plants species belonging to the two PFG with the seasonal availability of water and inorganic nitrogen to extend the definition of these PFG to their nitrogen use strategies.

Materials and methods

Study site

The study site (38° 27' 34" N, 9° 0' 20" W) is located in Serra da Arrábida in the Arrábida Natural Park, south of Lisbon, Portugal (a Natura 2000 site—PTCON0010 Arrábida/Espichel). Estimated nitrogen deposition is 5.2 kg ha⁻¹ year⁻¹ (2.9 kg NO_x+2.3 kg

NH_y—EMEP 2008). The place is situated on a south-facing slope of Jaspe Peak, a calcareous elevation, altitude 270 m. According to the climatic normal (1971–2000) mean annual precipitation was 730 mm; mean maximum temperature, 27.8°C (August); and mean minimum temperature, 8.1°C (January). Reported data refer to the nearest climatic station (Setúbal, 15 km distance—Instituto Nacional de Meteorologia e Geofísica). The soil is skeletal (15 cm depth), such that true profiles cannot be discerned. Silt predominates in the soil (57%), while clay and sand contents are 28% and 15% (silt-sand-loam—Correia 1988). Soil pH determined along the experimental period varied between 6.6 and 7.9 for the evergreen sclerophylls species and between 5.6 and 6.8 for the summer semi-deciduous (Cruz et al. 2008). The vegetation is a mixed sclerophyll scrub (Eunis class F5.2—Mediterranean maquis), which developed after a fire 18 years before this study.

Field sampling

The study site was a homogeneous area (50 m×50 m) representative of the surrounding vegetation, soil composition, slope, etc. The area was divided into 575 cells of 4 m² each. The dominant plant species in each cell was identified and three plant species belonging to the two plant functional groups were selected so that they were abundant and representative of the plant community. The evergreen sclerophylls included *Olea europaea*, *Arbutus unedo* and *Quercus coccifera*; while the chosen summer semi-deciduous species included *Cistus albidus*, *Cistus salvifolius* and *Rosmarinus officinalis*. Five cells per plant species were identified and further studied. Soil and green leaves were sampled monthly from September 2007 to June (for some parameters until August) 2008. Under the canopy (facing south) of each of the studied plant species, soil water content was measured between 10:00 and 12:00 by time domain reflectometry (TDR) and soil samples (2 cm diameter and 15 cm depth) were removed, sieved (2 mm) and stored at 4°C until analysis. Soil samples were analysed for inorganic N forms and concentrations. Nitrate and ammonium were extracted from the soil using 2 M KCl (1 g soil dry weight to 10 ml of KCl). The concentration of nitrate (in the soil and leaf) was determined by electrophilic substitution of salicylate acid (Matsumura and Witjaksono 1999) while that of

ammonium was determined using a modified Berthelot reaction (Cruz and Martins-Loução 2000).

Leaf sampling occurred between 10:00 and 14:00. All the leaves collected were fully-expanded, belonging to the third or fourth pair of leaves (the youngest fully-expanded leaf pair was considered the first), or otherwise indicated, and facing south. Samples were immediately wrapped in aluminium foil and kept in liquid nitrogen until analysis. In the case of NRA determined along the shoot, leaves were collected from the first (youngest) to the seventh (oldest) pair. At the same time, the third and fourth pair of leaves of *Olea europaea* and *Cistus albidus* were collected through the day, every 2 h (sunrise was at 6:58 and sunset at 7:32). Sampling along the shoot and along the day only took place at the end of April, when the leaves of all species are active.

Determination of NRA

NRA was determined *in vivo* according to Hageman and Hucklesby (1971), with the modification given by Gebauer et al. (1984) and *in vitro* according to Kaiser et al. (2000). The *in vivo* NRA determination was carried out in two steps. In the first step, whole leaves were cleaned with distilled water and then 100 mg of fresh material was cut into small pieces (~ 0.5 cm). The pieces of leaves were incubated for 90 min at 35°C in the dark with 5 ml of incubation buffer after vacuum infiltration (5 min) and nitrogen bubbling (10–15 min). The incubation buffer consisted of 100 mM sodium phosphate buffer pH 7.5, 1% iso-propanol and 200 mM KNO₃. The second step consisted of quantifying the nitrite produced, colorimetrically (spectrophotometer Tecan Spectra Rainbow A-5082) at 540 nm by addition to 50 µl of the reaction medium, 125 µl of 5% sulphanilamide in 3 N HCl and 125 µl of 0.1% N-naphthyethylenediamine HCl solution. The *in vivo* NRA was expressed as nmol g⁻¹ FW h⁻¹.

To determine the *in vitro* NRA, the extraction was performed using an extraction buffer (50 mM Hepes-KOH—pH 7.6–0.5% PVP, 0.02% BSA, 0.02% casein, 20 mM DTT, 10 µM FAD, 50 µM leupeptin, 2 mM pepablock, 10 mM MgCl₂) in the proportion of 1 g FW of plant material to 2 ml of extraction buffer. Three (out of the five) leaf samples were analysed for NRA. The recovered extracts were centrifuged at 5000 g, for 10 min at 4°C. The resulting crude extracts (650 µl) were desalinated using 5 mL

columns filled with Sephadex (G-25-150), centrifuged at 5000 g for 45 s, at 4°C. The desalinated extracts were then used to determine the *in vitro* NRA. The activity of NR is modulated by phosphorylation. In the presence of divalent cations phospho-NR forms a catalytically inactive complex by binding to a 14-3-3 protein. If cations are chelated by EDTA, NR becomes fully active (Kaiser et al. 2000). Therefore, to determine the potential activity (Pot), one aliquot of extract was pre-incubated for 15 min with a mixture of EDTA/AMP/PO₃²⁻. The reaction was initiated by addition of the substrates KNO₃ and NADH. The physiological reaction (Phys) was determined under limited concentrations of NADH and NO₃⁻ and the presence of divalent cations (Mg²⁺). Both reactions were stopped by addition of 125 µl (10 mM) zinc acetate. The produced nitrite was then quantified as for *in vivo* NRA. The *in vitro* NRA was expressed as µmol g⁻¹ FW h⁻¹. The enzyme activation state was determined as the ratio between the potential and the physiological rates. Although the NRA determined in the summer semi-deciduous species did not change, samples from the same plant species grown in the absence of ammonium showed increased NRA (data not shown). Thus, any absence of changes in summer semi-deciduous NRA would not result from inadequacy of the applied protocol.

