

SPECIES, SITE AND SEASONAL VARIATION IN LEAF-CHEMISTRY DIVERSITY OF WOODY MEDITERRANEAN PLANTS

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RÉSUMÉ. — *Variation selon l'espèce, le site et la saison de la diversité chimique foliaire de plantes ligneuses méditerranéennes.* — La présente étude analyse, dans les conditions du terrain, la diversité biochimique de 10 plantes ligneuses importantes, appartenant à 7 familles, du sud-est de l'Espagne. Sont décrites les différences entre espèces et entre sites (deux chaînes de montagne), de même que la variabilité saisonnière, de trois caractéristiques chimiques liées à la valeur nutritive des plantes (teneur en azote, phénols et tannins). Les résultats révèlent que les caractéristiques chimiques des plantes étudiées diffèrent selon l'espèce, la saison et le site. Cette grande variabilité chimique suggère que les stratégies propres aux espèces peuvent être autant sinon plus importantes que les réponses présumées découlant des théories habituelles pour caractériser la qualité chimique des plantes en tant qu'aliment. La végétation montagnarde méditerranéenne est hautement variable en ce qui concerne la distribution temporelle et spatiale des caractéristiques de la qualité nutritionnelle foliaire, en raison à la fois du processus interne de maturation des tissus, de la variation environnementale et des différences spécifiques. Toutes ces sources de variabilité créent un paysage chimique divers et changeant dans lequel les herbivores doivent sélectionner leur régime alimentaire, en fonction du temps et du site.

SUMMARY. — This study analyses the biochemical diversity, under field conditions, of 10 important woody-plant species of south-eastern Spain, belonging to 7 families. We describe differences between species and between sites (two mountain ranges), as well as the seasonal variability of three chemical characteristics related to plant nutritional value (nitrogen content, phenols and tannins). Our results reveal that the plants studied differed in their chemical characteristics between species, between times, and between sites. This high variability in the plant chemical features suggests that the species-specific strategies can be as much or even more important to characterize the chemical plant quality as food than the presumed responses depending on the current general theories. Mediterranean mountain vegetation is highly variable in the temporal and spatial distribution of leaf-quality characteristics, due both to the internal process of tissues maturation as well as to environmental variation and specific differences. All these sources of variability create a diverse and changing chemical landscape in which herbivores have to select their diet, not only between species, but also between different times and sites.

Plants are in general a poor-quality food resource, not only because they are low in nutrients and produce secondary defense compounds, but also because the temporal and spatial variability in their chemical characteristics makes them an unpredictable source of nutrients (Hartley & Jones, 1997). This condition highly determines the plant-herbivore interactions, since herbivores have to select how much, what, when and where to eat (Feeny, 1970). The

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variations in the nutritional characteristics of plants can be provoked by internal processes related to the plant growth, development, and reproduction, as well as to responses to environmental heterogeneity (Hartley & Jones, 1997). Many works have documented these patterns in nature. For instance, seasonal variability of plant chemicals is well known for diverse species (Dement & Mooney, 1974 ; Schultz *et al.*, 1982 ; Lebreton *et al.*, 1993). In other studies, plant quality and the accumulation of defense compounds have been related to canopy retention (Coley & Aide, 1991 ; Watson & Owen-Smith, 2002 ; Scogins *et al.*, 2004). Studies have also analysed the variations in plants traits and their effects in herbivores, between sites with different characteristics (e.g. productivity : Danell *et al.*, 1997 ; Turner, 1998).

According to these known and documented patterns of variation in nature, during the last 30 years several theories have tried to explain the grounds for these variations in plant chemical characteristics, and how these variations fit the probability of damage by herbivores. With respect to woody plants, this fit has usually been explained by the resource-driven hypothesis of plant defence (the carbon/nutrient balance (CNB) hypothesis, Bryant *et al.*, 1983 ; the growth/differentiation balance (GDB) hypothesis, Herms & Mattson, 1992). Following these theories, the synthesis of defence compounds is constrained by the resources availability (characteristics of the site) and the internal trade-offs in resource allocation between growth and defence (characteristics of the plant). Thus, a plant that grows quickly needs all its resources for growth, and cannot accumulate carbon-rich chemical defences against herbivores. This general framework can apply to many different situations : deciduous plants (which usually have faster growth rates) would have less defensive compounds and more nutrients than evergreen ones (e.g. Bryant *et al.*, 1988 ; Coley & Aide, 1991 ; Lebreton & Gallet, 2007) ; the same plant growing in sites with different nutrient availability would change their growth rate and accumulation of defensive compounds (Coley *et al.*, 1985) ; or plants during growth flushes would be less or more defended than the same plants once leaves have aged (Herms & Mattson, 1992).

