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Environmentally-induced changes in antioxidant phenolic compounds levels in wild plants

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Running title Antioxidant phenolic compounds levels in wild plants

Abstract

Different adverse environmental conditions cause oxidative stress in plants by generation of reactive oxygen species (ROS). Accordingly, a general response to abiotic stress is the activation of enzymatic and non-enzymatic antioxidant systems. Many phenolic compounds, especially flavonoids, are known antioxidants and efficient ROS scavengers *in vitro*, but their exact role in plant stress responses in nature is still under debate. The aim of our work is to investigate this role by correlating the degree of environmental stress with phenolic and flavonoid levels in stress-tolerant plants. Total phenolic and antioxidant flavonoid contents were determined in 19 wild species. Meteorological data and plant and soil samples were collected in three successive seasons from four Mediterranean ecosystems: salt marsh, dune, semi-arid and gypsum habitats. Changes in phenolic and flavonoid levels were correlated with the environmental conditions of the plants and were found to depend on both the taxonomy and ecology of the investigated species. Despite species-specific differences, principal

component analyses of the results established a positive correlation between plant phenolics and several environmental parameters, such as altitude, and those related to water stress: temperature, evapotranspiration and soil water deficit. The correlation with salt stress was, however, very weak. The joint analysis of all the species showed the lowest phenolic and flavonoid levels in the halophytes from the salt marsh. This finding supports previous data indicating that the halophytes analysed here do not undergo oxidative stress in their natural habitat and therefore do not need to activate antioxidant systems as a defence against salinity.

Keywords Dunes·Salt marshes·Gypsum habitats·Salt stress·Water stress·

Introduction

Flavonoids are a large group of plant polyphenolic compounds that includes more than 9,000 different molecules (Williams and Grayer 2004) with a common C6–C3–C6 structure consisting of two aromatic rings linked through a 3 carbon chain, usually organised as an oxygenated heterocycle. Flavonoids can be divided into several subfamilies according to the degree of oxidation of the oxygenated heterocycle: anthocyanins, flavones, flavonols, flavanones, chalcones, aurones, flavonons, isoflavonoids, biflavonoids, condensed tannins, etc. (Iwashina 2000). Flavonoids are merely a subgroup of the bigger family of phenolic compounds, which also include simple phenols, benzoic and cinnamic acids, coumarins, tannins, lignins, lignans or stilbenes. Phenolic compounds are ubiquitous in plants, which collectively synthesise tens of thousands of different chemical structures characterised by hydroxylated aromatic ring(s).

Plant phenolics fulfil a wide array of biological functions (Gould and Lister 2006; Pollastri and Tattini 2011; Truetter 2005, 2006). For example, they can be structural components of cell walls (e.g., lignins, hydroxycinnamic acids), participate in growth and developmental processes through the regulation of auxin transport (Brown et al. 2001) or, specifically flavonols, can function as plant hormones by stimulating pollen maturation and pollen tube growth (Ylstra et al. 1992; Napoli et al. 1999), but are mostly involved in the interaction of plants with their environment. Phenolic compounds act as signalling molecules in plant-microorganisms interactions (e.g., in the induction of nodulation in leguminous plants), are involved in plant defence

mechanisms against herbivores, and against bacterial, viral and fungal pathogens, or constitute attractants for pollinators and animals responsible for fruit and seed dispersal (Harborne and Williams 2000; Treutter 2005, 2006; Gould and Lister 2006; Cheynier et al. 2013). There is plenty of evidence that these secondary metabolites also participate in responses of plants to practically all types of abiotic stress: UV radiation, intense light, extreme temperatures, mineral nutrient imbalance, anoxia, ozone exposure, drought, salinity, heavy metals and herbicides (Winkel-Shirley 2002; Treutter 2005; 2006; Gould and Lister 2006; Pollastri and Tattini 2011; Di Ferdinando et al. 2012; and references therein).

Apart from elucidating the mechanisms mediating the multiple biological functions of phenolic compounds in plants, which is a topic of unquestionable interest, research into these compounds has vastly increased in recent years given their alleged beneficial effects on human health. Flavonoids, especially, but also some other phenolics, have been reported to possess a wide range of pharmacological activities, including antibacterial, antiviral, anti-inflammatory, antilipidemic, antidiabetic, neuroprotective, hepatoprotective and cardioprotective properties (Coman et al. 2012; Nechita et al. 2012; Kumar and Pandey 2013; Ravishankar et al. 2013; Romano et al. 2013). These properties have been related to the strong antioxidant character of many phenolic compounds and their capacity to scavenge 'reactive oxygen species' (ROS).

ROS are chemically reactive molecules continuously produced in plants as by-products of aerobic metabolism. They include, among others, highly reactive free radicals such as superoxide ($O_2^{\bullet -}$), hydroxyl ($OH^{\bullet}$) and perhydroxyl ($O_2H^{\bullet}$) radicals, singlet oxygen ($^1O_2$), molecular oxygen (O_2), ozone (O_3) or hydrogen peroxide (H_2O_2) (Takahashi and Asada 1988; Apel and Hirt 2004). When in excess, ROS are toxic compounds that oxidise amino acid residues in proteins, the unsaturated fatty acids in the cell membranes, and DNA molecules, thus causing cellular damage (Halliwell 2006). Under environmental stress conditions, the concentration of ROS may largely increase in plants, leading to oxidative stress (Van Breusegem and Dat 2006). Accordingly, one of the general responses to abiotic stress in plants is based on the activation of enzymatic and non-enzymatic antioxidant systems; many flavonoids and other phenolic compounds can be included in the latter category (Apel and Hirt 2004). Recently it has been found that the biosynthesis of antioxidant flavonoids is triggered especially under severe stress conditions, when the activities of antioxidant enzymes, considered the first line of defence against ROS, decline. Thus, flavonoids are regarded

as a secondary ROS scavenging system activated in plants under severe stress because of the depletion of primary antioxidant defence systems (Fini et al. 2011).

There are numerous *in vitro* experimental data which prove the antioxidant capacity of phenolic compounds, especially that of flavonoids, which inhibit the generation of ROS or can reduce ROS once formed (Pollastri and Tattini 2011; Bose et al., 2013, and references therein). Yet their functional role in abiotic stress tolerance mechanisms *in vivo* is still under debate (Hernández et al. 2008; Di Ferdinando et al. 2012). Experimental evidence for a biologically relevant antioxidant function in plants is limited to some individual flavonoids under particular experimental or developmental conditions. Moreover, flavonoid research has been based mostly on a few model plants, such as *Arabidopsis thaliana* and some crop species, all of which are rather sensitive to stress. Field data on wild species, which are adapted to environmentally stressful conditions in their natural habitats, are still very scarce.

We assumed, as a working hypothesis, that antioxidant phenolic compounds are indeed involved in the mechanisms of abiotic stress tolerance of plants in their natural habitats; that is, under ecologically relevant conditions. The objective of our work is to test this hypothesis by finding significant correlations between the levels of these secondary metabolites in the plants and the degree of environmental stress affecting them in the field. We are interested in establishing general patterns for the mechanisms of plant stress tolerance, rather than investigating the response of particular taxa to specific stress conditions. Yet we are also aware that species-specific differences in stress responses – as well as the multiple biological functions of phenolic compounds unrelated to environmental stress – may mask the specific effects of phenolics and flavonoids as antioxidants in abiotic stress tolerance mechanisms. Therefore, for this work we have selected a relatively large number of wild species (19), present in four distinct natural habitats and hence subjected to different types of environmental stress. In addition, plant samples were collected in three successive seasons during the year, in which the intensity of stress also varied. In the Mediterranean climate, characterised by hot, dry summers (Rivas-Martínez and Rivas-Saenz 1996-2009), the combination of drought, high temperatures, high solar radiation and increased soil salinity – in saline habitats – makes summer the most stressful period of the year, while temperatures in autumn and spring are mild and rainfall is generally abundant.

Therefore, we have determined phenolic and antioxidant flavonoid contents in the selected taxa, as well as a number of soil parameters and climatic data associated

with environmental stress, under a wide range of stressful conditions in the plants' natural habitats. We expected that the analysis of the results would allow us to establish a general and statistically significant correlation between the levels of these antioxidant compounds and the intensity of stress affecting the plants in the field, and also to get information about the relative importance of different environmental conditions on the induction of their synthesis.