Statistics

The repeated measures test (General Linear Model) was applied to assess the existence of significant interactions between time (month or hour) and plant species and PFG, for soil and plant parameters. The two-way ANOVA was applied to assess the existence of significant interactions between leaf pair number and PFG. In all cases there were significant interactions between the factors so that differences between PFG were examined for each sampling time and leaf pair number. Summary statistics of soil and plant parameters were compared (two-sided *t*-test, $p < 0.05$) for the different sample periods (and leaf pair number), and for PFG. Linear correlations between the soil nitrate and water availability, and plant parameters were also studied (Pearson's correlations). In all cases, preliminary analyses were performed to ensure there was no violation of the assumptions regarding the tests' application. SPSS software, version 19.0, was used for all tests.

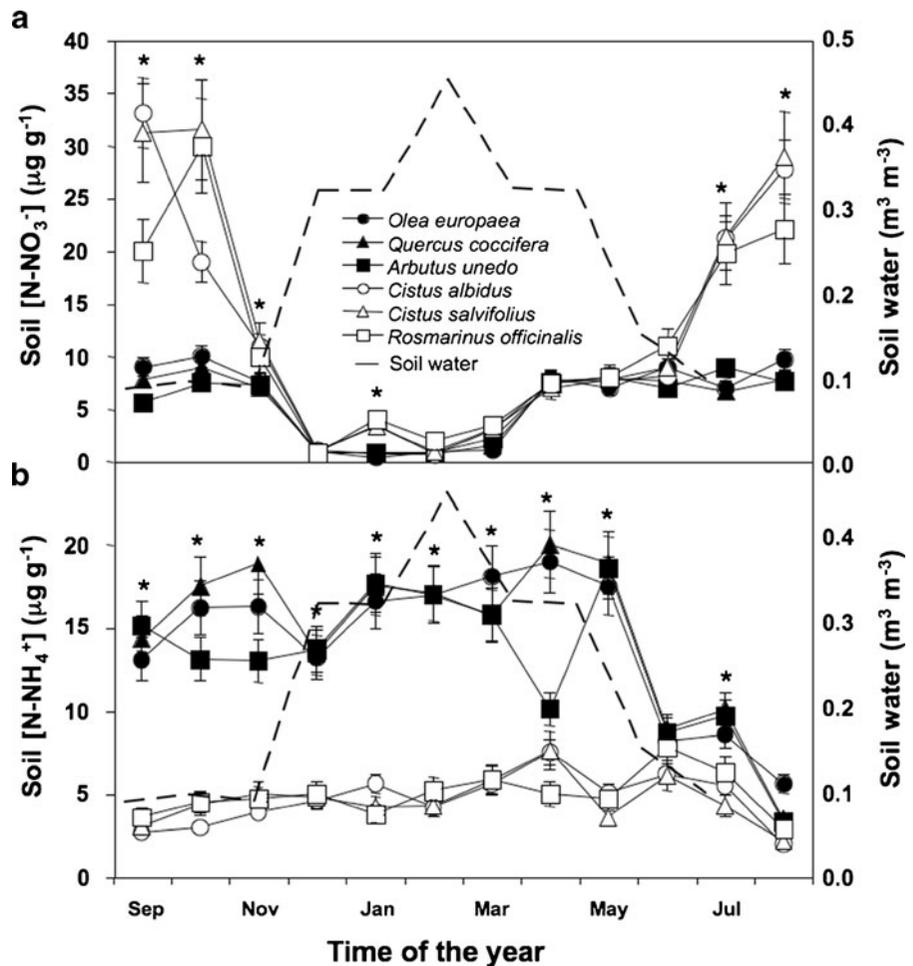
Results

The patterns of nitrate and ammonium availabilities along the year in the soil under the canopy of several plant species were quite distinctive of the two PFGs: summer semi-deciduous (*Cistus albidus*, *Cistus salvifolius* and *Rosmarinus officinalis*) and evergreen sclerophylls (*Olea europaea*, *Quercus coccifera* and *Arbutus unedo*—Fig. 1). The soil nitrate concentration under the canopy of evergreen sclerophylls species was low throughout the year ($< 10 \mu\text{g g}^{-1}$ —Fig. 1a). Under summer semi-deciduous species the soil ammonium concentration was low and constant along the year ($\sim 5 \mu\text{g g}^{-1}$ —Fig. 1b). During the winter and spring months, when water availability was higher, soil nitrate concentrations determined under the canopy of the studied species decreased to minimum levels. In the summer and autumn months, when water availability was lower, the soil patches occupied by the summer semi-deciduous species displayed higher nitrate concentrations than those determined under the canopies of the evergreen sclerophylls. In contrast, the ammonium concentrations determined in the soil patches occupied by summer semi-deciduous species were lower than those determined under the canopy of evergreen sclerophylls. Therefore, plant species belonging to the two PFG were associated with distinct soil nitrogenous environments.