There is a vast amount of information in this respect with plants from higher latitudes (e.g. *Betula* spp., Danell *et al.*, 1997 ; Lempa *et al.*, 2001 ; Riipi *et al.*, 2002). However, little is known regarding the extent of this pattern to Mediterranean-zone plants (but see Dement & Mooney, 1974 ; Glyphis & Puttick, 1988 ; Perevolotsky, 1994 ; Lebreton *et al.*, 1997 ; Baraza *et al.*, 2009). This is surprising, since Mediterranean basin has rich woody-plant diversity, a marked seasonal cycle constrained by a summer drought, and a high variability of habitats (Blondel & Aronson, 1999). Thus, Mediterranean vegetation includes all the sources of variability that can change plant quality. Furthermore, Mediterranean basin has been subject of high herbivore pressures for millennia, both from livestock and wild ungulates (Papanastasis & Peter, 1997). Although information is continuously growing (Perevolotsky, 1994 ; Massei & Hartley, 2000 ; Massei *et al.*, 2000 ; Baraza *et al.*, 2004 ; Lebreton & Gallet, 2007), before a general understanding of the nature, distribution, and impact of leaf-quality traits on herbivory will be possible in the Mediterranean area, a fuller knowledge of the temporal and spatial variation in plant quality of different species that share the same habitat (and the same herbivores) is necessary.

Hence, the objective of this study is the analysis, under field conditions, of the biochemical diversity of some of the most important Mediterranean woody species of south-eastern Spain. We examine between-species and between-mountain differences, as well as seasonal variability of three chemical characteristics related to plant nutritional value. Biochemical description of these Mediterranean species not only give more necessary information concerning the extent of secondary compounds in plants, but also can help us understand herbivory dynamics in these areas. Specifically, the questions we seek to answer are :

(1) How important are the differences in chemical characteristics between plant species that share the same habitat ?

(2) Are there differences between evergreen and deciduous species ?

(3) Do nitrogen concentrations decrease and chemical-defence compounds increase as leaves get older ?

(4) Are the chemical characteristics of the species consistent between different localities ?

MATERIAL AND METHODS

SPECIES AND AREAS

The study was carried out in the Mediterranean forests of two mountain ranges of the south-eastern Spain. Both mountains are protected areas, the National Park of Sierra Nevada and the Natural Park of Sierra de Huétor, with well-preserved vegetation. The site at Sierra Nevada (30SVG5505, 1850 m a.s.l.) is situated in a forest edge with sparse trees of Scots pine and Maple, thorny shrubs understory and open areas with grasses. In Sierra de Huétor (30SVG6123, 1500 m a.s.l.), the site is a sparse Holm oak (*Quercus ilex*) forest with an understory of Juniper (*Juniperus oxycedrus*) and *Rhamnus myrtifolius*, and deciduous shrubs located at ravines. In both areas, we selected the main tree and shrub species, according to size, coverage and varying degrees of herbivore damage (Baraza, 2004), expecting to find differences in secondary compounds concentration. The main ungulate herbivore in both areas is Spanish ibex (*Capra pyrenaica*), although herds of domestic goat (*Capra hircus*) and sheep (*Ovis aries*) are also common, and in Sierra Nevada the presence of free-ranging cow (*Bos taurus*) and horse (*Equus caballus*) is occasional.