Material and methods

Study areas

Four habitats affected by different environmental stressful conditions were selected: a salt marsh, a neighbouring littoral dune, a semi-arid inland habitat and a gypsum area at higher altitude. The dune and salt marsh habitats are located at El Saler (39º 21' N, 0º 19' W), in 'La Albufera' Natural Park near the city of Valencia, at 4 m a.s.l. The semi-arid habitat is situated in the area of Bétera (39º 39' 44'' N, 0º 28' 33'' W), at 220 m a.s.l. on calcareous soil. The gypsum habitat is located near Tuéjar (39º 47' 28'' N, 1º 04' 25'' W) at 600 m a.s.l.

Plant material and sampling design

Two experimental plots were selected in both the salt marsh (S1 and S2) and gypsum (G1 and G2) habitats, with different levels of soil salinity or gypsum contents, respectively; single plots were defined both in the dune (D) and the semi-arid (A) areas. Each plot covered 100 m^2 (10 m x 10 m). In the salt marsh, there is a gradient of salinity: soil electrical conductivity in plot S2, located in the central, more depressed area of the marsh, is higher than in plot S1. The selected plots in the gypsum habitat are located on a hill with south-western orientation and a variable slope of between 19º (G1) and 11.5º (G2). G1, at the top of the hill, is the driest spot, but contains less gypsum; G2, at the bottom of the hill, is the wettest, but contains more gypsum due to the runoff transport of soluble material that accumulates and precipitates in the lowest part of the slope.

Plant species were selected following two criteria: biotype and abundance. Since sampling was carried out on the same individuals throughout the year, only perennials

were suitable. Plant size was also considered – as sufficient plant material had to be collected from the same individual in successive samplings, without affecting its viability – as well as the inclusion of species characteristic of the different habitats belonging to different families, while avoiding endemic and threatened taxa. Most species analysed here had been included in some of our previous studies addressing different mechanisms of plant response to environmental stress, such as the control of ion transport, the accumulation of different osmolytes, or the activation of antioxidant systems (Gil et al., 2011; Boscaiu et al., 2013; Gil et al., 2014; Llinares et al., 2015). Table 1 shows the selected taxa and their presence in the different habitats and experimental plots.

To determine phenolic and flavonoid levels, samples of the selected species were collected from five individuals per plot; individual plants were labelled and used in successive samplings throughout the year. Plant material was collected in three different sampling dates (in spring, summer and autumn 2009), cooled on ice and transported to the laboratory. Green leaves were separated, and samples of 5-10 g from each individual were dried in an oven at 65ºC for several days until constant weight.

Climate analysis

To assess the climatic conditions previous to sampling in each area, data on the mean, maximum and minimum temperatures, rainfall and reference evapotranspiration (ETo) were collected on a daily basis from the nearest agroclimatological stations, located in Benifaió (less than 6 km from the salt marsh and dune areas), Bétera (10 km from the semi-arid zone) and Chulilla (18 km from the gypsum area). The mean temperature and the cumulative values for rainfall and ETo, were calculated from the daily data recorded during the 30 days prior to each sampling date.

Soil sampling and analysis

Three random soil samples were taken in each plot from a depth of 0-15 cm, three times during the study period and simultaneously with the plant material collection; that is, nine samples were analysed altogether per plot. Soil was sieved through 2 mm sieves and air-dried. In all the soil samples available P was extracted following Burriel-Hernando (1947) and was determined by colorimetry with ascorbic

205 acid (Kuo 1996). Available K was determined by flame photometry after ammonium
206 acetate extraction (Knudssen et al. 1982). pH and electrical conductivity were measured
207 in 1:2.5 soil water suspensions and 1:1 soil water extracts, respectively. All the soil
208 samples were analysed for oxidable organic carbon (OC) by the Walkey-Black method
209 (Nelson and Sommers 1982). Water holding capacity (WHC) was determined as the
210 water retained in a pressure chamber at 20 kPa. Gypsum content was determined by the
211 crystal water loss method (Nelson et al. 1978).
212 In each plot, three multiple sensors for soil moisture and temperature
213 measurements (5TE in the gypsum area, ECH_2O in the salt marsh, Decagon®), were
214 installed on the first sampling day, at depths of 10 cm (two) and 20 cm, and were
215 connected to a datalogger (EM50, Decagon®). In the semiarid area, four sensors for soil
216 water content and four sensors for temperature (ECT-S, Decagon®) were installed at
217 depths of 10 cm and 20 cm.
218
219 Determination of total phenolics and antioxidant flavonoids
220
221 Roughly 100 mg dry plant material were ground to a fine power in a mortar and
222 extracted with 80% methanol. Samples were shaken gently overnight at room
223 temperature. Supernatants were collected by centrifugation and stored at -20ºC until
224 used in the assays. Total phenolic compounds were assayed by a reaction with the
225 Folin-Ciocalteu reagent (Singleton and Rossi 1965; Marinova et al. 2005) using gallic
226 acid as a standard. The phenolic contents in the plant samples were expressed as ‘mg
227 equivalent of gallic acid per g dry weight’. Flavonoids were determined by a reaction
228 with $AlCl_3$ at a basic pH, as described by Zhishen et al. (1999), with catechin used as a
229 standard. This method is commonly used to quantify 'total flavonoid' contents in plant
230 and food samples (e.g., Kim et al. 2003; Nile and Khobragade 2010). Yet this is not
231 strictly correct, as the procedure is based on the nitration of aromatic rings bearing a
232 catechol group and only detects those phenolic compounds containing this chemical
233 structure, which include several subclasses of flavonoids – such as flavonols or
234 flavanols – but also other non-flavonoid phenolics, such as caffeic acid and derivatives
235 (Pekal and Pyrzyska 2014). Nevertheless, the method was chosen since the metabolites
236 determined by the reaction with $AlCl_3$ are all antioxidants and there is a good correlation
237 between their levels and the total antioxidant activity of the samples (Zhishen et al.
238 1999). To simplify, further on in the text we refer to the $AlCl_3$-reactive compounds,

collectively, as '*antioxidant flavonoids*' or simply '*flavonoids*', and express their contents as ‘mg equivalent of catechin per g dry weight’.

Statistical analysis

Data were analysed using the programmes SPSS for Windows, v.16.00 and Statgraphics XVI. The spatial and seasonal variations in the total phenolic and flavonoid contents in each species were analysed by one-way ANOVA and a multifactor analysis of variance. ANOVA requirements were checked by normality plots and by testing the homogeneity of variance of the residual means. When the ANOVA null hypothesis was rejected, *post hoc* comparisons were performed using the Tukey test. Pearson’s correlation coefficient was applied to determine the significance of environmental factors on phenolic and flavonoid contents. To reduce data variability, the multivariate approach of principal component analysis (Martens and Naes 1989) was used. The ecological variables that significantly correlated with the phenolic and flavonoid contents were subjected to the principal component option of Statgraphics XVI after a previous autoscale. Varimax rotation was applied to the data. After choosing the number of PC, data were projected onto the new reduced space of the two first principal components through the score plot. The new reduced space allowed the observation of a cluster of objects and an analysis of the factors involved (Sena et al. 2002).

Results

Climate and soil analysis

The temperature and precipitation data during the month previous to each sampling date are shown in Table 2. Seasonal variation of temperature in the sand dune and salt marsh area was lower than in the other experimental plots given their proximity to the sea. Both the lowest minimal and the highest maximal temperatures were recorded in the gypsum area, which is located inland and at a higher altitude, where the climate is more continental than at the other sites. The rainfall distribution during the study was irregular, with a summer drought period in all the selected areas, a typical feature of the Mediterranean climate (Rivas-Martínez and Rivas-Saenz 1996-2009). Only in spring was there significant rainfall in the month before the sample collection

date, while the months prior to the summer and autumn samplings were very dry. The greatest water deficit was recorded in summer in all the habitats.