The nitrate concentration in the leaves of summer semi-deciduous species was significantly higher than in evergreen sclerophylls except during the winter months when it reached minimum concentrations in both PFG (Fig. 2), thus resembling the temporal pattern of the soil nitrate concentration (Fig. 1a). Correlations between soil nitrate and leaf nitrate concentrations and between the availability of water and leaf nitrate concentration were stronger for summer semi-deciduous species than for evergreen sclerophylls (Table 1).

Irrespective of the plant species and the time of the year, leaf *in vivo* NRA was lower than *in vitro* NRA. Also, leaf NRA determined in summer semi-deciduous species was lower than in evergreen sclerophylls (Figs. 2b and 3). Summer semi-deciduous species displayed low and constant *in vivo* (Fig. 2b) and *in vitro* NRA (potential and physiological—Fig. 3a, b) throughout the year, while evergreen sclerophyllous exhibited differences: lower activities

Fig. 1 Relation between the plant species belonging to the two PFG (summer semi-deciduous—open symbols; and evergreen sclerophylls—filled symbols) and the concentration of nitrate (a) and ammonium (b) determined along the experimental period under each plant's canopy. There were significant interactions between PFG and time ($p < 0.05$). * refers to statistically significant differences between PFG (t -test $p < 0.05$). Symbols represent the mean ($n = 3$ plants per species) \pm SD

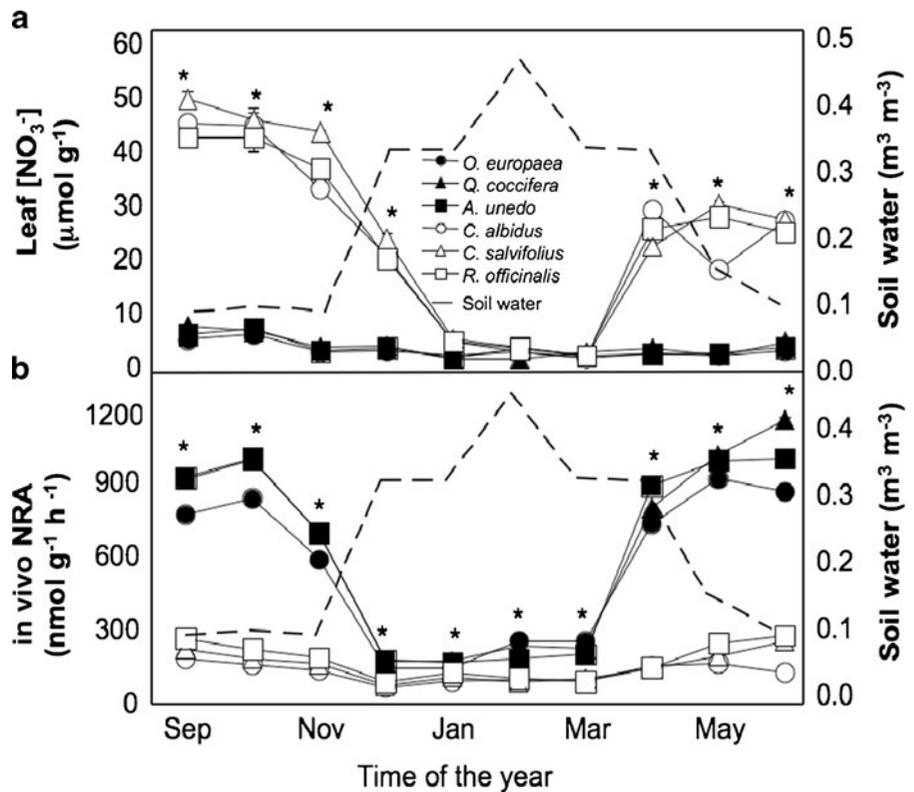


from November to April and higher activities during the rest of the year. Irrespective of the plant species, the patterns of in vivo NRA and in vitro NRA determined under non-limiting conditions (Potential activity—Fig. 3a) and those determined under limited reducing power conditions (Physiological activity—Fig. 3b) were very similar throughout the year and therefore there were no significant differences in the activation state of the NR (ratio between the physiological and the maximum activities—Fig. 3c). The patterns of the in vivo NRA (Table 2) and of the in vitro NRA determined along the year differed significantly according to the PFG.

The correlations between the soil nitrate and water availabilities, and plant parameters were assessed (Table 1). For the evergreen sclerophylls species, the strongest correlation was between soil nitrate and in vivo NRA. Therefore, expressing the in vivo NRA as a function of soil nitrate and ammonium concentra-

tions separated the studied plant species according to their PFG and also evidenced two clusters of data points from the evergreen sclerophylls: one cluster comprised the measurements made in winter/spring (closer to zero) and the other, in summer/autumn (higher values of NRA and soil nitrate concentrations—Fig. 4). The correlation (Table 1) between the in vivo NRA of the evergreen sclerophylls species and soil nitrate concentration and the significant effect of soil nitrate concentration on the in vivo NRA of the evergreen sclerophylls species (Table 2), points to a stimulation of the NRA by increasing soil nitrate availability. By contrast, and even though the range of soil nitrate concentration under the canopy of summer semi-deciduous species was greater than for the evergreen sclerophylls species, the stimulation of the NRA of the former was smaller (Fig. 4a and Table 1). Leaf in vivo NRA of summer semi deciduous species was significantly influenced by the interaction

Fig. 2 Leaf nitrate concentration (a) and in vivo NRA of the studied plant species belonging to the two PFG (summer semi-deciduous—open symbols; and evergreen sclerophylls—filled symbols) along the experimental period. There were significant interactions between PFG and time ($p < 0.05$). * refers to statistically significant differences between PFG (t -test $p < 0.05$). Symbols represent the mean ($n = 3$ plants per species) \pm SD



between sampling time (month) and the soil nitrate availability (Table 2).