The study plants included species of different biotypes : a deciduous tree, the Maple (*Acer opalus* subsp. *granatense*, Aceraceae) ; two evergreen trees, i.e. a broadleaf, Holm oak (*Quercus ilex*, Fagaceae) and a conifer, Scots pine (*Pinus sylvestris* var. *nevadensis*, Pinaceae) ; a deciduous shrub, Serviceberry (*Amelanchier ovalis*, Rosaceae) ; an evergreen shrub, Juniper (*Juniperus oxycedrus*, Cupressaceae) ; a leafless shrub, Piorno (*Genista versicolor*, Fabaceae) ; three deciduous thorny shrubs, Hawthorn (*Crataegus monogyna*, Rosaceae), Sloe (*Prunus ramburii*, Rosaceae), and *Rhamnus saxatilis* (Rhamnaceae) ; and a evergreen scrub, *Rhamnus myrtifolius* (Rhamnaceae). Some of these species are widespread in the Mediterranean area, being important components of Mediterranean forest while others are endemic of Penibetic Mountains, representing major elements of the forest in these areas. Overall, 10 species belonging to 7 families were analysed, 6 in each mountain range. Although all the species are present in both mountain ranges, the local abundances of the species differ widely, for which only two species, Serviceberry and Hawthorn, were selected for sampling at the two sites.

SAMPLING PROTOCOL

Samples were taken during the vegetative period, which in our study areas spans from middle May, with the bud burst, to the end of September, when leaves of deciduous species start to change colour, indicating the onset of senescence. From June to September of 2003, when all studied species present mature and not senescent leaves, current-year leaves of several twigs were monthly taken from five plants of each species. In all the cases, samples were collected each time from a different plant part in order to minimize the possible induced responses to the damage inflicted by the previous collection (Karban & Balwin, 1997 ; Orians & Jones, 2001). In the case of trees and bushes all samples were taken around 0.5-1.5 m in height, the usual feeding height for both wild and domestic goat, while for smaller scrubs samples were taken at the surface.

Samples were dried to constant weight in a stove at 45° C (at low temperatures, < 40 °C, enzymes may still function, leading to oxidation, whereas, at temperatures above 60 °C, heat damage and polymerization may occur ; Reed, 1995), ground to a particle size of 1 mm, and then stored for chemical analysis.

CHEMICAL ANALYSIS

Many chemical characteristics are related with plant nutritional value and herbivore intake (Danell & Bergström, 2002). Thus, the following variables were chosen, not only because they showed an effect on herbivores diet selection, but also for their simplicity, repeatability, and widespread use.

The following plant chemical characteristics were analysed :

1) Nitrogen content. N content was quantified by acid (sulphuric) digestion of 0.5 g dry grounded leaves/needles, distillation in a semi-automatic Kjeldahl distiller (Büchi B-324, Switzerland), and subsequent titration. N concentration is expressed as percentage of dry weight.

2) Phenolic compounds. Total phenols were analysed by the Folin-Ciocalteu method (Hódar & Palo, 1997) and condensed tannins were analysed by the proanthocyanidin assay (Waterman & Mole, 1994). No single method will give satisfactory results for quantitative analysis of phenolic compounds in plant tissues (Reed, 1995). Although there are problems with colorimetric analysis, caused by the variable structures of tannin polymers and the absence of satisfactory commercial standards (Makkar *et al.*, 1999 ; Appel *et al.*, 2001 ; Silanikove *et al.*, 2001), these procedures are considered adequate for relative comparisons (Yu & Dahlgren, 2000 ; Prior *et al.*, 2005, and references therein). For better comparisons, phenolic compounds were quantified against commercial standards, tannic acid for total phenols (TAE), and quebracho tannins (QTE) for condensed tannins (Waterman & Mole, 1994). However, the oxidative capacity of the particular phenols and tannins included in these standards are not equivalent to those included in plant material, so that standard equivalents must be considered merely as an expression of the total oxidative capacity of phenolic compounds in comparable units.

STATISTICAL ANALYSIS

The species and monthly variation of the chemical variables studied were analysed by repeated-measures variance analysis (rmANOVA) taking the species as between-groups factor and the month of the year as within-groups factor, for each site separately (Von Ende, 2001). For the two species present at both sites, we analysed the effect of site and species and monthly chemical variation by an rmANOVA, taking the species and site as between-groups factors and the month

of the year as a within-groups factor (Von Ende, 2001). When models were not comprised of independent orthogonal components (sphericity condition, by the Mauchly Criterion) the ϵ Greenhouse-Geisser correction values were used. All statistical analyses were performed with SAS JMP software (SAS Institute Inc., Cary, NC, USA).

RESULTS

There were significant differences between species for all the chemical characteristics analysed, in both Sierra Nevada and Sierra de Huétor (Tab. I). Monthly variation was also significant in all cases except for the tannin content of plants from Sierra Nevada (Tab. I), this pattern of monthly variation differing for each species (Tab. I).