The results of the soil analyses are summarised in Table 3, where the values shown are the means (± SD) of three random samples per plot collected in each of the sampling dates, in spring, summer and autumn of 2009. Gypsum was found only in the Tuéjar plots (G1 and G2), but with significant quantitative differences: the soil gypsum level in G2 was considerably higher than in G1. Organic matter (OM) content was very low in the sandy soils from the dune and salt marsh habitats (below 1%), and was higher in the semiarid area (more than 5% OM), which is related to its higher clay content. In the gypsum area, the OM percentage was relatively low, which is common in this parent material type (FAO 1990). The soil pH was basic in all the plots, although differences were found in the different experimental areas: the lowest values were measured in the gypsum zone, followed by the semi-arid plot, while the most alkaline soils were those of the dune and saline habitats. Water holding capacity was very low in the sandy soil habitats in comparison to the other sites. In the gypsum plots, it was slightly higher in G1 than in G2, which was in accordance with the higher gypsum content of the latter. Available phosphorous levels were extremely low in all the experimental plots, while those of potassium varied according to soil type. The highest available K values were found in the gypsum area, in plot G1 (at the top of the slope), located at the transition from the gypsum to the marl parent material. In the salt marsh, available K was almost 3-fold higher in S2 (more saline) than in S1. In this sandy soil type, with extremely low cation exchange capacity, K is found only in a soluble form and is transported with other soluble salts to the soil surface, where it accumulates in summer as water is lost through evaporation. For the aforementioned soil properties, in general, no significant seasonal variations were observed, except for the pH values in several plots, which might be explained by temperature effects on the soil microbiological activity. Also, the gypsum content in plot G2 was significantly higher in the soil samples collected in November than in the summer samples; this is probably due to accumulation of gypsum transported by October rains from the upper part of the hill, an explanation that is supported by the increase in soil moisture detected in October by the sensor installed in G2 (see Fig. 3).

Soil salinity (measured as the EC of soil aqueous extracts at a 1:1 soil-to-water ratio) is shown in Fig 1. $EC_{1:1}$ in the semi-arid area was very low, but the lowest value was recorded in the dune area – despite its proximity to the sea – due to leaching by rain

307 water of the salts accumulated on the soil surface. Conversely, soil salinity in the nearby
308 salt marsh was much higher because of water transport to the soil surface from the
309 shallow water table and accumulation of the dissolved salts by evaporation; there were,
310 however, clear differences between the two experimental plots defined in the salt marsh
311 regarding average salinity, which was much higher in the central part (S2) than on the
312 border of the marsh (S1). Despite the differences in gypsum content, soil electric
313 conductivity was similar in G1 and G2 (ca. 2.6 and 2.4 dS m^{-1}, respectively), indicating
314 that salinity is regulated by the relatively low gypsum solubility, which maintains the
315 soil solution saturated.
316 Soil salinity was found to be highly variable in the salt marsh, not only between
317 the two plots, but also within each one in the different samplings, contrary to what was
318 observed for other soil properties. As expected, the highest $EC_{1:1}$ values were recorded
319 in the salt marsh in summer – the warmest and driest season when evaporation and salt
320 concentration in the soil upper layers are maximal – reaching 12 dS m^{-1} in plot S2. In
321 the dune and semi-arid habitats, which showed much lower absolute values, soil salinity
322 increased slightly in summer. Significant differences in soil salinity were not found in
323 the gypsum area, neither seasonal nor within the G1 or G2 plots. However, $EC_{1:1}$ was
324 slightly higher in plot G1, since it contains more soluble $MgSO_4$, as reported previously
325 (Boscaiu et al. 2013).
326 The continuous variation of soil water content, registered from the first sampling
327 of plant material to the end of 2009 by the sensors installed in the experimental plots, is
328 presented in Figure 2. Only the soil moisture measurements obtained at 10 cm depth are
329 shown, as they better correlated with the plant contents of phenolic and flavonoid
330 compounds. The driest plots were those located in the dune area (panel D) and in the
331 semiarid zone in Bétera (panel A).
332 When comparing the two salt marsh plots, soil moisture in S2 was almost double
333 than in S1. Moreover, S2 was the only plot in which soil water content did not decrease
334 in summer, since it is located in the most depressed and humid area of the salt marsh.
335 Regarding the two gypsum plots, G2, located at the bottom of the hill, presented higher
336 values of moisture as compared to G1, as it collected run-off rainwater from the upper
337 part of the hill.
338
339 Phenolic compounds and antioxidant flavonoid contents
340

The salt marsh plants (Fig. 3) presented lower levels of total phenolics and antioxidant flavonoids than those from the dune, semi-arid and gypsum habitats (Figs. 4 and 5).

Considering together the three samplings of plant material for each analysed species present in the salt marsh, the mean amount of phenolic compounds ranged from 7.6 ± 2.5 mg eq. gallic acid g^{-1} DW in *Inula crithmoides* to 21.1 ± 4.3 mg g^{-1} DW in *Plantago crassifolia*. That of flavonoids varied from 4.0 ± 1.1 mg eq. catechin g^{-1} DW in *Juncus acutus* to 10.2 ± 4.5 mg g^{-1} DW in *Schoenus nigricans*. For the two species present in both plots (*Juncus maritimus* and *Sarcocornia fruticosa)*, the mean phenolic and flavonoid values were slightly higher in the plants from S2, although the only statistically significant difference was observed in the level of phenolic compounds in *J. maritimus*. Regarding seasonal variations, all the species except *I. crithmoides* had higher levels of phenolic compounds in summer, and the differences in *J. acutus*, *S. fruticosa* and *S. nigricans* were statistically significant. Flavonoid contcentrations also showed seasonal variations as their mean values increased in summer, and were statistically significant for *J. acutus*, *S. nigricans* and *P. crassifolia.*

For the species selected from the dune habitat (Fig. 4, 'D' panels), the contents of both groups of metabolites ranged from minimum mean values in *Ononis natrix* (22.64 ± 1.84 mg g^{-1} DW for total phenolic compounds and 7.82 ± 2.47 mg g^{-1} DW for antioxidant flavonoids) to maximum mean values in *Helianthemum syriacum* (66.61 ± 13.28 mg g^{-1} DW, and 34.84 ± 6.46 mg g^{-1} DW, respectively). For three of the selected taxa present in the dune habitat (*H. syriacum*, *O. natrix*, *T. capitatum*) it was not possible to determine seasonal changes in phenolic and flavonoid contents during the whole study period, since due to the extreme aridity of this environment these plants lost their leaves in summer. The robust shrub *Rosmarinus officinalis* was the only species for which it was possible to sample leaf material in all three seasons; in this case, significantly higher concentrations of phenolic compounds and flavonoids were measured in summer than in spring or autumn.

In the plants from the semi-arid habitat (Fig. 4, 'A' panels), both the mean phenolic and flavonoid contents ranged from low and similar values in the two *Stipa* species, to a maximum in *H. syriacum* for phenolics (63.35 ± 10.89 mg g^{-1} DW) and in *R. officinalis* for flavonoids (41.03 ± 15.16 mg g^{-1} DW). The highest concentrations of phenolic and flavonoid compounds were generally detected in summer, and seasonal

variation was significant in all the species, except those from the genus *Stipa*. Once again, it was not possible to collect the summer sample from *H. syriacum* and *Thymus vulgaris* plants because they had no leaves.

In the plants from the gypsum habitat (Fig. 5), the mean phenolic contents varied from 10.6 ± 2.3 mg g^{-1} DW in *Ononis tridentata* to 79.04 ± 20.87 mg g^{-1} DW in *Cistus clusii*, and the flavonoids went from a minimum of 3.8 ± 1.2 mg g^{-1} DW, again in *O. tridentata* to a maximum of 53.51 ± 31.27 mg g^{-1} DW in *R. officinalis*. Most species from this environment showed relatively high levels of both phenolics and flavonoids, except for *Gypsophila struthium* and *O. tridentata*, which are typical gypsophytes; that is, they grow exclusively in gypsum soils. In *C. clusii*, high contents of phenolic compounds were determined, but comparatively low amounts of flavonoids were measured. No obvious spatial variation was detected in the phenolic or flavonoid contents of the taxa present in both plots, except for the amount of flavonoids in *C. clusii*, which was higher in plot G2. A clear seasonal variation pattern was also observed for both groups of compounds, with summer peaks in all the species analysed, except *O. tridentata*. As with the dune and semi-arid habitats, some species from the gypsum area (*Anthyllis cytisoides*, *Plantago albicans* and *Helianthemum syriacum)* shed their leaves, so they were not sampled in summer (Fig. 5).

Principal component analysis

The environmental variables which correlated significantly by the Pearson coefficient with phenolic and flavonoid contents were subjected to a principal component analysis (PCA) for each habitat (Fig. 6).

In the salt marsh, two components with an Eigenvalue equal to or greater than 1 explained a cumulative percentage of variance of 77%. The plot of the loading vectors shows the relationship between variables (Fig. 6A). The first component (X-axis), which explained 52% of variance, is mainly related to climatic variables associated with water stress (mean air temperature, water deficit and previous rainfall) and, to a much lesser extent, with salinity. The second component, which explained a further 25%, is negatively related to soil humidity the loading vectors of the variables phenolics and flavonoids presented very small angles with the Y-axis, but in the positive part of the

graph, indicating a good negative correlation between changes in the levels these compounds and fluctuations of soil moisture.