A negative correlation was observed between the availability of water and nitrate concentration in the

leaves and NRA for all the studied plant species. This correlation was stronger for the summer semi-deciduous species than for the evergreen sclerophylls (Table 1).

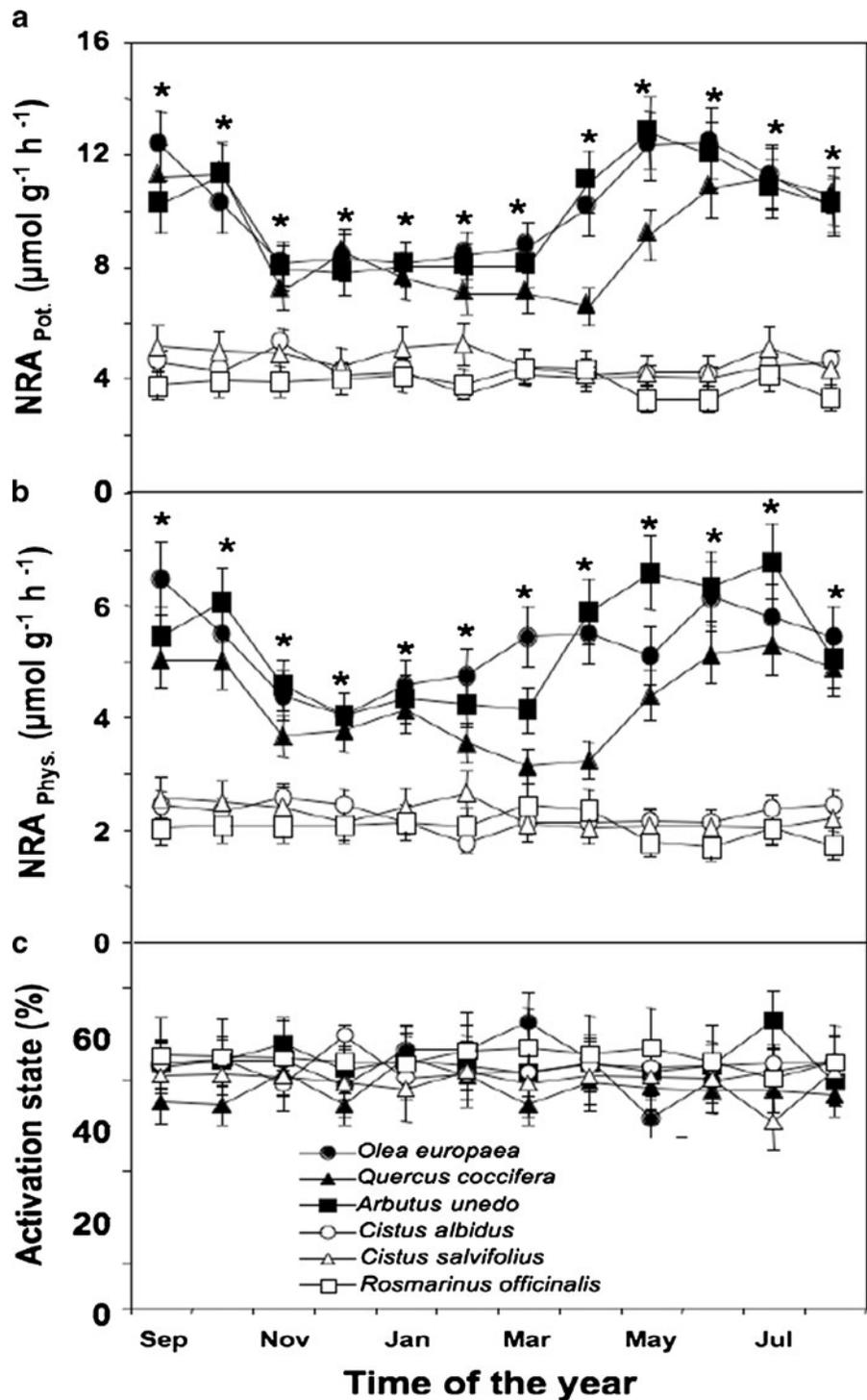
Table 1 Pearson's correlation coefficients between soil nitrate and water availability, and plant parameters during the year according to the studied plant species

PFG	Plant species		Leaf Nitrate	NRA in vivo	NRA Potential	NRA Physiological
	<i>O. europaea</i>	Soil nitrate	0.45 ^a	0.98 ^b	0.72 ^b	0.42 ^a
		Soil water	-.056 ^b	-.077 ^b	-.050 ^b	-.040 ^a
	<i>Q. coccifera</i>	Soil nitrate	0.48 ^b	0.95 ^b	0.56 ^b	0.46 ^b
		Soil water	-.0670 ^b	-.081 ^b	-.075 ^b	-.065 ^b
	<i>A. unedo</i>	Soil nitrate	0.13	0.90 ^b	0.85 ^b	0.89 ^b
		Soil water	-.050 ^b	-.080 ^b	-.059 ^b	-.053 ^b
	<i>C. albidus</i>	Soil nitrate	0.49 ^b	0.56 ^b	0.03	0.29
		Soil water	-.079 ^b	-.069 ^b	-.036 ^a	-.064 ^b
	<i>C. salvifolius</i>	Soil nitrate	0.83 ^b	0.69 ^b	0.29	0.147
		Soil water	-.087 ^b	-.086 ^b	-0.08	0.069
	<i>R. officinalis</i>	Soil nitrate	0.84 ^b	0.73 ^b	-.051 ^b	-0.22
		Soil water	-.085 ^b	-.088 ^b	0.63 ^b	0.38 ^a