TABLE I

Species and monthly variation effect on N-concentration (% N), total phenols (% TAE), and condensed-tannin (% QTE) concentration in Sierra Nevada

	% N		% TAE*		% QTE*	
	d.f. N/D	F	d.f. N/D	F	d.f. N/D	F
Sierra Nevada						
Species	5/24	10.09	5/24	168.82	5/24	90.84
Model (Between)	5/24	10.09	5/24	168.82	5/24	90.84
Month	3/72	62.04	1.9/46.4	7.20	2.2/52	4.04
Month \times Species	15/72	9.99	9.7/46.4	2.99	10.8/52	5.09
Model (Within)	15/72	9.99	9.7/46.4	2.99	10.8/52	6.35
Sierra de Huétor						
Species	5/24	64.13	5/24	26.16	5/24	186.57
Model (Between)	5/24	64.13	5/24	26.16	5/24	186.57
Month	3/72	13.92	3/72	10.99	3/72	15.96
Month \times Species	15/72	0.38	15/72	5.09	15/72	10.78
Model (Within)	15/72	0.38	15/72	5.09	15/72	10.78

* ϵ Greenhouse-Geisser correction values were used. Significant F values after Bonferroni correction ($P < 0.017$) are in bold.

The three chemical indicators analysed show contrasting results between species at the two study sites. For instance, nitrogen content can double from the poorest to the richest plant species (Figs. 1 & 2), and these differences are even more pronounced for total phenols or condensed tannins (Figs. 1 & 2). In Sierra Nevada, Piorno was the species with the highest N concentration in June and September, but in July and August it was Maple (Fig. 1). In Sierra de Huétor, Juniper always showed the lowest nitrogen concentration (Fig. 1). Sloe, Serviceberry and *R. myrtifolius* in general presented higher nitrogen concentration than did Holm oak, Hawthorn or *R. saxatilis* (Figs. 1 & 2). Overall, evergreen species registered a lower nitrogen concentration at the beginning of the season, but there was no clear pattern for its content in defence compounds, since variation on phenolic compounds seems much more species-specific.

The monthly variation of nitrogen content steadily declined for all species, except for Piorno in Sierra Nevada from August to September. On the contrary, the monthly variation of total phenols and condensed tannins displayed a less clear pattern in both mountain ranges. Thus, in Sierra Nevada Hawthorn and Serviceberry showed a continuous increase of total phenols, whereas Maple and Sloe did not reveal clear tendency, and in Piorno this parameter decreased from June to July and increased to September. Scots pine maintained a constant phenol concentration, the lowest of all the species (Figs. 1 & 2). In Sierra de Huétor, Juniper showed a progressive increase in total phenols, while Holm oak and *R. saxatilis* maintained constant values from June to August and underwent decreases in September. In Serviceberry