The PCA performed for the dune habitat (Fig. 6B) indicated a similar pattern of the loading vectors. The first component, which explained 58% of the variance, was related to atmospheric variables causing water stress in the plants, and the second one, explaining an additional 24% of the variance, showed a positive correlation with phenolics and flavonoids variation and a negative one with soil moisture.

The PCA for corresponding to the semiarid habitat is shown in Fig. 6C. The first two components explained about 85% of the variance. Phenolics and flavonoids variations were negatively correlated with the previous month's rainfall. Contrary to the salt marsh and the dunes, where the water table is located near the soil surface and supplies additional water to the rooting zone, in this plot soil is shallow with very little capacity of store water, so water deficit is controlled by external supply (rainfall).

The analysis for the gypsum habitat (Fig. 6D) indicated a strong positive correlation of phenolics and flavonoids with soil salinity and a negative correlation with the soil humidity at 10 cm depth. Salinity was seasonally constant in the gypsum zone, but it was dependent on the position of the plots along the slope, so that slightly higher EC values were determined in plot G1, at the top of the hill, as compared to plot G2, at the bottom; water availability, on the contrary, was bigger in G2. Since variability in phenolics and flavonoids is mostly seasonal, we consider that their positive correlation with salinity is indirect, due to the lower soil moisture in the more saline plot. Therefore, in all these habitats, considered independently, variation of antioxidant flavonoids and phenolics contents depends mostly on the degree of water stress affecting the plants.

A joint analysis of the four habitats confirmed the results obtained in each experimental zone, regarding the correlation between drought and phenolics contents. In this general PCA (Fig. 7), the first component, which explained 35% of variance, correlated positively with the climatic variables associated with water stress (ETo, water deficit and temperature) and also with salinity, but to a much lesser extent.

The second component, which explained a further 24%, related to altitude – a new variable introduced when considering all plots together – and presented very small angles with the loading vectors of the variables phenolics and flavonoids, indicating a close positive correlation. Gypsum in soil showed similarly high correlations with the levels of these metabolites in the plants, but probably due to the fact that these plots are

located at the highest altitude of all the study areas, not because of a direct effect of gypsum stimulating the synthesis of phenolic compounds. Although the ecological variables explained only about 63% of variance, this value can be considered highly significant since, for this study, we selected a relatively large number of species (19), with clear quantitative differences in phenolic and flavonoid contents. Therefore, the genetic variability of the plants most likely contributes, and to a great extent, to the unexplained causes of variance in the joint PCA. Moreover, considering the multiple biological functions of these secondary metabolites, factors not related to environmental stress, such as interactions with herbivores or pathogens and developmental cues, are also likely to influence phenolic and flavonoid contents in the plants.

Discussion

The results reported here indicate high interspecific variation for the accumulation of total phenolic compounds and antioxidant flavonoids in the selected plant species, with the highest levels found in taxa from the families *Labiatae* (*R. officinalis, T. capitatum* and *T. vulgaris*) and *Cistaceae* (*H. syriacum* and *C. clusii*), and the lowest in the plants from the families *Juncaceae* and *Poaceae*. This homogeneity within a family is not a general feature since a wide variation was found among the four legume species (*A. cytisoides, D. pentaphyllum, O. natrix* and *O. tridentata*) included in this study. These species-specific differences obviously hamper the analysis of the general function of these compounds in the mechanisms of plant tolerance to abiotic stresses. However, taxonomic considerations and direct comparisons among species are beyond the aims of this study. The major contribution of our work was to detect, in all species analysed here, common patterns of variation in the levels of total phenolics and antioxidant flavonoids, associated to changes in specific stressful environmental conditions.

The PCA analysis of our results shows a significant positive correlation of total phenolic and antioxidant flavonoid contents with the factors related mainly to water stress: high temperature, evapotranspiration and water deficit, which are represented in the first axis, while there is a significant negative correlation with soil moisture. These data are in agreement with many studies carried out on specific taxa, which strongly supports the notion that the synthesis of antioxidant phenolic compounds is induced

under water stress and should contribute to the drought tolerance of plants in their natural habitats.

Tattini et al. (2004) found that efficient *in vitro* ROS scavengers, such as flavonoids and hydroxycinnamates, play a role in responses to drought and high solar radiation in *Ligustrum vulgare*. Drought significantly increased the level of quercetin, a flavonol, in white clover, and higher contents of this compound were measured in genotypes better adapted to water deficit conditions (Ballizany et al. 2012). In *Cistus clusii*, one of the species analysed in the present work, Hernández et al. (2004) reported an increase in flavanol levels in plants collected from the field in summer, and in plants subjected to experimental drought treatments. Flavonols were also found to increase under drought conditions in other taxa, such as *Crataegu*s *laevigata* and *C. monogyna* (Kirakosyan et al. 2003). Recently, metabolome and transcriptome profiling in wild-type and several *Arabidopsis thaliana* mutants has also provided evidence that flavonoid overaccumulation is key to enhance tolerance to oxidative and drought stress in this model species (Nakabayashi et al. 2014).

The correlation with salinity, on the contrary, is very weak, although it is known that root zone salinity enhances the biosynthesis of flavonoids (Agati et al. 2011). From all the taxa included in our study, those halophytic species present in the salt marsh showed the lowest phenolic and flavonoid levels, which were significantly lower than those of the species growing in the nearby dune habitat with practically identical altitude and climatic conditions. It seems unlikely that lack of accumulation of these antioxidant compounds in the salt marsh species is due to genetic differences with the taxa present in other habitats, but could be explained assuming that they do not undergo oxidative stress secondary to salt stress, at least not under the specific conditions of their natural habitat during the course of the present study. These results, therefore, support the notion that the salt-tolerant species included in our study possess efficient mechanisms to avoid excessive ROS production, mediated by the control of ion transport and the accumulation of compatible solutes; that is, they do not activate the synthesis of antioxidant compounds simply because they do not need them as a defence mechanism against soil salinity in their environment. We proposed this hypothesis based on previous results, which revealed a lack of significant differences in the levels of malondialdehyde – MDA, a product of membrane lipid peroxidation considered an excellent marker of oxidative stress (Shulaev and Oliver 2006) – and in the specific activity of antioxidant enzymes in some of the halophytic species included in the

present work, in response to major seasonal changes in soil salinity in the salt marsh (Gil et al. 2014).

When we compared the four habitats, the joint PCA detected altitude as the main single factor that best correlated with increased phenolics and antioxidant flavonoid levels. Many authors have found larger amounts of phenolic compounds and flavonoids in plants sampled at higher altitudes (Bachereau et al. 1998; Zidorn et al. 2005; Spitaler et al. 2008; Rieger et al. 2008; Murai et al. 2009). Other reports indicate, more specifically, a significant increase in the ratio of dihydroxy B-ring substituted flavonoids in tissues and organs exposed to excess solar radiation (Tattini et al. 2000). Ortho-dihydroxy flavonoids – those containing a catechol group, such as flavonols or flavanols – are threefold to fourfold better radical scavengers than other flavonoids (Rice-Evans et al. 1996), and are detected by reaction with $AlCl_3$, together with some phenolics other than flavonoids, such as caffeic acid, also bearing this group. There is overwhelming evidence on the good correlation of $AlCl_3$-reacting phenolics contents with the total antioxidant activity in the samples (Kim et al. 2003; Rohman et al. 2010; Nile et al. 2010, Hajimahmoodi et al. 2013). In addition, the presence of an OH group in the 3-position of the flavonoid skeleton promotes the ability of flavonols and other flavonoids to chelate transition metal ions increasing the antioxidant capacity (Pollastri and Tattini 2011 and references therein).

There are numerous reports describing a positive correlation between flavonoid accumulation and UV exposure (e.g., Stapleton and Walbot 1994; Lavola 1998; Bieza and Lois 2001; Jaakola et al. 2004, among many others). As the atmosphere filters mainly UV radiation, an increase in altitude brings about a higher UV to total solar radiation ratio (Blumthaler et al. 1997). Therefore, the altitude-dependent increase in antioxidant flavonoids and total phenolic compounds can be partly attributed to the higher UV radiation that affected the plants.