^a Correlation is significant at the 0.05 level (2-tailed)

^b Correlation is significant at the 0.01 level (2-tailed)

Fig. 3 Temporal pattern of NRA_{Pot} (a), NRA_{Phys} (b) and the NR activation state (c) of the plant species belonging to the two PFG (summer semi-deciduous—open symbols; and ever-green sclerophylls—filled symbols). There were significant interactions between PFG and time ($p < 0.05$). * refers to statistically significant differences between PFG (t -test $p < 0.05$). Symbols represent the mean ($n=3$ plants per species) \pm SD



Since the potential for NRA changes with leaf age (Gratani and Bombelli 2000), we assessed which leaves could potentially contribute to nitrate reduction. Again, and based on the pattern of the in vitro

NRA along the leaf pairs, the studied plant species were separated according to their PFG (Fig. 5). The in vitro NRA determined on the youngest and second leaf pairs were very low for all the studied plant

Table 2 Statistical analyses on leaf in vivo NRA for the studied plant species (all and according to their PFG—evergreen sclerophylls and summer semi-deciduous) of the time of sampling (month), soil nitrate concentration and PFG

Variable	All studied species			Leaf in vivo NRA Evergreen sclerophylls			Summer semi-deciduous		
	df	F	P	df	F	P	df	F	P
Month	8	30.5	<0.001	8	29.7	<0.001	8	1.4	0.27
Soil [NO ₃ ⁻]	75	–	–	45	2.9	<0.001	45	0.6	0.91
Month × Soil [NO ₃ ⁻]	38	0.2	0.99	20	0.3	0.99	20	3.0	0.02
PFG	1	300.3	<0.001						
PFG × Month	2	1.5	0.40						
PFG × Soil [NO ₃ ⁻]	7	0.8	0.68						
PFG × Month × Soil [NO ₃ ⁻]	2	2.8	0.08						

A repeated-measures ANOVA ($p < 0.05$ are shown in bold type) was conducted for leaf in vivo NRA

species. However, the in vitro NRA determined on the third and fourth leaf pairs of the evergreen sclerophylls were approximately twice the NRA measured in the leaves of the summer deciduous species. Of the remaining analysed leaf pairs, the in vitro NRA of the summer semi-deciduous species were significantly higher than that of the evergreen sclerophylls.

Although it was not possible to follow the diurnal patterns of in vitro NRA in all the studied plant species, it differed between *Cistus albidus* (summer semi-deciduous) and *Olea europaea* (evergreen sclerophylls—Fig. 6). At the beginning (6–10 am) and the end (5–7 pm) of the light period, the in vitro NRA of *C. albidus* was significantly higher than that of *O. europaea*. However, during the middle of the light period (11 am to 3 pm) the in vitro NRA of *O. europaea* was significantly higher than that of *C. albidus*.

Based on the analysed parameters, two patterns of nitrate reduction were evident: one shown by *O. europaea*, *Q. coccifera* and *A. unedo*, the three studied evergreen sclerophylls species; and the other by *C. albidus*, *C. salvifolius* and *R. officinalis*, the three summer semi-deciduous species.

Discussion

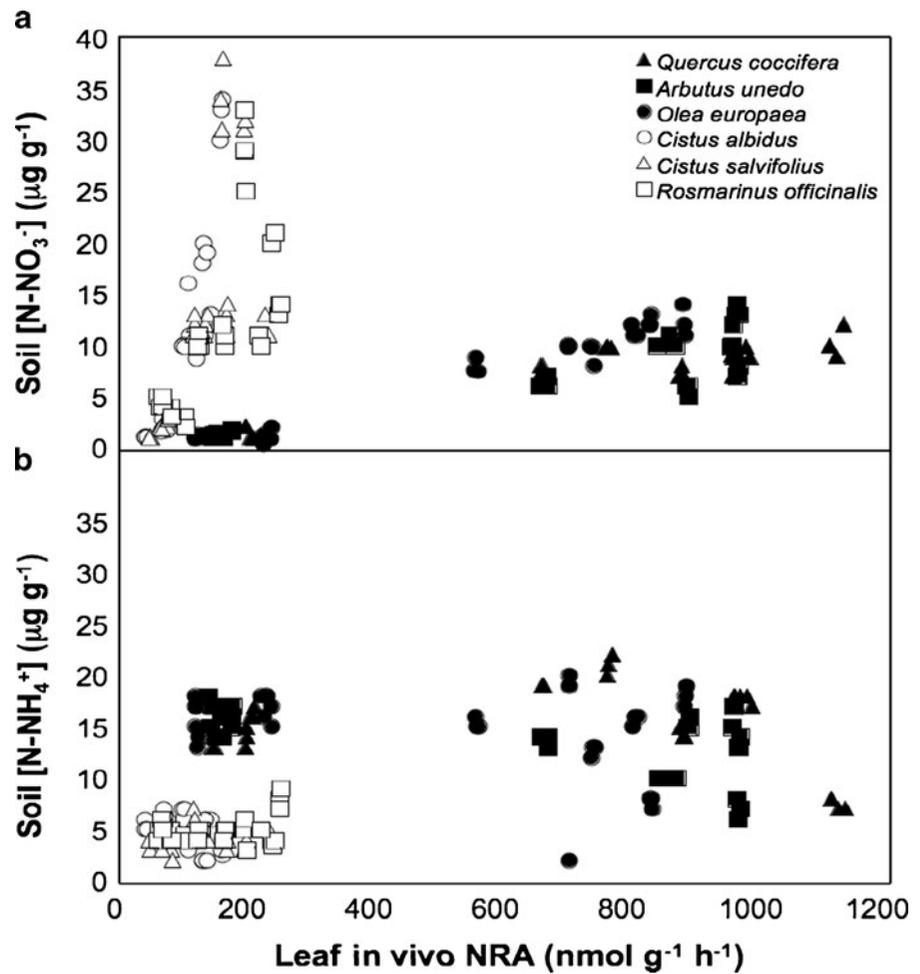
Soil heterogeneity at the scale of plant species has been described for Mediterranean soils (Gallardo et al. 2006; Rutigliano et al. 2009). Moreover, Cruz et al. (2008) showed that the two main Mediterranean plant functional groups (PFG), summer semi-deciduous and