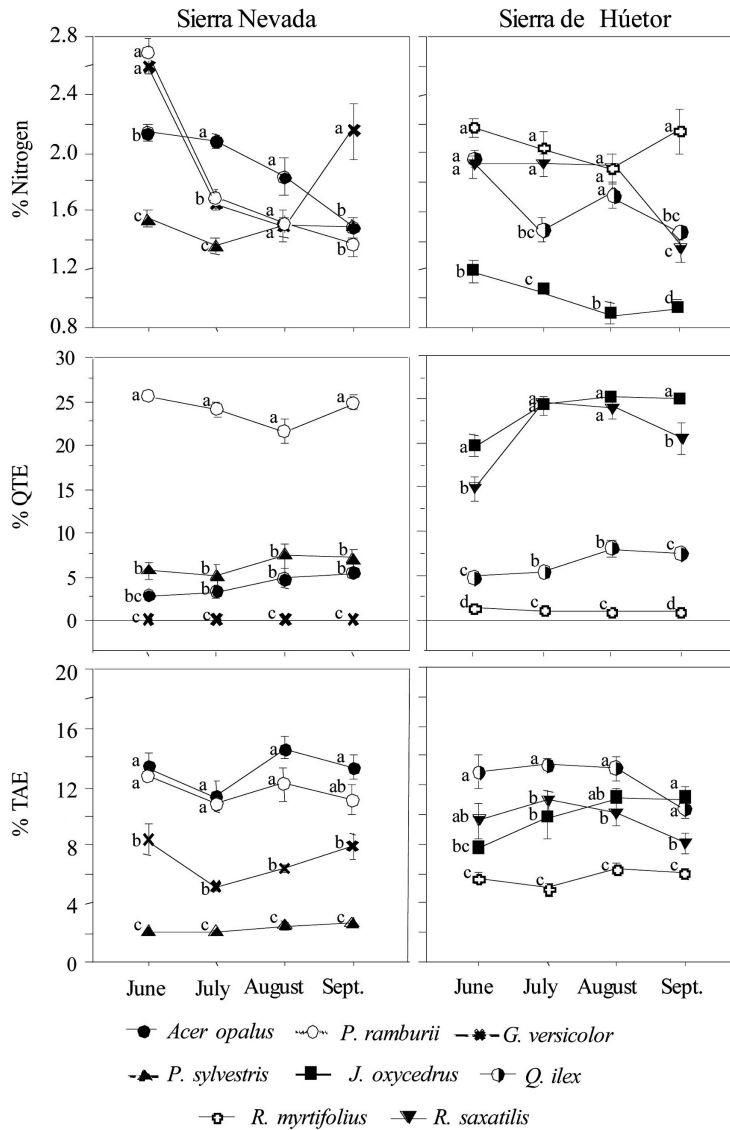


Figure 1. — Monthly variation of chemical characteristics of Mediterranean woody plant species analysed in Sierra Nevada (left) and Sierra de Húetor (right) : % Nitrogen, condensed tannins (% QTE), and total phenols (% TAE). Symbols correspond to means (five samples per month and species) and vertical lines to standards errors. Symbols with different letters indicate significant differences between species within a month (*a posteriori* Tukey-Kramer HSD test).

total phenols increased to August but decreased to September (Fig. 2). *Rhamnus myrtifolius* and Hawthorn showed the lowest total phenols, whereas Holm oak showed the highest (Figs. 1 & 2).

Condensed tannins were found to be less variable than total phenols between months, and showed a slight increase during the summer (Figs. 1 & 2) with some exceptions. For example, in Sierra de Húetor, *Rhamnus saxatilis* showed an important increase in condensed tannins from June to July, whereas Hawthorn presented the opposite trend (Figs. 1 & 2). In *Rhamnus saxatilis*, Juniper, and Sloe, condensed tannins were higher than in the rest of species. Hawthorn and Serviceberry also had higher condensed tannins than in Holm oak, Scots pine

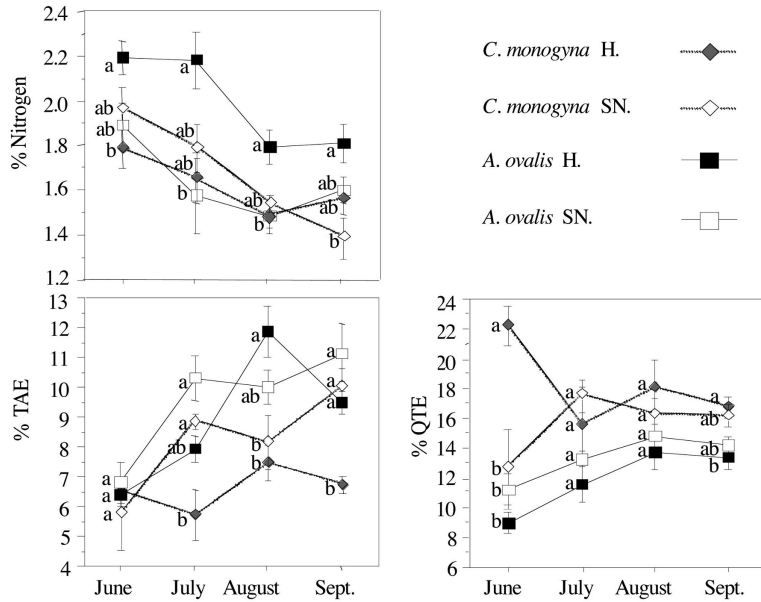


Figure 2. — Monthly variation of chemical characteristics of the two species analyzed in both mountains : Sierra Nevada (SN) and Sierra de Huétor (H) : % Nitrogen, condensed tannins (% QTE), and total phenols (% TAE). Symbols correspond to means (five samples per month and species) and vertical lines to standards errors. Tukey symbols with different letters indicate significant differences between species and sites within a month (*a posteriori* Tukey -Kramer HSD test).

and Maple, with Piorno and *Rhamnus myrtifolius* being the species with the lowest condensed tannins (Figs. 1 & 2).