The molecular basis of this correlation is known as it has been described in different plant species that the expression of chalcone synthase, the first enzyme in the flavonoid biosynthesis pathway, is transcriptionally activated by UV light (e.g., Kaulen et al. 1986; Koes et al. 1989; Schulze-Lefert et al. 1989) as are other genes involved in the metabolism of phenolic compounds (e.g., Winkel-Shirley 2002; Park et al. 2013 and references therein). Yet UV radiation is not a pre-requisite for the accumulation of flavonoids, since many other light qualities and non-light signals also stimulate their biosynthesis (Jenkins 2009); actually, CHS is regulated by a variety of external and

endogenous stimuli, ranging from light and temperature to metabolites and plant growth regulators (Jenkins et al. 2001; Bilger et al. 2007). It should also be considered that many other environmental factors change with altitude, such as precipitation, mean and extreme temperatures, duration of snow cover and solar radiation (Körner 1999).

The role of flavonoids in UV protection was first demonstrated in studies with *Arabidopsis* mutants (Li et al., 1993), and later it was strongly emphasised by other authors, such as Rozema et al. (1997) or Burchard et al. (2000). Besides a proposed function in direct UV-B screening (Rozema et al. 2002), flavonoids play other roles in photoprotection, especially due to their ability to scavenge ROS and behaving as signal molecules mediating the oxidative stress-induced activation of signaling cascades controlling cell growth and differentiation (Pollastri and Tattini 2011; Agati et al. 2013).

Albert et al. (2009) analysed flavonoid levels in *Arnica montana* plants cultivated in a climate chamber which reproduced the conditions of UV B-radiation and temperature at sub-montane (600 m) and high-montane (1400 m) altitudes. These authors found significant changes in flavonoid profiles, with an altitude-dependent increase in the degree of hydroxylation of the B ring, changes that were comparable to those observed in nature when the plants were exposed to lower temperature, but not to higher UV radiation. In the present study, an increase in phenolic or antioxidant flavonoid contents in the selected taxa could not be related to a decrease in temperature, but on the contrary to its increase. This pattern of response is related to the climate type. Of the four analysed habitats, the gypsum area is located at the highest altitude and has also the most continental climate, with the highest temperatures in summer. In the Mediterranean area, climate is especially harsh in summer, when an increase in temperature is associated with drought and other stresses, such as total solar radiation, which also increases significantly with the altitude. In fact, light stress, together with summer drought, is considered as the most important challenge for plants in Mediterranean ecosystems (Di Ferdinando et al. 2014).

Conclusions

We have compared the levels of total phenolic compounds and antioxidant flavonoids in a relatively large number of plant species from different families growing under varied environmental conditions: in three different seasons and four different natural habitats. Based on the statistical analysis of our results, a significant correlation

of these antioxidants with environmental factors, mostly altitude and water stress (but not with soil salinity), could be established. Our data strongly support a general and relevant role of these compounds in mechanisms of tolerance to at least some abiotic stresses in the plants' natural habitats, despite species-specific genetic differences, and the multiple biological functions of plant phenolics, which can mask their effects on environmental stress responses as antioxidants.

Author Contribution Selection of experimental plots and plant species and field samplings of plant material have been carried out by M. Boscaiu, P. Donat and O. Mayoral. Soil samplings and soil pre-treatments have been carried out by I. Bautista, A. Lidón and C. Lull. Installation of soil moisture and temperature sensors and processing of the data have been carried out by J.V. Llinares and A. Lidón. Analyses of plant material have been performed by O. Vicente and M. Boscaiu. Soil analyses have been performed by I. Bautista and C. Lull. Statistical analysis of the data has been realized by I. Bautista. M. Boscaiu, I. Bautista, C. Lull and A. Lidón have collaborated in the elaboration of the manuscript. O. Vicente has been responsible for the general supervision of the work.

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811

812

813 **Table 1** Selected taxa and their location in the study areas

Taxa under study	**Family**	**Abb.[a]**	**Habitat**	**Sampling zone**
Inula crithmoides L.	Asteraceae	Ic	Salt marsh	S2
Juncus acutus L.	Juncaceae	Ja	Salt marsh	S1
Juncus maritimus Lam.	Juncaceae	Jm	Salt marsh	S1, S2
Plantago crassifolia Forssk.	Plantaginaceae	Pc	Salt marsh	S1
Sarcocornia fruticosa (L.) A. J. Scott	Amaranthaceae	Sf	Salt marsh	S1,S2
Schoenus nigricans L.	Cyperaceae	Sn	Salt marsh	S1
Ononis natrix L.	Fabaceae	On	Dune	D
Teucrium capitatum L.	Lamiaceae	Tc	Dune	D
Plantago albicans L.	Plantaginaceae	Pa	Gypsum	G1
Anthyllis cytisoides L.	Fabaceae	Ac	Gypsum	G1
Cistus clusii Dunal	Cistaceae	Cc	Gypsum	G1, G2
Ononis tridentata L.	Fabaceae	Ot	Gypsum	G1, G2
Gypsophila struthium Loefl.	Caryophyllaceae	Gs	Gypsum	G2
Dorycnium pentaphyllum Scop.	Fabaceae	Dp	Semiarid	A
Stipa tenacissima L.	Poaceae	St	Semiarid	A
Stipa offneri Breistr.	Poaceae	So	Semiarid	A
Thymus vulgaris L.	Lamiaceae	Tv	Semiarid	A
Helianthemum syriacum (Jacq.) Dum.-Cours.	Cistaceae	Hs	Various	A, D, G1, G2
Rosmarinus officinalis L.	Lamiaceae	Ro	Various	A, D, G1, G2

814 [a] *abbreviation*

815

Table 2 Seasonal changes in climatic variables in the sampling zones. Variables were calculated from daily values during the month previous to each sampling data, obtained from the meteorological station nearest to each site

Variable	Sampling dates								
	S and D			A			G		
	30.04.09	01.07.09	30.11.09	06.05.09	31.07.09	18.12.09	29.04.09	13.07.09	11.12.09
Mean temperature (ºC)	12.5	21.0	14.3	14.9	25.9	11.5	11.6	24.8	11.7
Maximum temperature (ºC)	18.5	27.4	21.1	22.4	31.6	18.7	17.7	33.3	18.3
Minimum temperature (ºC)	10.5	18.8	12.5	7.9	19.8	7.0	5.5	16.4	5.1
Cumulative rainfall (mm)	68.3	1.0	1.4	37.6	3.6	1.0	51.0	5.8	9.8
Cumulative reference evapotranspiration (mm)	99.9	172.7	73.4	98.4	154.1	36.5	111.0	184.7	73.8

S = Salt marsh, D = Dune, G = Gypsum and A = Semiarid

Table 3 Mean values and standard deviations (n = 3) of the indicated soil properties in the six selected plots and the three sampling seasons. Values followed by the same Latin letter within a column are not significantly different among the seasons for each plot. Values followed by the same Greek letter within a line are not significantly different among the plots for the same sampling season (P > 0.05; ANOVA followed by LSD). OM: organic matter; WHC: water holding capacity.