evergreen sclerophylls, significantly affected soil superficial characteristics (e.g. soil pH, organic matter, nitrification potential, etc.) in distinct ways. Accordingly, concentrations of nitrate and ammonium in the soil through the year were in the range found in other Mediterranean ecosystems (Gallardo et al. 2006; Cruz et al. 2008), and clearly reflected the two PFG co-existing at the site: summer semi-deciduous—*Cistus albidus*, *Cistus salvifolius* and *Rosmarinus officinalis*; and evergreen sclerophylls—*Olea europaea*, *Quercus coccifera* and *Arbutus unedo*, i.e. the two main PFG in Mediterranean ecosystems, have distinct soil nitrogenous environments (Fig. 1). This may be related to group-specific resource requirements and the use of distinct soil nitrogen pools, decreasing the competition between co-existing groups for limiting nutrients (Kahmen et al. 2006).

The relative abundance of soil nitrate and ammonium depends on the balance between production and consumption. When nitrate is not directly added to the soil (by fertilization or deposition), nitrification is the main process of soil nitrate production. It has been reported that plant species from later successional phases (e.g. evergreen sclerophylls) inhibit soil nitrification (Cruz et al. 2008), which could at least partly explain why nitrate concentrations under the canopies of those species were lower than those of ammonium (from November to May—Fig. 1a).

The temporal pattern of nitrate concentration in leaves of both PFG (Fig. 2) followed that of the soil (Fig. 1a) especially in the summer semi-deciduous species (Table 1), showing that when nitrate was more abundant in the soil (September and October and July

Fig. 4 Relation between all the determinations of leaf in vivo NRA and the respective concentrations of soil nitrate (a) and ammonium (b) along the experimental time and according to the plant species (summer semi-deciduous—open symbols; and evergreen sclerophylls—filled symbols; see Table 1 for correlations). Symbols represent the individual values ($n=3$ measurements \times 9 sampling times per species)

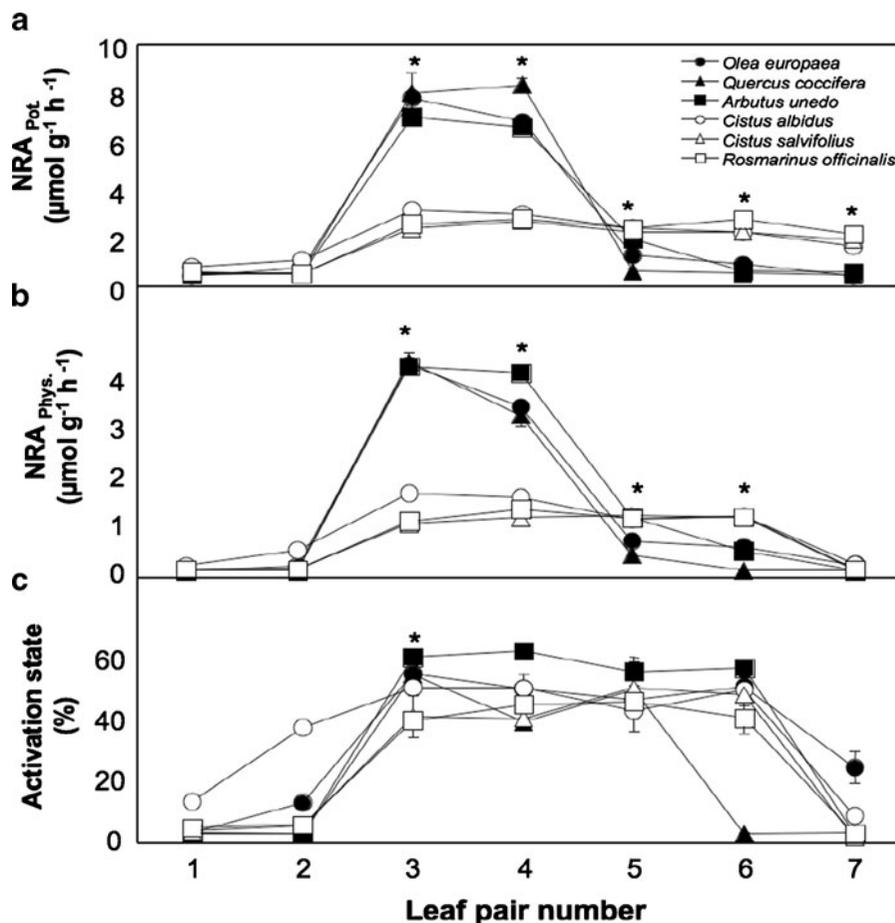


and August), it could be detected in the shoot. Thus, leaf nitrate concentration (Fig. 2) may be an indicator of soil nitrate availability (Fig. 1a). In general, within this time period and irrespective of the PFG, there was an inverse relation between the concentration of nitrate (in the soil and in the leaves) and the water availability (Table 1 and Figs. 1a and 2a). This inverse relation may result from nitrate losses (e.g. leaching and/or runoff) or uptake by the biota (soil microbial community and vegetation).