The two species analysed at both sites showed little differences between sites. The nitrogen content reflected a significant effect of the site, of the species, and of the interaction between sites and species (Tab. II). While Hawthorn had similar nitrogen content in both mountains, Serviceberry in Sierra de Huétor presented greater nitrogen content than in Sierra Nevada (Fig. 2). However, total phenols and condensed tannins did not show significant differences between sites (Tab. II).

TABLE II

Values of site, species, and monthly variation effect on N-concentration (% N), total phenols (% TAE), and condensed tannins (% QTE) concentration in *Crataegus monogyna* and *Amelanchier ovalis* of Sierra Nevada and Sierra de Huétor

	% N		% QTE *		% TAE	
	df N/D	F	df N/D	F	df N/D	F
Site	1/16	10.99	1/16	0.12	1/16	4.03
Species	1/16	13.27	1/16	14.91	1/16	11.59
Site × Species	1/16	17.53	1/16	3.61	1/16	0.71
Model (between)	3/16	13.93	3/16	6.21	3/16	5.45
Month	3/14	24.79	1.9/31.5	4.01	3/14	14.12
Month × Site	3/14	0.60	1.9/31.5	3.95	3/14	2.85
Month × Species	3/14	0.63	1.9/31.5	4.85	3/14	10.93
Month × Site × Species	3/14	4.31	1.9/31.5	7.01	3/14	1.25
Model (Within)	9/48	1.22	5.9/31.5	3.95	9/48	4.67

* ε Greenhouse-Geisser correction values were used. Significant F values after Bonferroni correction ($P < 0.017$) are in bold. df N/D indicates the degrees of freedom numerator/denominator.

DISCUSSION

Our results reveal that the plants studied differed in their chemical characteristics between species, between times, and between sites. All these sources of variability create a diverse and changing chemical landscape in which herbivores have to select their diet.

The taxonomic disparity of the species studied (10 species belonging to 7 families) greatly contributes to the between-species differences found. The special features of each species (gymnosperm vs. angiosperm, evergreen vs. deciduous) provide a diverse background in which the chemical diversity is a logical consequence. For instance, according to the resource-based theories (Bryant *et al.*, 1983 ; Herms & Mattson, 1992), evergreen species present greater concentrations of stable compounds of high molecular weight and little mobility, such as condensed tannins and fibre, than do deciduous ones (Bryant *et al.*, 1988). Therefore evergreens cannot afford the loss of these components every year, and this in turn causes a lower nitrogen concentration than in deciduous species (Coley, 1987 ; Escudero & Manzano, 1987 ; Cornelissen *et al.*, 1997), so that evergreen leaves must be more defensive than deciduous ones. However, several of the species studied here contradict the hypotheses : *Rhamnus myrtifolius*, an evergreen scrub, presented lower condensed tannins and total phenols, and a higher nitrogen concentration than in deciduous species, including the congenetic *Rhamnus saxatilis* ; meanwhile, Holm oak showed a higher nitrogen concentration and less condensed tannins than in deciduous shrubs, although it presented more total phenols. Furthermore, some plants can develop defences that are not characterized by chemical analyses. For example, *Rhamnus myrtifolius* reacts after browsing, producing smaller leaves and a higher shoot density (personal observations ; see Massei *et al.*, 2000 for *Phillyrea angustifolia* and Massei & Hartley, 2000 for *Olea europaea* var. *sylvestris*, for similar cases), and this may reduce the foraging efficiency of ungulates feeding on this species (Shipley *et al.*, 1998). Thus, our result showed plant defence strategies as species-specific since there is not total correspondence with any of the current general theories (Hamilton *et al.*, 2001). Moreover, in Mediterranean areas, with high plant-species biodiversity, these species-specific strategies involve high biochemical diversity, which could determine the relationship between herbivores and plants (Lebreton *et al.*, 1997 ; Provenza *et al.*, 2003 ; Lebreton & Gallet, 2007 ; Baraza *et al.*, 2009).