		Plot						
		Salt marsh		Dune	Semiarid	Gypsum		*ANOVA p-value (plot)*
Property	Date	S1	S2	D	A	G1	G2	
Gypsum (%)	Spring 09	–	–	–	–	33.97± 20.90a,α	71.28 ± 9.30ab,β	*0.0476*
	Summer 09	–	–	–	–	44.23 ± 6.59a,α	54.69 ± 24.45a,α	*0.5139*
	Autum 09	–	–	–	–	36.99 ± 19.87a,α	97.45 ± 11.14b,β	*0.0101*
	ANOVA p-value (season)					*0.7612*	*0.0494*	
OM (%)	Spring 09	0.53 ± 0.06a,α	0.43 ± 0.06a,α	0.43 ± 0.06a,α	4.47 ± 0.87a,βγ	4.93 ± 1.86a,γ	3.00 ± 0.53a,β	*0.0000*
	Summer 09	0.97 ± 0.25b,α	0.83 ± 0.32a,α	0.83 ± 0.32a,α	6.83 ± 4.04a,β	3.20 ± 0.40a,α	2.97 ± 1.48a,α	*0.0100*
	Autumn 09	nd	nd	nd	4.63 ± 0.81a,β	3.53 ± 0.83a,αβ	1.80 ± 1.01a,α	*0.0221*
	ANOVA p-value (season)	*0.0438*	*0.1212*	*0.1212*	*0.4607*	*0.2498*	*0.3654*	
pH	Spring 09	9.08 ± 0.10b,γ	9.03 ± 0.08 a,γ	9.03 ± 0.08 a,γ	8.42 ± 0.02b,β	7.82 ± 0.05a,α	7.74 ± 0.03a,α	*0.0000*
	Summer 09	8.59 ± 0.12a,γ	8.91 ± 0.14a,δ	8.91 ± 0.14a,δ	8.15 ± 0.18a,β	7.89 ± 0.03ab,αβ	7.85 ± 0.10ab,α	*0.0000*
	Autum 09	8.89 ± 0.12b,γ	8.92 ± 0.09a,γ	8.92 ± 0.09a,γ	8.50 ± 0.03b,β	7.95 ± 0.04b,α	8.00 ± 0.11b,α	*0.0000*
	ANOVA p-value (season)	*0.0048*	*0.3557*	*0.3557*	*0.0151*	*0.0149*	*0.0243*	
WHC (%)	Spring 09	3.90 ± 0.22a,α	4.32 ± 0.84a,α	4.32 ± 0.84a,α	23.04 ± 3.28a,β	25.77 ± 7.84a,β	24.89 ± 2.17b,β	*0.0000*
	Summer 09	4.13 ± 1.62a,α	4.20 ± 2.65a,α	4.20 ± 2.65a,α	24.98 ± 1.96a,β	22.89 ± 2.43a,β	19.49 ± 6.79ab,β	*0.0000*
	Autum 09	3.59 ± 0.82a,α	4.51 ± 3.05a,α	4.51 ± 3.05a,α	24.94 ± 1.93a,γ	25.16 ± 5.70a,γ	15.43 ± 3.16b,β	*0.0000*
	ANOVA p-value (season)	*0.8252*	*0.9866*	*0.9866*	*0.5775*	*0.8182*	*0.1065*	

$P_{Burriel}$ (mg kg_{ss}^{-1})	Spring 09	2.57 ± 1.59a,αβ	1.24 ± 0.16a,α	1.24 ± 0.16a,α	2.48 ± 0.84b,αβ	2.63 ± 1.18b,αβ	4.16 ± 2.33b,β	*0.1969*
	Summer 09	0.99 ± 0.18a,α	1.01 ± 0.54a,α	1.01 ± 0.54a,α	1.09 ± 0.07a,α	1.03 ± 0.09a,α	1.19 ± 0.35a,α	*0.9293*
	Autum 09	0.92 ± 0.20a,α	0.81 ± 0.25a,α	0.81 ± 0.25a,α	1.35 ± 0.17ab,α	0.97 ± 0.27a,α	2.06 ± 0.31ab,β	*0.0055*
	ANOVA p-value (season)	*0.1267*	*0.3936*	*0.3936*	*0.0550*	*0.0457*	*0.0890*	
$K_{assimilable}$ (mg kg_{ss}^{-})	Spring 09	31.24 ± 7.04a,α	20.24 ± 2.08a,α	20.24 ± 2.08a,α	168.23 ± 15.65a,γ	266.85 ± 76.87a,δ	155.92 ± 44.59a,βγ	*0.0010*
	Summer 09	49.40 ± 35.79a,αβ	22.94 ± 4.40a,α	22.94 ± 4.40a,α	244.77 ± 53.88b,γ	192.69 ± 56.94a,βγ	183.32 ± 129.57a,βγ	*0.0682*
	Autum 09	29.35 ± 4.62a,α	35.18± 8.99b,α	35.18± 8.99b,α	229.19 ± 27.61ab,βγ	239.88 ± 139.42a,γ	115.11 ± 63.61a,αβ	*0.0057*
	ANOVA p-value (season)	*0.4858*	*0.0446*	*0.0446*	*0.0872*	*0.6612*	*0.6493*	

Figure captions

Fig. 1 Soil salinity measured as EC of soil water extract 1:1 in the salt marsh (plots S1 and S2), dune (D), semi-arid (A) and gypsum (plots G1and G2) areas for each sampling date.

Fig. 2 Daily soil moisture measured with sensors at 10 cm depth in the salt marsh (plots S1 and S2), dune (D), semi-arid (A) and gypsum (plots G1and G2) areas. Arrows indicate the soil and plant material sampling dates.

Fig. 3 Seasonal variation of phenolic and flavonoid(*) contents of the species sampled in the two salt marsh plots (S1 and S2): *Juncus maritimus* (Jm), *J. acutus* (Ja), *Sarcocornia fruticosa* (Sf), *Plantago crassifolia* (Pc), *Schoenus nigricans* (Sn). Bars indicate the mean values and standard deviations calculated in 5 individuals per plot and season, and per species. (*)Antioxidant flavonoids and another phenolic with a cathecol group.

Fig. 4 Seasonal variation of phenolic and flavonoid(*) contents of the species sampled in the dune (D) and the semi-arid (A) areas: *Helianthemum syriacum* (Hs), *Rosmarinus officinalis* (Ro), *Ononis natrix* (On), *Teucrium capitatum* (Tc*), Dorycnium pentaphyllum* (Dc), *Stipa offneri* (So), *S. tenacissima* (St), *Thymus vulgaris* (Tv). Bars indicate the mean values and standard deviations calculated in 5 individuals per plot and season, and per species. (*)Antioxidant flavonoids and another phenolic with a cathecol group.

Fig. 5 Seasonal variation of phenolic and flavonoid(*) contents of the species sampled in the two plots of the gypsum area (G1 and G2): *Cistus clusii* (Cc), *Helianthemum syriacum* (Hs), *Ononis tridentata* (Ot), *Rosmarinus officinalis* (Ro), *Anthyllis cytisoides* (Ac), *Plantago albicans* (Pa), *Gypsophila struthium* (Gs). Bars indicate the mean values and standard deviations calculated in 5 individuals per plot and season, and per species. (*)Antioxidant flavonoids and another phenolic with a cathecol group.

Fig. 6. Biplots from the principal component analysis model showing the relationship between the ecological variables correlated with phenolic (Phe) and flavonoid (Fla) contents in the saltmarsh habitat (A), dune habitat (B), semiarid habitat (C) and gypsum habitat (D). Water deficit (WD), previous month mean temperature (T), rainfall (R), soil moisture at 10 cm depth (Hum10) and electrical conductivity in the 1:1 soil water extract (Sal).

872 **Fig. 7**. Biplot from the principal component analysis model showing that the relationship
873 between the ecological variables correlated with phenolic (Phe) and flavonoid (Fla) contents:
874 water deficit (WD), altitude (Alt), gypsum (G), previous month mean temperature (T), rainfall
875 (R), soil moisture at 10 cm depth (Hum10), water deficit (WD) and electrical conductivity in the
876 1:1 soil water extract (Sal).
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879 Figure 1

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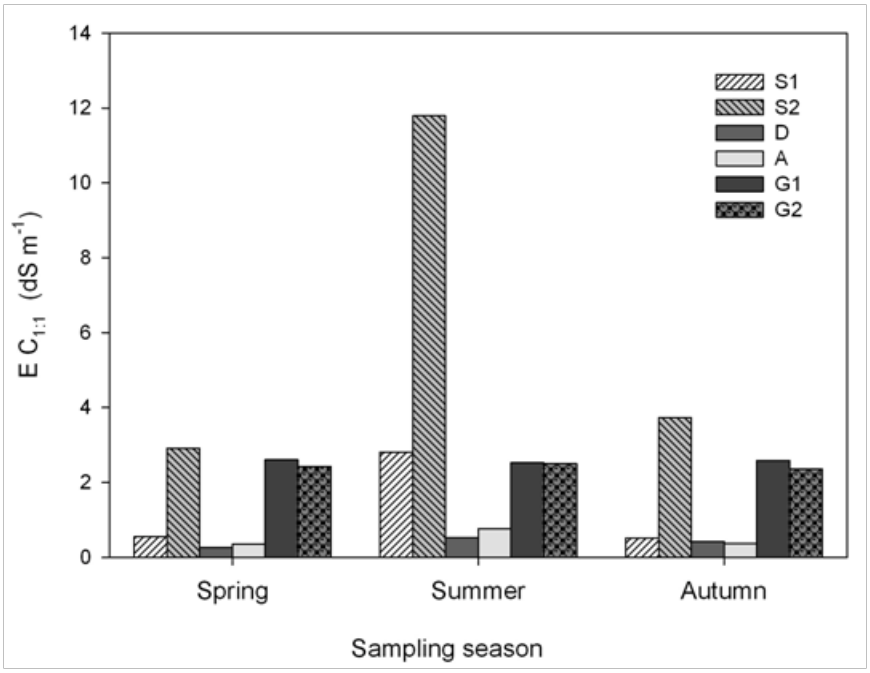