As not many data are available for in vitro leaf NRA determined under field conditions, in vivo NRA was also determined (Fig. 2b). All the studied plant species displayed in vivo leaf NRA (Fig. 3a) comparable with a wide range of Mediterranean geophytes (Arslan and Güleriyüz 2005) and shrub species (Arslan et al. 2009), but lower than those of plant species characteristic of other calcareous habitats (*Poterium sanguisorba* and *Scabiosa columbaria*—

Havill et al. 1974). This may be due to local nutrient availability (Fitter and Hay 2002) and/or the relative growth rates experienced by these plant species in their habitats (Poorter et al. 1995; Craine 2009). However, in vivo NRA is mainly limited by the availability of nitrate and reducing power (Kaiser et al. 2000), so that although they followed the same temporal patterns, in vitro NRA (Fig. 3) was higher than in vivo NRA (Fig. 2a). Given that, in higher plants, NRA is rapidly modulated by environmental conditions (Kaiser and Spill 1991, Kaiser and Huber 2001), NRA is expected to change along the year. This temporal response of NRA to environmental conditions was observed in evergreen sclerophylls but not in summer semi-deciduous species, which indicates that the temporal pattern of NRA also differentiated between summer semi-deciduous and evergreen sclerophyllous species (Table 2—Karavatas and Manetas 1999).

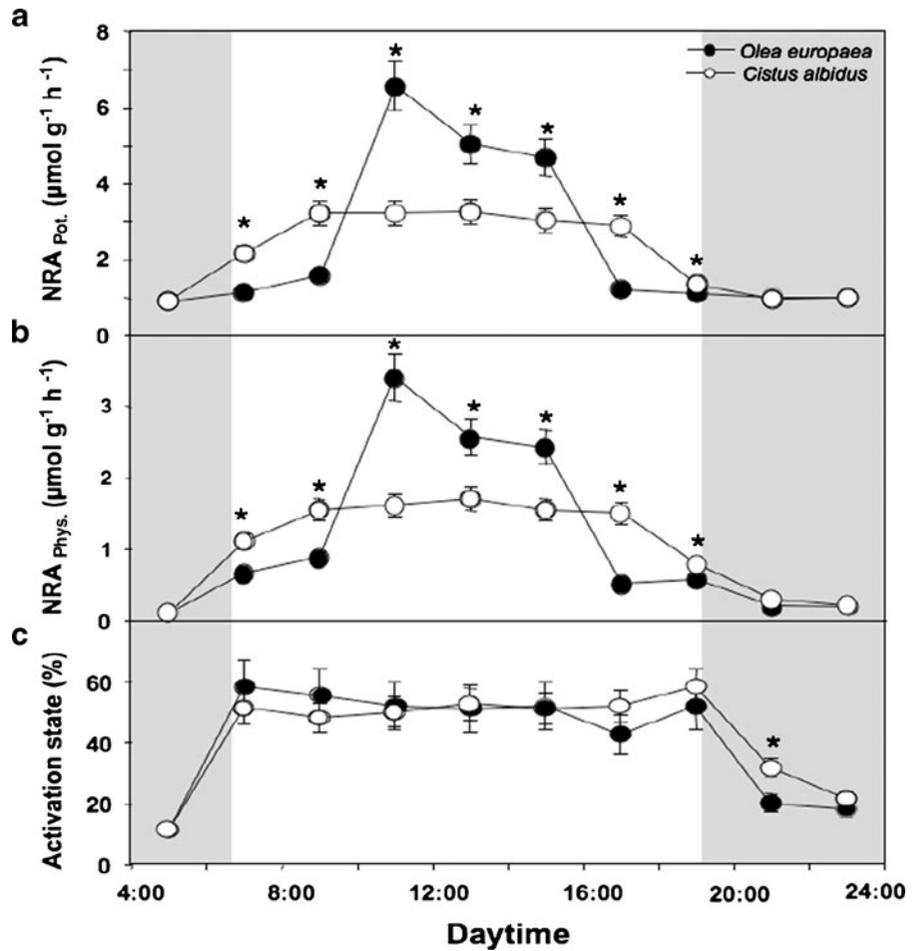
Fig. 5 Comparison between the in vitro NRA_{Pot} (a), NRA_{Phys} (b) and the NR activation state (c) along the branches of the plant species belonging to the two PFG (summer semi-deciduous—open symbols; and evergreen sclerophylls—filled symbols). There were significant interactions between PFG and time ($p < 0.05$). * refers to statistically significant differences between PFG (t -test $p < 0.05$). Symbols represent the mean ($n = 3$ plants per species) \pm SD



Since NR is a substrate-inducible enzyme (Kaiser et al. 2000, 2001; Arslan and Güleriyüz 2005), the NRA of a plant has been assumed to reflect the long-term nitrate supply to the plant so that in ecological studies NRA can indicate nitrate availability (Lee and Stewart 1978; Arslan and Güleriyüz 2005; Sakar et al. 2010). This was the case for the evergreen sclerophylls species, but not for the summer semi-deciduous species (Tables 1 and 2). Surprisingly, even though summer semi-deciduous species occupied nitrate-rich soil patches and had more nitrate in the leaves than the evergreen sclerophylls (Fig. 1a), in vivo and in vitro NRA determined in the former was lower than in the latter (Figs. 2b and 3). In fact, the NRA of summer semi-deciduous species responded less to increases in nitrate availability than the evergreen sclerophylls (Tables 1 and 2 and Figs. 1a and 4). However, as NR can facilitate the transduction of many environmental stimuli into metabolic activity (Kaiser and Spill 1991, Kaiser and Huber 2001,