A well-known cause of within-species differences are seasonal chemical changes. Some of the species studied here showed a decline in nitrogen concentration and a slight increase in condensed tannins, similar to that described by other authors (Dement & Mooney, 1974 ; Riipi *et al.*, 2002). Meanwhile, total phenolics, in most of the cases, shows no constant increase through the season, in contrast with results reported by Feeny (1970) and Dement & Mooney (1974) for other plant species, but similar to those reported by Schultz *et al.* (1982). These between-species differences could be explained by genetic differences that not only determine chemical characteristics but also by the way in these characteristics change over the vegetative period (Schultz *et al.*, 1982 ; Oiser & Lindroth, 2001). Chapin III *et al.* (1987) found major differences between species in the seasonal changes of carbohydrate storage, depending on leaf lifespan and biotype of the plant. In our case it is not possible to combine deciduous or evergreen species in groups of different seasonal-variation pattern, perhaps because they not only differ in their biotype but also in their phylogeny. This high seasonal-variation divergence increases the marked diversity in chemical characteristics found between the species studied. In other words, Mediterranean woody vegetation provides a multivariate chemical environment for herbivores, allowing them to select not only between species, but also between different times to find the most adequate diet.

Another important source of significant chemical differences within a species is site differences. For example, Serviceberry presented a significantly higher N concentration in Sierra de Huétor than in Sierra Nevada. Although the two populations analysed are similar in exposure and altitude, without remarkable environmental differences between sites, different soil nitrogen concentration or genetic differences between populations could explain the foliar nitrogen differences found on Serviceberry. Chemical differences between same-species populations would be caused by multiple factors, such as environment differences between growth sites

(Chapin III *et al.*, 1987 ; Castro-Diez *et al.*, 1997 ; Cunningham *et al.*, 1999 ; Thomas & Schafellener, 1999) or genetic variations (Oiser & Lindroth, 2001 ; Andrew *et al.*, 2007). Differences in plant quality between sites are obvious when analysing the same species along gradients of conditions, as altitude (Hengxiao *et al.*, 1999 ; Fernández-Calvo & Obeso, 2004), exposure (Pettorelli *et al.*, 2001), length of the growing season (Skarpe & van der Wal, 2002), or latitude (Pennings *et al.*, 2001), emphasizing the need to consider all sources of variation in plant quality that potentially can affect herbivores populations (Pettorelli *et al.*, 2001 ; Danell & Bergström, 2002).

Although the leaf habit, growth rate, and resource availability can affect the concentration of defence compounds in plants, our results highlight the necessity of taking into account the phylogeny and evolutionary history of each species in order to understand the nature and concentration of the chemical defence that plants present (Augner *et al.*, 1991 ; Hamilton *et al.*, 2001). This is more necessary in very diverse systems, such as Mediterranean ones, with not only a high number of species but also very diverse phylogenies. For example, our study was centred in the ten most abundant woody species in the study areas, involving seven different families. Moreover, some authors argue that the long history of intensive exploitation of Mediterranean vegetation by human activity – heavy grazing, woodcutting, and frequent fires – may be crucial in modelling the chemical defence of the plants (Dafni, 1991 ; Perevolotsky, 1994), making it necessary to take into account this particular characteristic of Mediterranean vegetation in order to understand the plant-herbivore relationship in these ecosystems.

In summary, Mediterranean mountain vegetation is highly variable in the temporal and spatial distribution of leaf-quality characteristics, due not only to internal processes of tissue maturation but also to environmental variation and specific differences (Hunter & Price, 1992 ; Hartley & Jones, 1997 ; Danell & Bergström, 2002 ; Lebreton & Gallet, 2007). Herbivores can respond to this variable environment by two strategies : 1) by consuming each species when its nutritive value is maximal, or 2) by extending their dietary consumption to all the available plants at any moment (Illius & Gordon, 1993 ; Baraza *et al.*, 2009). Our study suggests more support to the second strategy in that the high diversity in chemical characteristics found between and within the study species, creates a multivariate chemical environment that allows generalist herbivores to consume multiple species at different times. This leads to the maintenance of the high chemical diversity despite the high herbivore pressure for millions of years in the Mediterranean area.

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