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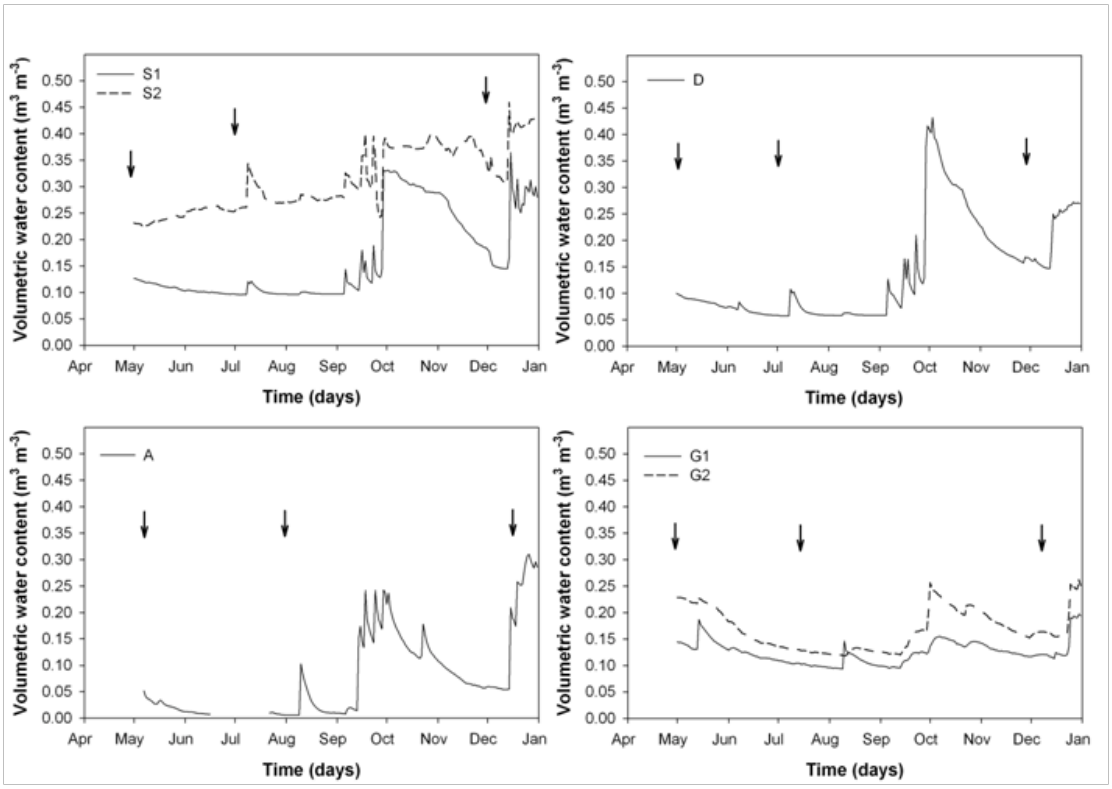
883 Figure 2



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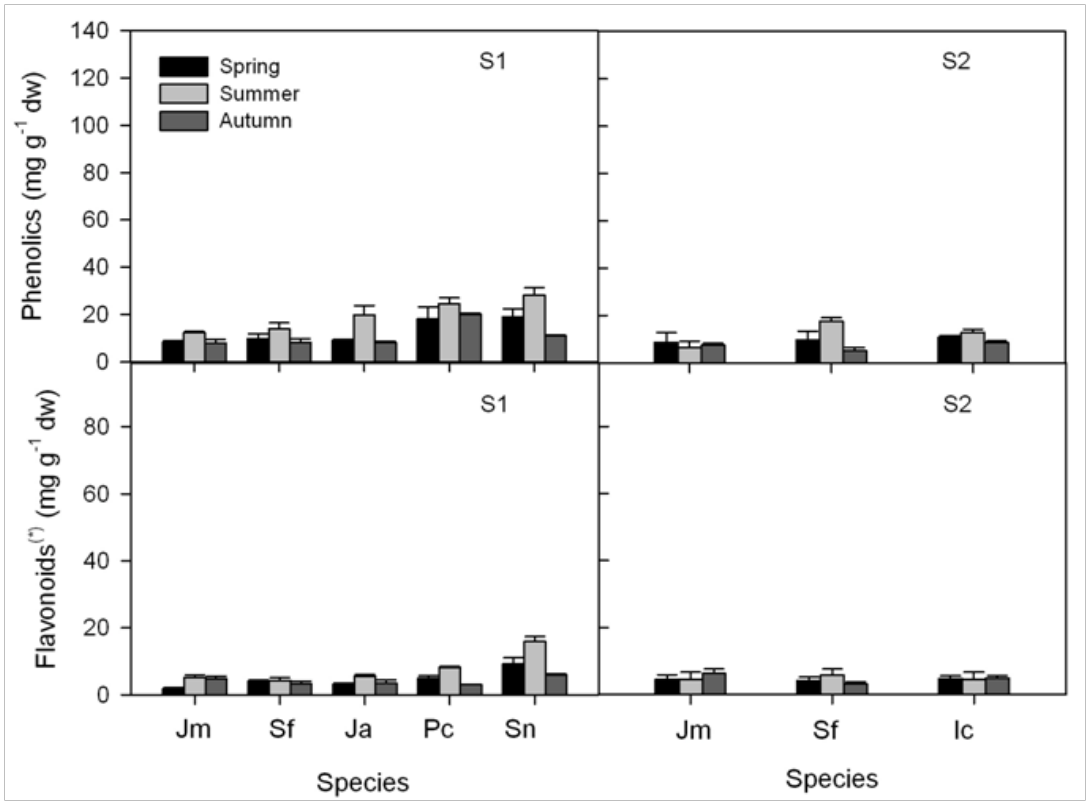
887 Figure 3



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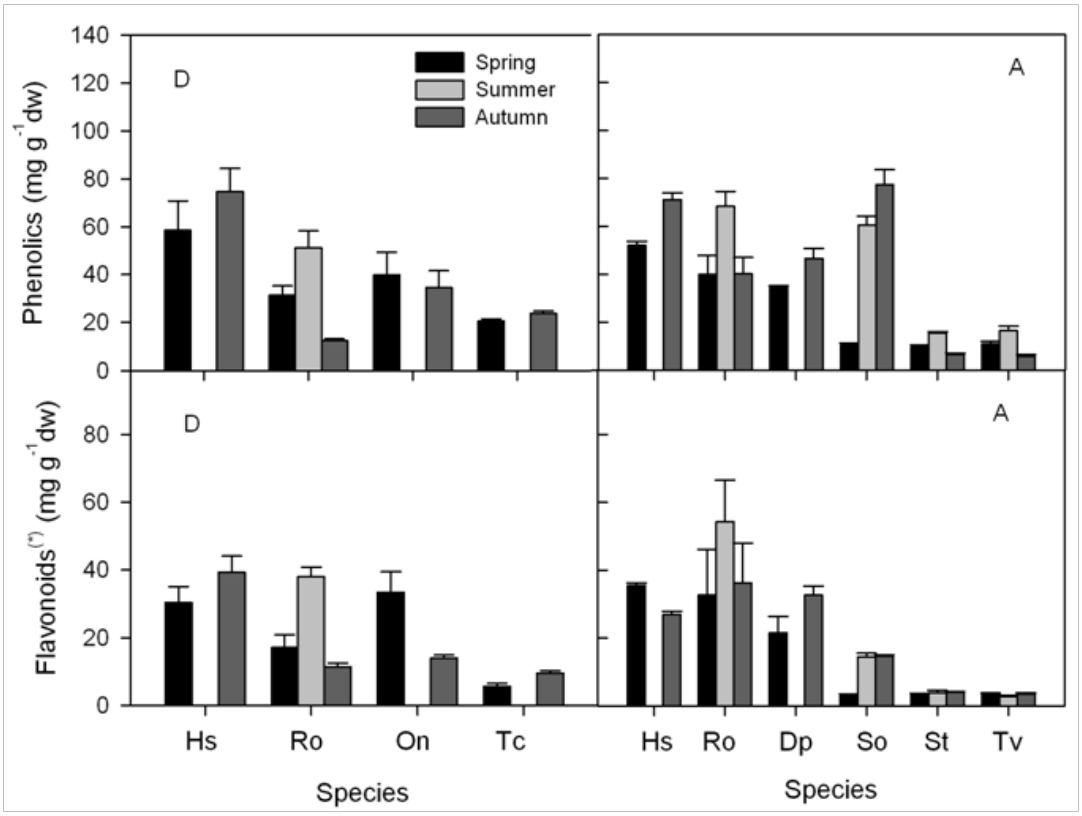
891 Figure 4