Kaiser et al. 2002), it is possible that besides the stimulation of NRA by nitrate (Figs. 1a and 2a), there was, in the summer semi-deciduous species, a predominant inhibitory signal. The fact that nitrate accumulated in the leaves of summer semi-deciduous species (Fig. 2a) and that ammonium concentrations in the soil under the canopy of these species were below $8 \mu\text{g g}^{-1}$ (Figs. 1b and 4b) suggest that ammonium can be an inhibitory signal. Ammonium can affect the metabolism of nitrate because most steps in plants' nitrate assimilatory pathway are nitrate-inducible but ammonium, or its metabolic products, can inhibit the reduction of nitrate (Orebamjo and Stewart 1975; Oaks et al. 1977; Emmett 2007) through inhibition of NR synthesis. This hypothesis should be further assessed as a potential mechanism contributing to the natural replacement of summer semi-deciduous species by evergreen sclerophylls during plant succession (Werner et al. 1999, 2001). Non-exclusively, the two PFG also differ in rooting

Fig. 6 Diurnal pattern of the in vitro NRA_{Pot} (a), NRA_{Phys} (b) and the NR activation state (c) according to the plant species: *Cistus albidus* (summer semi-deciduous) and *Olea europaea* (evergreen sclerophylls). Sunrise occurred at 7 am and sunset at 7 pm. There were significant interactions between PFG and time ($p < 0.05$). * refers to statistically significant differences between PFG (t -test $p < 0.05$). Symbols represent the mean ($n=3$ plants per species) \pm SD



depths; summer semi-deciduous tend to have a superficial root system while evergreen sclerophylls have a more complex root system with both superficial and deeper roots (Correia 1988; Canadell et al. 1996). Although a small fraction of root biomass might be found at depths below 1 m, the functional significance of those roots is important for ecosystem water and carbon fluxes and nutrient cycling (Canadell et al. 1996). Deep roots improve water uptake and increase the probability of survival in Mediterranean communities (Lloret et al. 1999). Also, in the Brazilian Cerrado, deep roots have also been shown to access nitrate that had been leached (Canadell et al. 1996). Thus, the deeper root system of evergreen sclerophylls may enable them 'escape' the summer drought. On the contrary and given that higher nitrate availability coincided with lower water availability (Fig. 1a), summer semi-deciduous may not have been able to reduce the nitrate due to their shallow root system.

The pattern of NRA along the twig was different for the two PFG (Fig. 5), which may be related to their phenology, i.e., the life span of the summer semi-deciduous leaves is less than 1 year while that of evergreen sclerophylls is 1 to 2 years (Correia 1988; Oliveira and Peñuelas 2004). This corresponds to a substantial carbon cost in nitrate assimilation so that NRA along the twig needs to be linked to the metabolic activity of the leaves, in particular the carbon balance (Foyer et al. 1998) and availability of reducing power. The greater importance of the third and fourth pairs of leaves relative to the others in evergreen sclerophylls species may result from a combination of factors and is in agreement with the slow relative growth rate of these species (Chapin et al. 2002). Summer semi-deciduous species displayed lower NRA but the relative contribution of each pair of leaves to the nitrate reduction was more uniform, suggesting a more homogeneous contribution of all

the leaves to the plant metabolism, which is characteristic of plants from the initial stages of succession and with higher relative growth rates.

The NRA of summer semi-deciduous and evergreen sclerophylls species also differed throughout the day (Fig. 6). The diurnal pattern of NRA may result from the complex mechanisms regulating the activity of the NR and its interactions with other enzymes and metabolites such as soluble sugars, amino acids, malate and nitrate concentrations in the roots and in the xylem and phloem (Matt et al. 2001b). The differences in the patterns of diurnal activities will have drastic consequences on the partitioning of the newly assimilated carbon (Matt et al. 2001a; Cruz et al. 2003).

Conclusions

Altogether, the experimental data are in agreement with other preliminary studies made in the same area (Cruz et al. 2008), which together suggest that each of the PFG is composed of a number of physiologically similar plant species, as suggested by Kummerow (1973). Therefore, and given that phenology and rooting depth of the plant species appeared to have influenced NRA, the use of NRA as an indicator of the nitrate availability has to take into consideration the time of the year, the plant species and its PFG.

In the study area, evergreen sclerophylls and summer semi-deciduous co-exist. However, in later phases of succession, evergreen sclerophylls tend to dominate to the detriment of the summer semi-deciduous species. This may be accompanied by a higher occupation of the soil space by roots of evergreen sclerophylls, with the concomitant decrease of nitrate and increase of ammonium concentrations in the soil (Chapin et al. 2002). It is possible that in earlier phases of the ecological succession, when summer semi-deciduous species dominate, their leaf NRA could be a more robust indicator of the nitrate supply (Lee and Stewart 1978; Arslan and Güleriyüz 2005; Sakar et al. 2010).

Based on the estimates of increased nitrogen deposition for the Mediterranean Basin (Galloway et al. 2004; Phoenix et al. 2006), it is possible that the ecological nitrogen niche occupied by summer semi-deciduous species will become narrower, with the concomitant lengthening of that of evergreen sclero-

phylls. However, more research is needed to understand how the Mediterranean maquis will respond to increased nitrogen deposition, namely to different forms and doses.

Finally, the observed differences between plant species belonging to the two PFG may be the consequence of evolutionary trade-offs, and represent specializations of the endemic species to increase their chances of getting access to nitrogen, so that different and complementary nitrogen strategies can contribute to the benefits of PFG diversity on ecosystem functioning (Kahmen et al. 2006).

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