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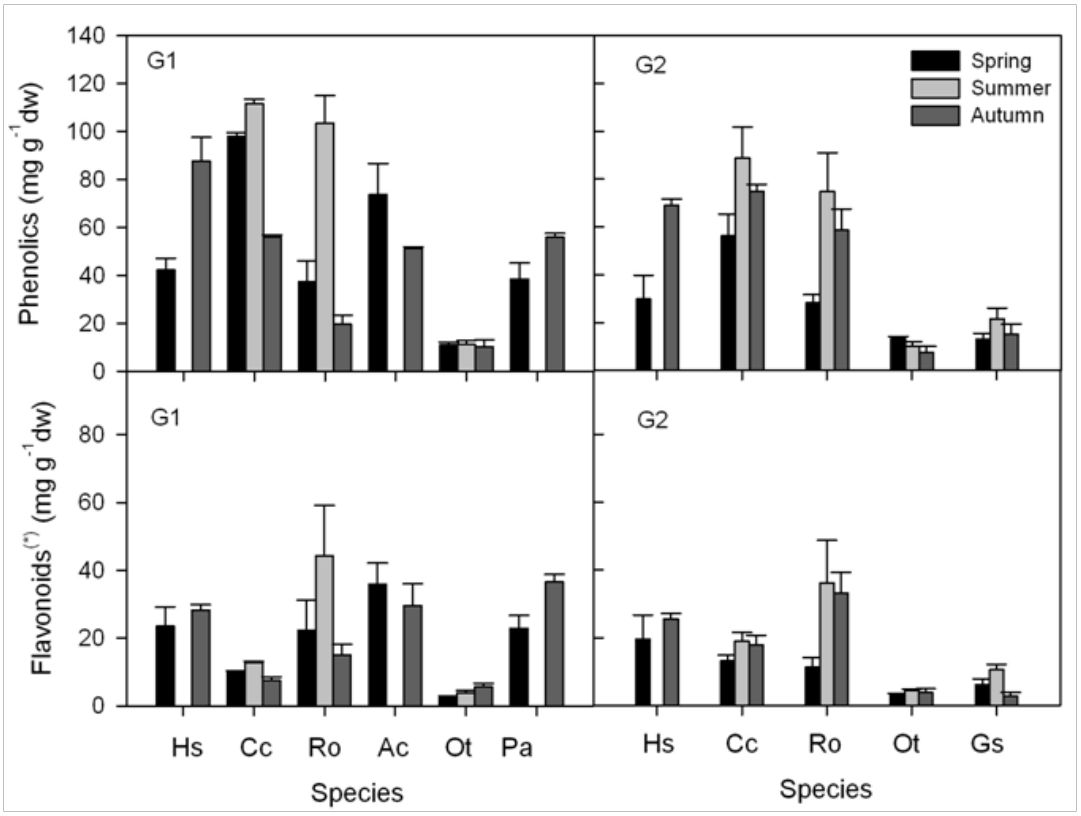
895 Figure 5



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900 Figure 6

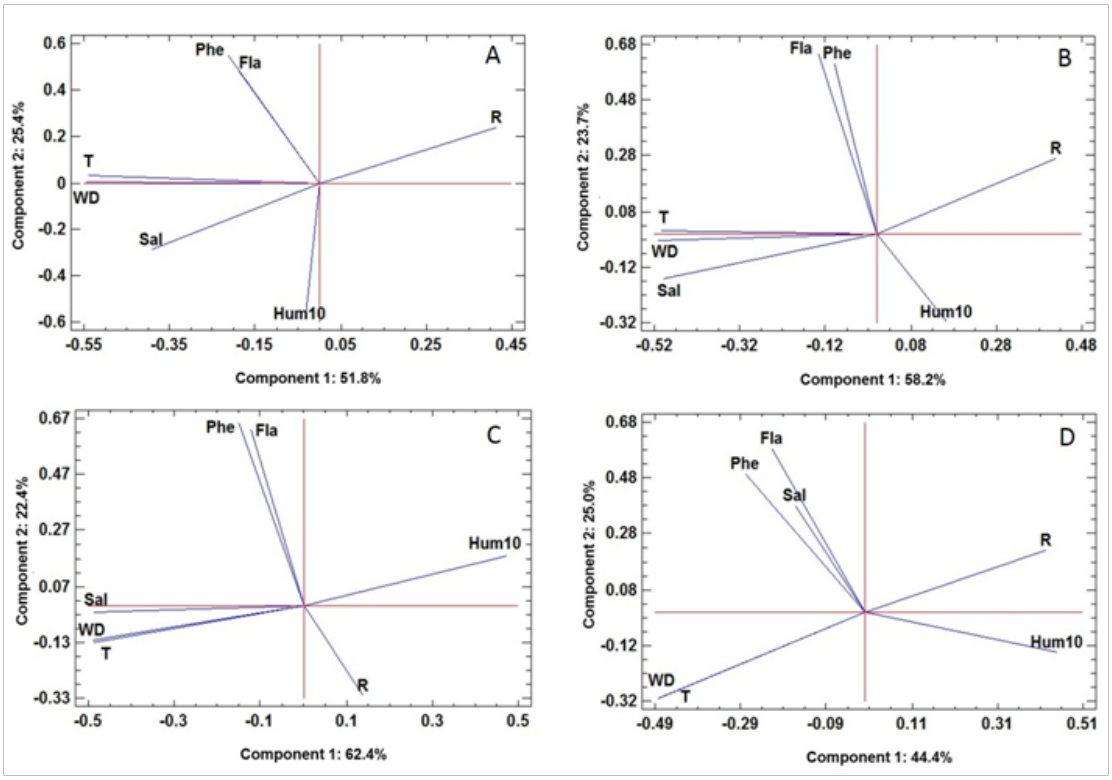

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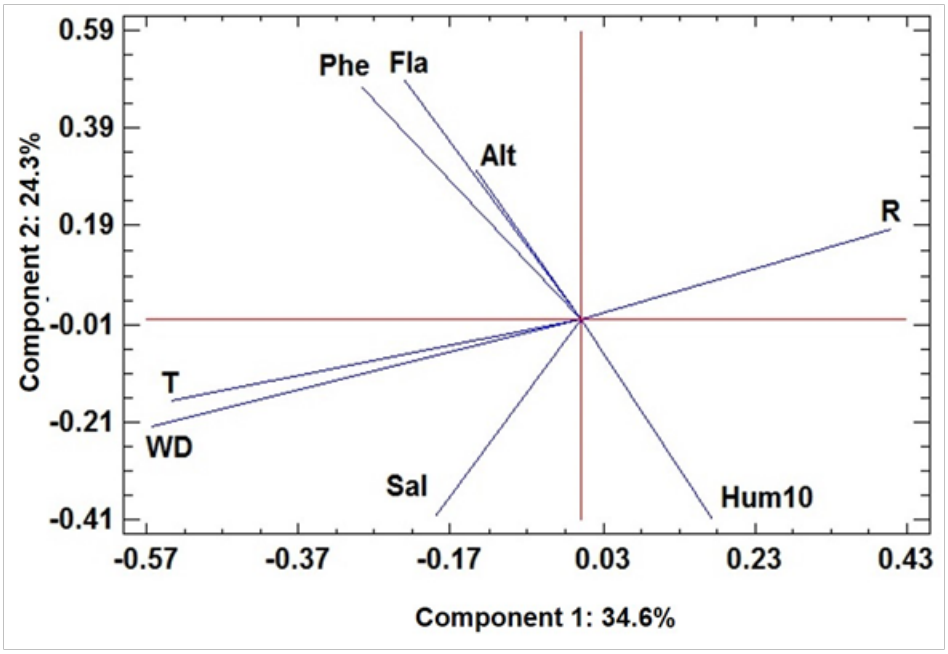
Figure 7


0.59
0.39
0.19
-0.01
-0.21
-0.41
Component 2: 24.3%
Phe
Fla
Alt
R
T
WD
Sal
Hum10
-0.57
-0.37
-0.17
0.03
0.23
0.43
Component 1: 34.6%